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Tıbbi Biyoloji ve Genetik Anabilim Dalı

İLERİ EVRE PROSTAT KANSERİ HÜCRE HATLARINDA *TRAIL*'A DİRENÇLİLİK MEKANİZMALARININ ARAŞTIRILMASI

Ahter Dilşad ŞANLIOĞLU

Doktora Tezi

Tez Danışmanları
Doç.Dr. Salih ŞANLIOĞLU
Dr. Bahri KARAÇAY

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2) Surface TRAIL decoy receptor-4 expression is correlated with TRAIL resistance in MCF7 breast cancer cells **Ahter D. Sanlioglu**, Ercument Dirice, Cigdem Aydin, Nuray Erin, Sadi Koksoy, Salih Sanlioglu. *BMC Cancer* Vol 5(1):54, 2005.

3) Current progress in adenovirus mediated gene therapy for patients with prostate carcinoma. **Ahter D. Sanlioglu**, Turker Koksal, Mehmet Baykara, Guven Luleci, Bahri Karacay, Salih Sanlioglu. *Gene Ther Mol Biol* Vol 7, 113-133, 2003.

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Tez Danışmanı: **Doç.Dr. Salih ŞANLIOĞLU**
Akdeniz Üniversitesi Tıp Fakültesi
Tıbbi Biyoloji ve Genetik Anabilim Dalı

Üye : **Prof. Dr. Güven LÜLECİ**
Akdeniz Üniversitesi Tıp Fakültesi
Tıbbi Biyoloji ve Genetik Anabilim Dalı

Üye : **Prof. Dr. Engin YILMAZ**
Hacettepe Üniversitesi Tıp Fakültesi
Tıbbi Biyoloji Anabilim Dalı

Üye : **Doç. Dr. İbrahim KESER**
Akdeniz Universitesi Tıp Fakültesi
Tıbbi Biyoloji ve Genetik Anabilim Dalı

Üye : **Doç. Dr. Türker KÖKSAL**
Akdeniz Üniversitesi Tıp Fakültesi
Üroloji Anabilim Dalı

ONAY:

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Sağlık Bilimleri Enstitüsü Müdürü

ÖZET

Adenoviral yolla aktarılan TRAIL aracılı gen tedavi yaklaşımları, ileri evre prostat kanserlerinde apoptozisi indükleyici güçlü potansiyel terapötik yaklaşımlar olarak değerlendirilmektedir. Ancak bazı prostat kanser hücrelerinin TRAIL aracılı apoptozise dirençli olduğunun gözlenmesi nedeniyle, TRAIL dirençlilik mekanizmalarının araştırılması ve bu dirençliliği kıracak yeni yaklaşımların geliştirilmesi, TRAIL'ın ileride etkili bir tedavi yaklaşımı olarak kliniğe kazandırılması açısından oldukça önemlidir.

Bu nedenlerden dolayı çalışmamızda üç farklı ileri evre prostat kanser hücre hattında (DU145, PC3, LNCaP) TRAIL'a dirençlilikten sorumlu olabilecek mekanizmalardan olan hücre içi bazal ve TRAIL aracılığıyla indüklenen NF-kB aktivitesinin, ve TRAIL ölüm ve yalancı reseptör sentez oranlarının hücrelerde TRAIL duyarlılığı ile ilişkisi araştırıldı. Bunun yanında, prostat kanserlerinde ilk kez denenen Ad5hTRAIL ve AdIKKβKA ikili vektör sisteminin hücrelerde gözlenen TRAIL dirençliliğini kırmaktaki etkinliği araştırıldı.

Sonuçlarımız, TRAIL yalancı reseptör kompozisyonunun ve hücre içi NF-kB aktivitesinin prostat kanser hücrelerinde TRAIL dirençliliğine katkıda bulunan iki önemli faktör olduğunu göstermiştir. Bunun yanında, IKKβKA aracılı IKK inhibe edici stratejilerin, hücrelerde TRAIL dirençliliğini kırmada, dirençlilik mekanizmasına bağımlı olmaksızın etkili olduğu görülmüştür. Araştırmamız, prostat kanser hücrelerinde TRAIL yalancı reseptörlerinin sentez seviyesi ile TRAIL dirençliliği arasında anlamlı bir ilişki açığa çıkarmıştır. Bulgularımız, Ad5hTRAIL ve AdIKKβKA ikili vektör sistemi ile prostat kanser hücrelerinde TRAIL dirençliliğinin kırılabileceğini gösteren ilk çalışma olması açısından da önemlidir. Bu bulgular ışığında, IKK inhibisyonu altında adenoviral yolla TRAIL gen aktarımı, ileri evre prostat kanserinde TRAIL'ın terapötik indeksini genişletmede değerli bir yaklaşım olacaktır.

Anahtar Kelimeler: TRAIL, prostat kanseri, gen tedavisi, NF-kB, adenoviral vektörler.

ABSTRACT

Adenovirus-mediated gene therapy approaches using TRAIL arose as powerful potential therapy modalities in advanced prostate carcinoma. However, as TRAIL resistance was reported in some prostate cancer cells, investigation of resistance mechanisms to TRAIL in advanced prostate carcinoma cells and development of new therapeutic approaches are crucial for TRAIL to be used efficiently in clinical settings in the future.

For this reason, unstimulated and TRAIL-induced NF-kB activities and TRAIL death and decoy receptor patterns were investigated as possible resistance mechanisms to TRAIL in three different advanced prostate carcinoma cell lines (DU145, PC3, LNCaP). Furthermore, efficiency of a dual vector approach including Ad5hTRAIL and AdIKKβKA in breaking down the TRAIL resistance in cells was investigated.

Our results revealed that TRAIL decoy receptor composition and intracellular NF-kB activity are two important factors leading to TRAIL resistance in advanced prostate cancer cells. Furthermore, IKKβKA-mediated IKK inhibiting strategies were efficient in breaking down the resistance to TRAIL in cells independent of the resistance mechanisms. Our investigation has shown a significant correlation between decoy receptor expression levels and TRAIL resistance in advanced prostate cancer cells. Furthermore, this is the first study showing that a dual vector strategy with Ad5hTRAIL and AdIKKβKA is efficient in eliminating TRAIL resistance in advanced prostate cancer cells. Consequently, this dual vector approach appears to be a novel potential treatment modality to increase the theraupetic index of TRAIL in advanced prostate cancers.

Key Words: TRAIL, prostate cancer, gene therapy, NF-kB, adenoviral vectors.

TEŞEKKÜR

Doktora çalışmam boyunca yol göstericiliği ve desteği için, ve tez çalışmamda üniversitemiz Tıp Fakültesi Hastanesi'nde kurmuş olduğu Gen Tedavi Ünitesi'nin imkanlarından yararlanmamı sağladığı için sayın danışman hocam Doç.Dr. Salih ŞANLIOĞLU'na,

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Ek-3. Current progress in adenovirus mediated gene therapy for patients with prostate carcinoma. **Ahter D. Sanlioglu**, Turker Koksal, Mehmet Baykara, Guven Luleci, Bahri Karacay, Salih Sanlioglu. *Gene Ther Mol Biol*, Vol 7, 113-133, 2003.

SİMGELER ve KISALTMALAR DİZİNİ

AAH : Atipik Adenomatöz Hiperplazi

AMACR : Alpha-Methylacyl-GA Racemase

AR : Androjen Reseptörü

BPH : Benign Prostatik Hiperplazi

CAPB : Cancer of Prostate and Brain

CAR : Coxsackie Adenovirus Receptor

c-FLIP : Cellular FLICE-Inhibitory Protein

CGH : Comparative Genomic Hybridization

CMV : Cytomegalovirus

EGF : Epidermal Growth Factor

EGFP : Enhanced Green Fluorescent Protein

ELAC2 : elaC homolog 2 (E.coli)

ETV6 : ETS (Erythroblastosis) Variant 6

FGF : Fibroblast-derived Growth Factor

FBS : Fötal Bovine Serum

FISH : Fluorescent In Situ Hybridization

IAP : Inhibitors of Apoptosis Protein

IFN-γ : Interferon-gamma

IGF : Insulin-like Growth Factor

IKK : IkBα Kinase

İEPCa : İleri Evre Prostat Kanseri

HCP1	: High-Affinity cAMP-specific Phosphodiesterase Gene
HKLK3	: Human Glandular Kallikrein 3
NF-kB	: Nuclear Factor-kB
OSPCa	: Organa Sınırlı Prostat Kanseri
PACP	: Prostatic Acid Phosphatase
PIN	: Prostatik Intraepitel Neoplazi
PSA	: Prostat Spesifik Antijen
PSMA	: Prostat Spesifik Membran Antijeni
PTEN	: Phospatase and tensin homologue deleted on chromosome Ten
Rb	: Retinoblastoma
RNASEL	: Ribonuclease L
RT-PCR	: Reverse Transcription-Polymerase Chain Reaction
SCID	: Severe Combined Immuno Deficiency
TİB	: Transrektal İğne Biyopsisi
TNF	: Tumor Necrosis Factor
TRAIL	: TNF-Induced Apoptosis Inducing Ligand
TRUS	: Transrektal Ultrason

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GİRİŞ VE AMAÇ

İleri evre prostat kanserinin tedavisinde kullanılan geleneksel tedavi metodlarından olan kemoterapi, etkinliğini p53 tümör baskılayıcı protein aracılığıyla gerçekleştirir. Ancak ileri evre prostat kanserlerinin önemli bir bölümünde p53 geninde mutasyonlar olduğu bilinmektedir. TNF ailesi üyesi ölüm ligantlarının (TNF-α, FasL, vb.) ise, apoptozisi p53 bağımsız yollarla indükleyebildikleri bildirilmiştir. Bu nedenle, bu ligantların ileri evre prostat kanserinin tedavisinde kullanımları gündeme gelmiştir. Ölüm ligantları aracılığıyla prostat kanser hücrelerinde apoptozisin indüklenmesini amaçlayan başarılı çalışmalar yapılmış olmasına rağmen, ciddi yan etkilerle karşılaşılması, bu moleküllerin sistemik kullanımlarını sınırlayan önemli bir faktör olmuştur. Bir başka TNF ailesi üyesi olan TRAIL'ın ise, diğerlerinden farklı olarak tümör hücrelerinde apoptozisi indüklerken normal hücrelerde apoptotik etki göstermediği bildirilmiştir. Bu nedenle, TRAIL'ın sistemik kullanımının güvenli olacağı düşünülmüş, ve bu yaklaşımın güvenli olduğu birçok çalışma ile kanıtlanmıştır. TRAIL ile ilgili tüm bu umut verici gelişmelere rağmen, bazı prostat kanser hücrelerinin TRAIL'a dirençli olduğu bildirilmiştir. Bu nedenle, henüz tam olarak bilinmeyen TRAIL dirençlilik mekanizmalarının açığa kavuşturulması ve direnciliği kıracak yeni tedavi metodlarının geliştirilmesi, TRAIL'ın prostat gen tedavisine kazandırılabilmesi açısından kritik öneme sahiptir.

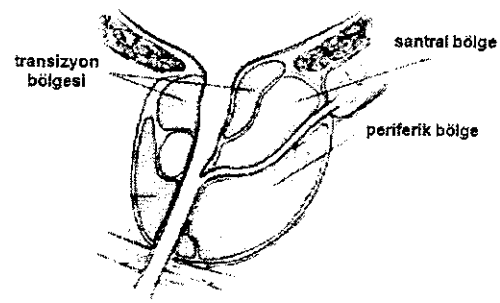
İleri evre prostat kanserinin, hastalığın tedavide en çok zorlanılan aşaması olması nedeniyle, bu çalışmada ileri evre prostat kanser hücre hatları (DU145, PC3, LNCaP) kullanıldı. TRAIL'a dirençli hücre hatlarında, dirençliliğe sebep olabilecek mekanizmalar olan bazal ve TRAIL aracılığıyla indüklenen NF-kB aktivasyon seviyesinin, ve TRAIL ölüm ve yalancı reseptör sentez oranlarının hücrelerde TRAIL duyarlılığı ile ilişkisinin açığa çıkarılması amaçlandı. Bunun yanında, prostat kanserlerinde ilk kez denenen bir ikili vektör (Ad5hTRAIL ve AdIKKβKA) sisteminin, hücrelerde gözlenen TRAIL direnciliğini kırmaktaki etkinliğinin araştırılması hedeflendi.

GENEL BİLGİLER

Prostat kanseri, prostat bezinde çoğunlukla adenokarsinoma olarak ortaya çıkan, heterojen ve genellikle çok odaklı olarak seyreden bir hastalıktır [1]. Batılı ülkelerde erkekler arasında en yaygın kanserdir, ve erkeklerde kanser sebebiyle ölümlerde ikinci sırada yer almaktadır [2]. Amerika Birleşik Devletleri'nde her yıl ortalama 200,000 kişiye prostat kanseri tanısı konmaktadır [3, 4]. Prostat spesifik antijen (PSA) taramaları, dijital rektal muayene, ve lokalize prostat kanserinin erken tedavisi gibi metodlarla son yıllarda prostat kanserinden ölüm oranlarında azalma sağlanmıştır [4]. Ancak tüm çabalara rağmen, bugün yetişkin bir erkek bireyin yaşamı süresince prostat kanserine yakalanma riskinin 5'te 1 olduğu, bu vakalar arasında metastatik prostat kanseri nedeniyle ölüm riskinin ise 30'da 1 olduğu bildirilmiştir [5]. Bu bulgular ışığında, özellikle ileri evre prostat kanserinde hastaların yaşam sürelerini uzatabilmek için mevcut tedavi yöntemlerini tamamlayıcı yeni tedavi stratejileri geliştirilmesi gerekmektedir.

2.1. Prostat Bezinin Yapısı

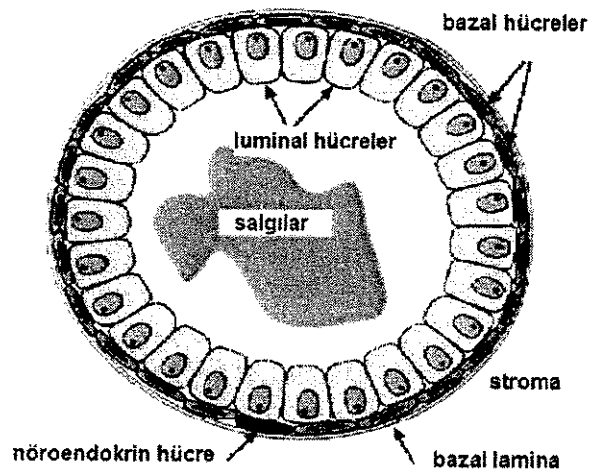
İnsanda prostat bezi, mesanenin hemen altında yer alır. Prostat bezinin orta kısmından, idrar boşaltımında rol alan üretra geçer. Prostatı oluşturan hücrelerin yaptığı salgı, seminal sıvının bir bölümünü oluşturur. McNeal, insan prostatı içinde beş farklı morfolojik bölge tanımlamıştır: periferik bölge, transizyon bölgesi, santral bölge, preprostatik sfinkter ve anterior fibromüsküler stroma [6, 7]. Şekil 2.1'de, bahsedilen farklı bölgelerden üçü gösterilmiştir.



Şekil 2.1. İnsan prostat bezinin şematik gösterimi (sagital kesit) [8]

Prostat epiteli içinde, morfolojik özellikleri, fonksiyonel durumları ve karsinogenezle ilişkileri açısından en az üç farklı hücre tipi ayırt edilebilir (Şekil 2.2). En yaygın olarak bulunan hücre tipleri, salgı yapan luminal hücrelerdir. Bu hücre tipleri androjen bağımlıdır ve prostatik salgı maddelerini üretir. Moleküler seviyede, luminal hücreler androjen reseptör sentezi, sitokeratin 8 ve 18, ve hücre yüzey belirteçi CD57 ile tanımlanabilir [9-13]. İkinci önemli epitelyal hücre tipi, bazal hücrelerdir. Bu hücreler, luminal hücreler ile bazal membran arasında bulunur,

ve prostat dokusunda luminal hücreleri çevreleyen bir katman oluşturur. Bazal hücreler, sitokeratin 5 ve 14, CD44, ve düşük seviyede androjen salgılar, ancak prostatik salgı proteinlerini üretmezler [9-14]. Bazal hücrelerin aynı zamanda, potansiyel kök hücreler olabilecekleri düşünülmektedir. Bu hücreler, muhtemel kök hücre profiline uygun olarak, DNA hasarına karşı koruyucu moleküller sentezler. Bu moleküllere örnek olarak anti-apoptotik Bcl2 proteini verilebilir [14, 15]. Üçüncü prostatik epitelyal hücre tipi ise, embriyonik kökeni tam olarak bilinmeyen nöroendokrin hücrelerdir. Bu hücrelerin, luminal hücrelerin büyümesini sağlayan parakrin sinyaller ürettikleri düşünülmektedir [16, 17]. Nöroendokrin hücreler, androjen bağımsız hücrelerdir. Bazal katmanda dağınık olarak bulunurlar ve kromogranin A, serotonin, ve çeşitli nöropeptitler sentezlerler [8].

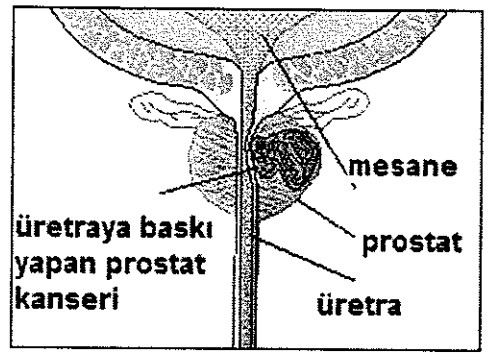


Şekil 2.2. İnsan prostat bezini oluşturan hücre tiplerinin şematik olarak gösterilmesi [8]

Prostat dokusunda tanımlanan farklı hücre tiplerinin köken ilişkilerinin bilinmesi, prostat kanserinin oluşum mekanizmasının açığa çıkarılması açısından önemlidir. Prostat kanser hücreleri genellikle bazal hücrelere spesifik molekülleri sentezler. Ancak prostat kanserinde bazal hücre katmanı kaybedilir [18, 19]. Ayrıca, Prostat Spesifik Antijen (PSA) sadece luminal hücreler tarafından salgılandığından, kanser hücreleri en azından kısmen luminal fenotipe sahiptir. Bu duruma muhtemel bir açıklama olarak, neoplastik transformasyonda, luminal hücrelerin bazal hücre fenotipine benzeyen daha az farklılaşmış bir hücre tipine dönüştüğü ileri sürülmüştür [8].

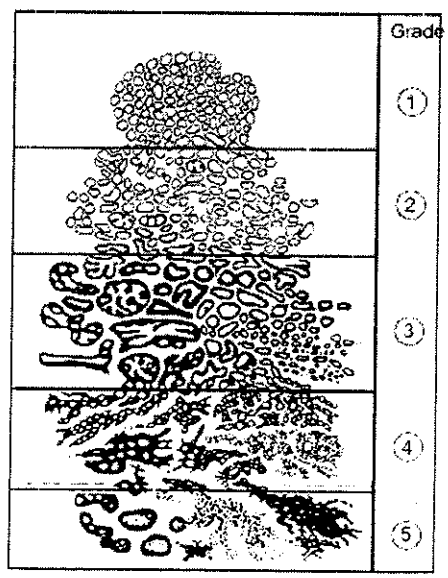
2.2. Prostat Kanserinin Tanımı ve Sınıflandırılması

Prostat kanseri, tümörün baskısı sonucu sık idrara çıkma, hematüri, hematospermi ya da postejakülatör ağrı gibi semptomlarla ortaya çıkan bir kanserdir. (Şekil 2.3) İleri evrede lenf nodlarına, kemiğe ya da diğer organlara metastazı tipiktir.



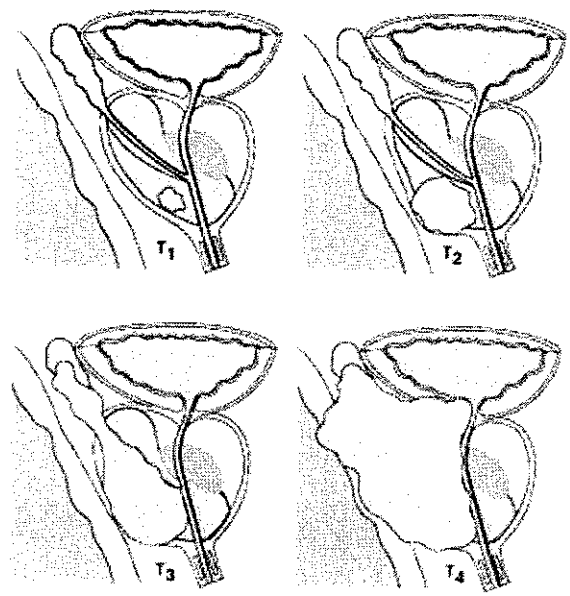
Şekil 2.3. Prostat kanserinin prostat bezinde yerleşiminin şematik olarak gösterilmesi.

Prostat tümörleri çoğunlukla adenokarsinomlardır. Prostat adenokarsinoma, prostat bezinin epitel hatlarından köken alır. Radikal prostatektomi örneklerinin incelenmesi, olguların %85'inde çok merkezli gelişim olduğunu göstermiştir. Prostat kanseri oldukça heterojen bir kanserdir. Prostat kanser dokusunun histolojik incelemesi sonucunda, aynı alanda benign bezler, prenoeplastik odaklar (PIN), ve farklı derecelerde neoplastik odaklar birarada görülebilir (Şekil 2.6). Bu heterojenite ile ilgili olarak, Gleason bugün iyi bir prognostik indikatör olarak kabul görmüş olan bir derecelendirme sistemi geliştirmiştir [20]. Bu sistemde glandüler yapı değerlendirilir. Gleason dereceleri 1 ile 5 arasında değişmekte olup, en sık görülen derece ile ikinci sıklıkta görülen derecenin toplamı Gleason skor'unu oluşturur (Şekil 2.4). Gleason skor 2 ile 10 arasında değişmektedir. Birinci derece tümörler normale yakın bir özellik gösterirken, 5 derecede herhangi bir glandüler yapı görülmemektedir. Küçük ve iyi diferansiye tümörler (1. ve 2. derece) genelde organa sınırlı iken, büyük (4 cm^3'ten büyük) ve kötü diferansiye tümörler (4. ve 5. derece) genellikle lokal ileri evre ya da metastatiktirler. Gleason skor 2-4 iyi diferansiye tümör, Gleason skor 5-7 orta derecede diferansiye tümör, Gleason skor 8-10 ise kötü diferansiye tümörü temsil eder [21]



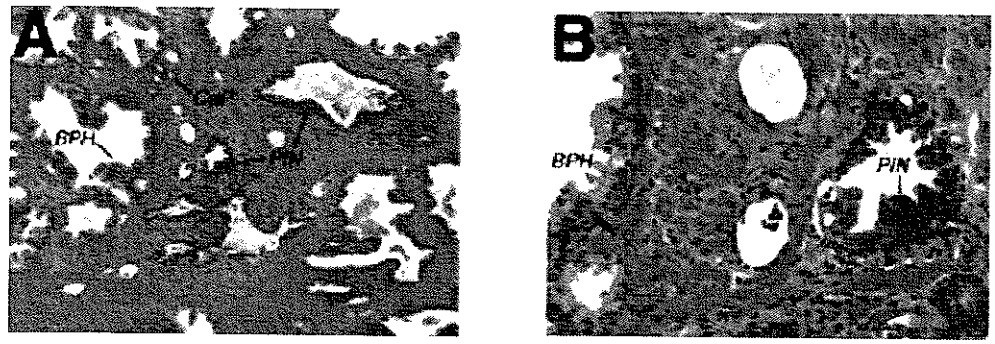
Şekil 2.4. Gleason derecelendirme sistemi [22].

Prostat kanserinin evrelendirilmesinde, Tumor, Nod, Metastaz (TNM) sistemi de sıklıkla kullanılmaktadır. Bu sistemde, lokal tümör büyümesi, T1 ile T4 arasında dört evrede tanımlanır. T1 evresinde tümör dijital rektal muayene veya ultrasonla tespit edilemez, ancak transüretral rezeksiyon veya PSA testi sonrası biyopsi ile tanımlanabilir. T4, tümörün komşu organları tuttuğu ileri evresini temsil eder. Nodal evreler (N0-N1) ve metastatik evreler (M0-M1C), hastalığın sırasıyla lenf nodlarına ve uzak bölgelere klinik yayılımını (metastaz) tanımlar [22]



Şekil 2.5. TNM evrelendirme sistemine göre lokal prostat tümör büyümesinin dört farklı evresi. Noktalı olarak gösterilen kısımlar, tümör dokusunu temsil etmektedir [22]

Prostat kanseri, genel anlamda Organa Sınırlı Prostat Kanseri (OSPCa) ve İleri Evre Prostat Kanseri (İEPCa) olarak sınıflandırılabilir. Prostat kanseri, genellikle periferik bölgede oluşur. İEPCa ise çoğunlukla kemiğe ve lenf noduna olmak üzere metastazla sonuçlanır. Bunun yanında erkeklerde, yaş ilerledikçe sıklığı artan, prostat bezinde büyüme ile karakterize olan iyi huylu bir hastalık olarak ifade edilen Benign Prostatik Hiperplazi (BPH) gelişebilir. BPH genellikle transizyon bölgesinde ortaya çıkar. Bazal katmanın genişlemesi ve stromal hiperproliferasyon ile tipiktir [8].



Şekil 2.6. İnsan prostat dokusunun histolojik incelemesi. A ve B panellerindeki görüntüler hematoksilen-eozin boyama ile elde edilmiştir. (A) Fotoğrafta prostat dokusunun heterojenitesi, aynı alanda bulunan Benign Prostat Hiperplazi (BPH), Prostatik Intraepitel Neoplazi (PIN), ve iyi diferansiye adenokarsinoma (CaP) ile görülebilmektedir. (B) Panel A'dan bir bölgenin mikroskop altında yüksek büyütmede görüntüsü verilmiştir.

2.3. Prostat Kanserinin Epidemiyolojisi

2.3.1. Yaş

Prostat kanserinin gelişiminde, yaş çok önemli bir faktördür. Yaşa bağlı olarak prostat kanseri nedeniyle ölüm oranı artar. Erkeklerde 65 ila 70 yaşlarında 100.000'de 358 olan prostat kanseri insidansı, 80 yaş ve üzerinde 100.000'de 1035'e çıkar. Her iki grupta ölüm oranları ise sırasıyla %21.2 ve %40 olarak bildirilmiştir [21].

2.3.2. Coğrafya ve Çevresel Faktörler

Prostat kanseri insidansının coğrafik dağılımı oldukça heterojendir. Örneğin Tayland'ta prostat kanserinden ölüm oranı 100.000'de 0.1 iken, Batı Hindistan'ın bazı bölgelerinde 100.000'de 30'dur. Hastalığın genel insidansı da oldukça çeşitlilik gösterir. Prostat kanserinin görülme sıklığı Çin'de 100.000'de 0.8 iken, Kuzey Amerika'da yaşayan zenci populasyonda 100.000'de 100'dür. Dünya genelinde gözlenen bu farklılıkların açıklamasında, çevresel faktörlerin ırksal faktörlerden tam olarak ayırt edilemeyeceği açıktır, ancak göç eden toplumlar üzerine yapılan çalışmalar, çevresel faktörlerin ırksal orijinlerden daha önemli olduğunu ortaya koymuştur [21]. Örneğin Asya kökenli ülkelerde prostat kanseri insidansı çok düşükken, Amerika Birleşik Devletleri'nde prostat kanseri insidansı bu ülkelere göre önemli oranda yüksektir. Amerika Birleşik Devletleri'nin Asya kökenli ülkelerden yüksek oranda göç aldığı bilinmektedir. Bu nedenle, diyet ve çevresel faktörlerin, diğer epitel kanserlerde olduğu gibi, prostat karsinogenezinde de anahtar rol oynadığı kabul edilmektedir [8].

2.3.3. Ailesel Yatkınlık

Kalıtsal faktörler, prostat kanserlerinin oldukça küçük bir yüzdesinden (%10) sorumlu tutulmakta ve erken yaşta ortaya çıkan prostat kanseri ile ilişkilendirilmektedir. Ancak pozitif aile hikayesi, prostat kanseri için en güçlü epidemiyolojik risk faktörleri arasında sayılmaktadır. Birçok kromozomal bölgenin prostat kanserine yatkınlıkta rol aldığı düşünülmektedir [8]. Prostat kanserine

yatkınlığın poligenik mekanizmalar içerdiği, ve Mendelian kalıtımın farklı modelleri, eksik penetrans ve etnik varyasyonlar aracılığıyla gerçekleştiği düşünülmektedir. Linkaj analizi yoluyla, 1, 10, ve 17 no.lu kromozomlarda ve X kromozomunda birçok lokus prostat kanserinde ailesel yatkınlık ile ilişkilendirilmiştir. Özellikle 1 no.lu kromozom üzerinde çalışılmıştır. Bu kromozomun muhtemel prostat kanseri yatkınlık genlerini barındırabilecek en az 3 alt bölge (HCP1, PACP, ve CAPB) içerdiği ileri sürülmüştür [23]. Tablo 2.1'de prostat kanserine yatkınlık genleri olarak tanımlanan genlerin listesi verilmiştir.

Tablo 2.1. Prostat kanseri (PCa) gelişimi ile ilgili başlıca yatkınlık genleri.

Gen ismi	**Yatkınlık ile ilgili özellikleri**
Andojen Reseptörü (AR)	CAG tekrarlarının PCa'ne yatkınlık ile ilgili olduğu düşünülmektedir.
MSR1 (Makrofaj Süpürücü Reseptör)	Yedi önemli gen mutasyonu bulunmuştur.
ELAC2	Mutasyon taşıyıcılarında PCa geliştirme riski yüksektir,
BRCA2	PCa'nin erken evrelerinde rol aldığı düşünülmektedir.
RNASEL (ribonükleaz L'yi kodlayan gen)	Mutant allel açısından heterozigot erkeklerde PCa geliştirme riski %50 artar.
ETV6 (tümör supresör gen)	Mutasyonel inaktivasyon PCa ile sonuçlanabilir.
AMACR/P504S (alfa metil-CoA Racemase)	Tanımlanan onyedi dizi varyantı, PCa'ne yüksek riskle ilgili olabilir.

2.3.4. Steroid Hormonların Rolü

Steroid hormon reseptör sinyal yolları, prostat karsinogenezinin her evresinde önemli rol oynamaktadır. Erkeklerde androjen/estrojen oranının yaşla birlikte azalması, prostat kanserinin ortaya çıkmasında önemli bir faktör olarak düşünülmektedir [8].

2.4. Prostat Kanserinde Tanı Yöntemleri

Prostat kanseri heterojen bir hastalıktır ve özellikle ileri evrede ve metastatik hastalıkta mevcut tedavi yöntemleri, hastaların yaşam sürelerini uzatmada yetersiz kalmaktadır. Bu nedenle prostat kanserinin erken teşhisi, tedavide yüksek başarı açısından son derece önemlidir [24]. Bu amaçla farklı tarama metodları kullanılmaktadır [25, 26]:

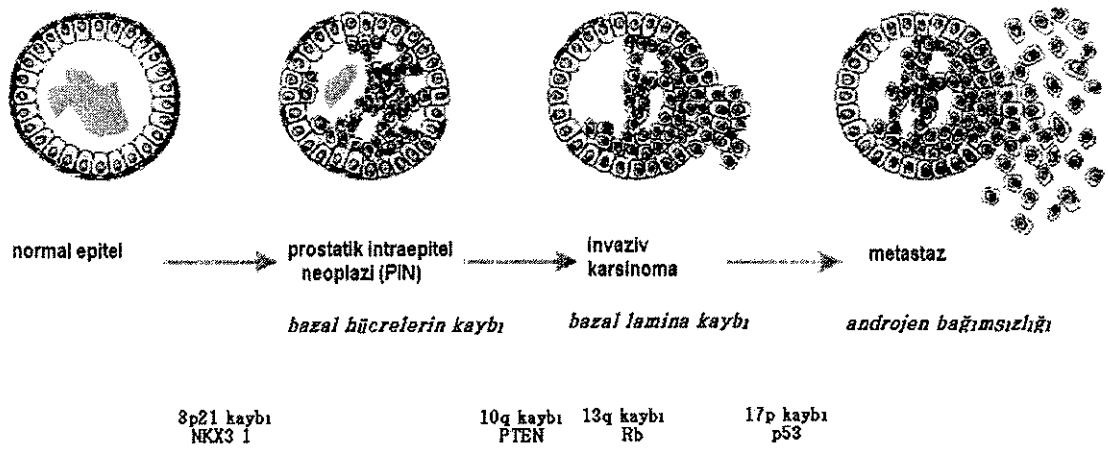
Dijital Rektal Muayene: Dijital rektal muayene, prostat kanseri taramalarında rutin olarak kullanılan bir tekniktir. Şüpheli dijital rektal muayene, serum prostat spesifik antijen (PSA) düzeyleri ile birlikte değerlendirildiğinde taramalarda prostat kanseri tanı oranını yükseltir. Elli yaş üzeri gönüllü erkeklerle yapılan iki çalışmada, dijital rektal muayene, 0,5 m^3'ten büyük intrakapsüler tümörlerin teşhis edilme ihtimalini 1,5 ila 2 kat, ekstrakapsüler tümörlerin teşhis edilme ihtimalini ise 3 ila 9 kat artırmıştır [27].

Prostat Spesifik Antijen (PSA): PSA, 33 kDa ağırlığında bir prostatik salgı glikoproteinidir. Kromozom 19 üzerinde hKLK3 geni tarafından kodlanan, tripsin ve kimotripsin benzeri aktiviteye sahip bir serin esterazdır. PSA sentezi androjen bağımlıdır, ve androjenler yokluğunda azalır. Prostat kanserinde, serum PSA seviyeleri, benign prostatik hiperplazidekine oranla yüksektir [21]. Ancak farklılaşmamış androjen bağımsız prostat kanser hücreleri PSA üretemez. Bu durum, hormona dirençli hastaların bir kısmında PSA artışının görülmemesini açıklamaktadır [28]. Tek başına çok güçlü diagnostik değeri olmamasına rağmen, Gleason skoı [29] ve klinik evrelendirme [30] ile birlikte değerlendirildiğinde, PSA seviyesinin ölçülmesi prostat kanserlerinin histolojik derecesini belirlemede değerli bir yöntemdir [31].

Diğer Tanı Yöntemleri: Prostat kanserinin tanısında, dijital rektal muayene ve serum PSA düzeylerine bakılması yanında Transrektal Ultrason (TRUS) ve eşliğinde Transrektal İğne Biyopsisi (TİB) de yaygın olarak kullanılan yöntemlerdendir. Yüksek PSA seviyesi belirlenen veya dijital rektal muayenesinde normal dışı durum gözlenen hastalara, genellikle transrektal ultrason biyopsisi uygulanır [21].

2.5. Prostat Kanserinin Gelişiminde Moleküler Mekanizmalar

Prostat kanserinin ortaya çıkışından ve progresyonundan sorumlu mekanizmalar tamamen açığa çıkarılamamıştır, ancak tümör progresyonunda birçok değişik faktörün (sinyal iletim yollarında, anjiogenez ve adhezyon moleküllerinde değişiklikler gibi) önemli rol oynadığı düşünülmektedir (Şekil 2.7).



Şekil 2.7. İnsanda prostat kanseri gelişim basamakları. Gelişim aşamaları, spesifik kromozom bölgelerinin ve aday tümör baskılayıcı genlerin kaybı ile gerçekleşir [8].

2.5.1. Prostat Kanserinin Erken Evreleri

Prostat kanserlerinde gerçekleştirilen histopatolojik çalışmalar sonucu, insan prostat kanserinin primer öncüsü olduğuna inanılan spesifik bir lezyon tipi tanımlanmıştır (Şekil 2.6). Prostatik Intraepitelyal Neoplazi (PIN) adı verilen bu lezyon tipi, atipik hücresel özellikler ve nükleer polimorfizm ile belirgindir, ve düşük dereceli ve yüksek dereceli formların arasında bir geçiş formu olarak nitelendirilmektedir. Yüksek dereceli PIN'in, erken invaziv karsinomanın hemen

önceki aşamasını temsil ettiği düşünülür. PIN lezyonları, prostat epitelinde görülen BPH ve Atipik Adenomatöz Hiperplazi (AAH) gibi diğer bazı histopatolojik abnormalitelerinden yapısal ve sitolojik olarak ayırt edilebilmektedir. BPH ve AAH, PIN'in tersine, prostat kanserinin öncü evreleri olarak düşünülmemektedir [32].

Prostat kanserinde sıklıkla görülen moleküler düzeyde değişikliklerden biri, 8. kromozomun spesifik bölgelerinde gerçekleşen delesyonlardır. Bu tür düzensizlikler, FISH, CGH, ve allelik dengesizlik analizi teknikleri ile prostat tümörlerinin %80'inde, bunun yanında kolorektal ve akciğer kanserlerinin belirli bir yüzdesinde de belirlenmiştir [33, 34]. Delesyonların özellikle 8p12-21 ve 8p22'ye karşılık gelen iki bölgede meydana geldiği bildirilmiştir [35]. 8p12-21 kaybının prostat karsinogenezinde erken evrede ortaya çıktığı ve hem PIN hem de erken invaziv karsinomada tanımlandığı bildirilirken, 8p22 delesyonlarının ise geç dönemde meydana geldiği bildirilmiştir. 8p12-21 lokusunda bulunan NKX3.1 homeobox geni, prostat kanseri gelişimi ile ilgili aday gen olarak bildirilmiştir [36].

2.5.2. Prostat Kanserinin Progresyonu

Prostat kanserinin progresyonunda rol oynadığı düşünülen farklı kromozomal düzensizlikler bildirilmiştir. Örneğin 10q kromozomal bölgesinde, daha çok prostat karsinomada tanımlanmış olan, PIN lezyonlarında ise nadir olarak görülen delesyonlar bildirilmiştir. 10q23 bölgesinde bulunan PTEN/MMAC1 tümör baskılayıcı geni, prostat kanser progresyonunda rol aldığı düşünülen genler arasındadır. Prostat kanserlerinin yanında glioblastoma, meme, ve endometrial kanserler gibi birçok farklı kanser türünde 10q23 bölgesinde kayıplar bildirilmiştir [8]. PTEN proteininin kaybının, insan prostat karsinogenezinde anahtar rol oynadığı düşünülmektedir. Bu bulgu ile uyumlu olarak, Vliestra ve arkadaşları, birçok prostat kanser hücre hattında ve ksenograftta PTEN kaybı olduğunu göstermişlerdir [37].

Prostat kanserinin gelişiminde önemli rol oynadığı düşünülen bir başka faktör, Retinoblastoma (Rb) geninin bulunduğu 13q kromozomal bölgesinde meydana gelen kayıplardır. Prostat kanserlerinin %50'sinde, 13q bölgesinde delesyonlar bildirilmiştir [38]. Bookstein ve arkadaşları, *Rb* eksikliği olan prostat kanser hücrelerine retrovirüs aracılı gen transferi yoluyla *Rb* proteinini kodlayan gen aktardıklarında tümör oluşumunun engellediğini göstermişlerdir [39]. Sonraki çalışmalarda, organa sınırlı ve ileri evre prostat kanserlerinde de *Rb* proteininin yokluğu bildirilmiştir [40]. *Rb* proteininin, özellikle androjene cevapta prostat hücrelerinde apoptozisi düzenleyici rol oynadığı bildirilmiştir. Ancak bir çok diğer çalışmada *Rb* gen mutasyonları gösterilememiştir [38, 41]. Bu durum, prostat karsinonogenezinde, 13q'da *Rb* geni dışında diğer bir genin delesyonunun daha önemli olabileceğini göstermektedir.

Diğer birçok tümörde olduğu gibi, prostat kanserinde de hücre döngüsünün kontrolü ile ilgili genlerde meydana gelen değişikliklerin hastalığın progresyonunda belirgin rol oynadığı düşünülmektedir. Normal şartlarda, prostat epitelinde oldukça düşük oranda gerçekleşen hücre çoğalması, yine düşük oranda gerçekleşen apoptozis ile dengede tutulur [42]. Buna karşın, PIN ve erken invaziv karsinomalarda hücre

çoğalma oranında 7 ila 10 kat artış olduğu, ileri evre ve/veya metastatik prostat kanserlerinde de apoptozis oranında yaklaşık %60 azalma olduğu gösterilmiştir. Bu nedenle, organa sınırlı hastalığın ilerlemesinde hücre döngüsünün kontrolünün kaybedilmesinin önemli rol oynadığı, apoptoziste meydana gelen düzensizliklerin ise ileri evre kanserde daha önemli olduğu düşünülmektedir. Hücre döngüsünü düzenleyici rol oynayan genler arasında, CDK4 inhibitörü olan $p27^{kip1}$'de fonksiyon kaybı, prostat tümörlerinde sıklıkla görülmektedir, ve prognostik belirteç olarak fonksiyon görebileceği bildirilmiştir [8]. *p27kip*[1] geni, 12p12-13.1 bölgesinde bulunmaktadır [43]. Bu bölgenin ileri evre prostat kanserinde sıklıkla delesyona uğradığı bildirilmiştir [44].

Yüksek dereceli PIN'den erken invaziv karsinomaya geçişte en kritik faktörlerden birinin yaşlanma olduğu bilinmektedir. Yaşlanma ile hastalık ilerleyişi arasındaki bu güçlü ilişki, prostat epitelinin düşük proliferasyon oranından ve transformasyonun oluşabilmesi için geçmesi gereken süreden kaynaklanabilir. PIN lezyonları ve prostatik karsinomada telomer uzunluklarının azaldığı ve telomeraz aktivitesinin arttığı gözlenirken, BPH'ta benzer bir durumdan söz edilmemektedir [45]

2.5.3. Metastatik Hastalığa Geçiş

Prostat kanserinde metastatik hastalığa geçişle birlikte androjen bağımlılığının kaybolması sık görülen bir durumdur. Prostat kanser hücrelerinin başlangıçtaki androjen bağımlılıklarını nasıl kaybettikleri tam olarak açığa kavuşturulmamıştır. Önceleri androjen bağımsız tümör gelişiminin androjen reseptör (AR) mRNA'sının ve proteininin kaybı ile gerçekleştiği düşünülmekteydi [46]. Ancak sonraları, AR proteininin primer tümörlerde, tekrarlayan lokal tümörlerde, ve hatta metastatik tümörlerde oldukça homojen olarak sentezlendiği gözlenmiştir [47-49]. Bu bulgular, kanser hücrelerinin AR sentezinin azaltılmasından bağımsız bir mekanizma ile androjen bağımlılığını kaybettiklerini göstermektedir. Hücrelerin androjen bağımsızlığı edinmesinde birçok mekanizma rol oynayabilir. AR aktivitesinde, fonksiyonunda, ve/veya özgünlüğünde değişiklikler meydana gelebilir. Prostat kanser hücre hatlarında ve primer tümörlerde yapılan çalışmalarda, androjen reseptör geninde hormon bağlama bölgesinde mutasyonlar bildirilmiştir [50, 51]. AR aktivitesini etkileyebilecek diğer mutasyonlara örnek olarak, CAG tekrarlarının amplifikasyonu ve tüm genin amplifikasyonu verilebilir. CAG tekrarlarının uzunluğu, androjen fonksiyonu ile ters orantılıdır [52]. Tüm genin amplifikasyonunun ise, hastalığın tekrarı ile ilişkili olabileceği düşünülmektedir. Son olarak, androjen kaynağının sınırlı olduğu durumlarda (androjen sentezini inhibe edici tedavi yaklaşımlarında olduğu gibi), hormon bağımlılıklarının, androjen reseptörünün IGF, FGF, ve/veya EGF gibi büyüme faktörleri ile sinerjistik aktiviteleri ile aşılabileceği bildirilmiştir [8, 53].

İleri evre prostat kanserinde ve metastatik hastalıkta, p53 tümör baskılayıcı genini içeren 17p kromozomal bölgesinin delesyonu bildirilmiştir [54, 55]. p53'ün immunositokimyasal lokalizasyonunun belirlendiği veya direk olarak mutasyon analizi yapılan çalışmalarda, ileri evre prostat kanserinde ve tekrarlayan metastatik hastalıkta p53 geninde çeşitli mutasyonlar tanımlanmıştır [8, 56, 57].

2.6. Prostat Kanserinin Tedavisinde Kullanılan Mevcut Yaklaşımlar

Prostat kanserinde tanıda belirlenen histolojik derecelere göre hastalar farklı stratejiler ile tedavi edilmektedir. Hastalığın organa sınırlı olarak seyrettiği erken evrelerde, tedavide genellikle operasyon ve/veya radyasyon tedavisi uygulanır [58, 59]. Tümörün artık prostat bezi ile sınırlı olmadığı, ancak metastatik yayılıma dair belirtiye rastlanılmayan aşama, lokal ileri evre olarak tanımlanır. Lokal ileri evre hastalığın tedavisinde amaç, metastatik yayılım ve doku invazyonu riskini düşürmektir. Bu evrede, tedavide genellikle radyoterapi ve hormon tedavisinin birlikte kullanıldığı yaklaşımlardan yararlanılır. Ancak, hastalık erken evrede teşhis edilemediğinde ya da agresif formlarda, seminal vesiküllerin lokal invazyonu ile karakterize olan ileri evrelere geçiş, ve çoğunlukla kemiğe olmak üzere metastaz gerçekleşir. Metastatik hastalığın tedavisinde amaç, hastanın yaşam süresini uzatabilmek, ve metastazın kemik ağrısı gibi başlıca semptomlarını önleyerek ya da kontrol altına alarak hastanın yaşam kalitesini artırmaktır. Bu evrede hastalara genellikle hormon tedavisi uygulanır [22]. Androjen sentezini bloke edici ajanlarla muamele, hastalığın gelişimini bir süre geriletse de, bu tümörlerin hemen hemen hepsinde androjen yokluğunda tümör progresyonu devam eder [60, 61]. Bu aşamada hastalığın progresyonunu yavaşlatmak amacıyla sitotoksik kemoterapi veya glukokortikoidlerle birlikte uygulanan radyoterapiden yararlanılır [22]

Prostat kanserinin tedavisinde sıklıkla yararlanılan radyoterapi [62] ve kemoterapi [63], kanser hücrelerini apoptozis yoluyla ölüme götürme prensibi ile çalışır. Bu tedavi metodlarının apoptotik etkinliği, p53 tümör baskılayıcı proteininin varlığını gerektirir [64]. Ancak diğer birçok tümörde olduğu gibi, ileri evre prostat kanserlerinde de, p53 geninde ilgili proteinin fonksiyonunu inhibe edici mutasyonlar bildirilmiştir [8, 56, 57]. Sonuç olarak, fonksiyonel bir p53 genine sahip olmayan tümörler, hem kemoterapiye hem de radyoterapiye dirençlilik gösterir [65].

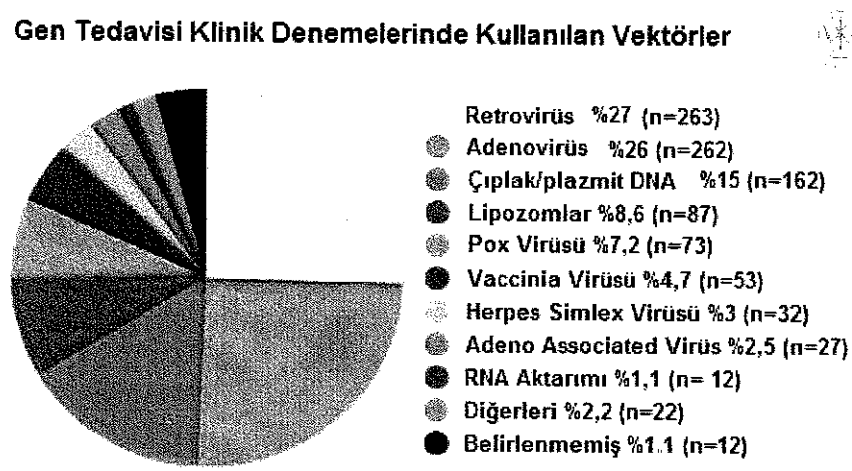
2.7. Prostat Kanserinde Gen Tedavisi

Prostat kanserinde mevcut tedavi yöntemlerinin hastaların yaşam sürelerini uzatmada yeterli olmaması [66], yeni tamamlayıcı tedavi yaklaşımlarının geliştirilmesini gerektirmektedir. Bu sebeplerden dolayı, prostat kanserinde gen tedavisi çalışmaları başlamıştır [67]. Son yıllarda, prostat kanserinde gen tedavisi alanında önemli gelişmeler kaydedilmiştir. Viral ve viral olmayan yöntemlerle daha etkin gen transferi, doku spesifik DNA promotor mekanizmalarının geliştirilmesi, ve yeni terapötik genlerin keşfi, bu gelişmelere örnek olarak verilebilir [68]. Prostat dokusu, gen tedavi uygulamaları için ideal bir dokudur. Gen transfer vektörlerinin kolay ulaşabileceği bir doku olmasının yanında, kendine özgü antijenlere sahiptir (örn. PSA, PSMA). Prostat kanserinde gen tedavisi için mevcut stratejiler, immunotedavi ve sitolitik/pro-apoptotik yaklaşımları içermektedir [69]. Bu yaklaşımların başarısında önemli bir faktör, etkin gen transferidir. Viral yöntemler, kanser gen tedavisi çalışmalarında oldukça yaygın olarak kullanılmaktadır. Birçok diğer kanserde olduğu gibi, prostat kanserine karşı gen tedavi stratejilerinin geliştirilmesinde de, adenoviral vektörler etkin gen tedavi vektörleri olarak tercih edilmektedir

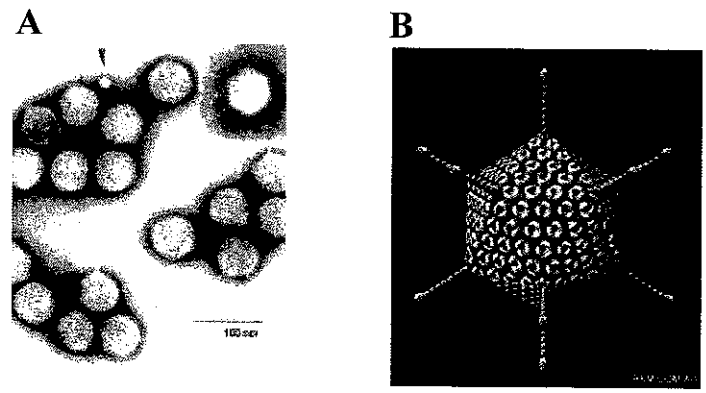
2.7.1. Adenoviral Vektörler

Prostat kanseri gen tedavisi çalışmalarında tedavi edici genlerin hücrelere aktarımı için bugüne kadar çeşitli farklı viral vektörler kullanılmıştır. Bunlara örnek olarak lentivirüsler [70], herpes simplex virüsler [71], adeno associated virüsler [72], ve adenovirüsler [73] verilebilir. Semliki Forest virüsü ve Sindbis virüsü gibi diğer bazı virüs türlerinin de prostat kanser hücrelerine gen aktarımında etkinliği denenmiştir [73]. Ancak bu virüsler, prostat kanser hücrelerini etkin bir şekilde transdüksiyona uğratmakta etkisiz kalmıştır. Antijenik özellikleri ve doku transdüksiyon özellikleri nedeniyle adenovirüsler, kanser gen tedavisi yaklaşımlarında ve klinik denemelerde tercih edilen viral vektörler haline gelmiştir (Şekil 2.8). Bunun yanında, doku spesifik promotorların keşfi, adenovirüsleri klinikte kullanım için daha uygun hale getirmiştir [74].

Bugüne kadar 49 farklı Adenovirüs serotipi izole edilmiş ve 6 farklı grup altında toplanmıştır. Gen transfer vektörleri olarak en sık kullanılanlar, C alt grubunun 5 ve 2 numaralı serotipleridir [75].



Şekil 2.8. Gen tedavisi klinik denemelerinde kullanılan vektörler ve yüzdeleri [76]

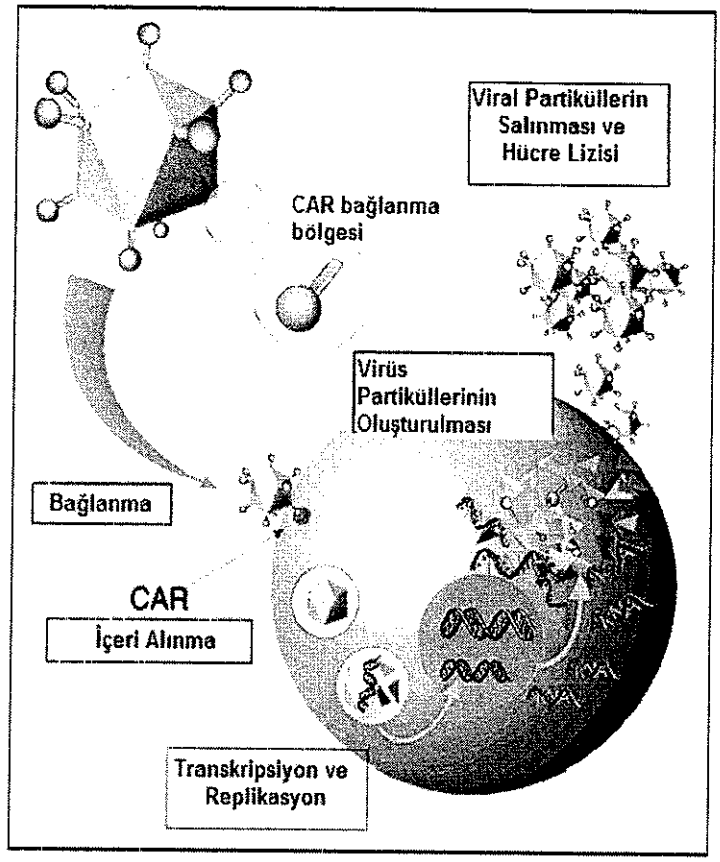


Şekil 2.9. Adenovirüsler. (A) Adenovirüslerin Elektron Mikroskopu altında görüntüsü [77]. (B) Adenovirüsün şematize edilmiş görüntüsü [78].

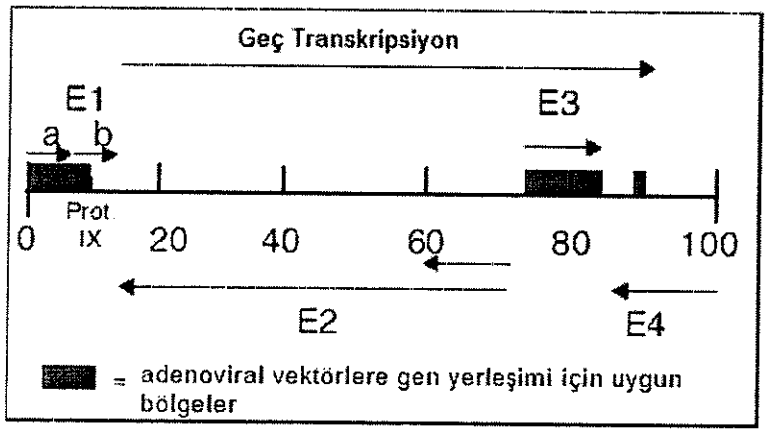
Adenovirüsler, yaklaşık 70-100 nm çaplı bir protein kapsitten, ve kapsid içinde yaklaşık 36 kb uzunluğunda tek kopya çift zincirli DNA'dan oluşur. Adenovirüsler, konakçı hücre yüzeyine Coxsackie Adenovirus Receptor (CAR) reseptörleri aracılığıyla bağlanır. CAR reseptörlerine bağlanan adenovirüsün reseptör aracılı endositoz yoluyla hücre içine alınabilmesi için, aynı zamanda αvβ integrinlere bağlanması gerektiği gösterilmiştir (Şekil 2.10). Hücreye girdikten sonra dış kapsidin ayrışması ile virüs endozomdan kaçar ve sitoplazmaya salınır. Daha sonra viral DNA nukleus içine bırakılır. Viral DNA replikasyonu, enfeksiyondan yaklaşık 7 saat sonra başlar. Adenoviral genom nukleus içine bırakıldıktan sonra, erken adenoviral genlerin (E1-E4) sentezi başlar. Öncelikli olarak sentezlenen proteinler, E1a ve E1b genleri tarafından kodlanan proteinlerdir. Bu proteinler diğer viral genlerin sentezini ve genom replikasyonunu yönetirler. DNA replikasyonu başladıktan sonra viral transkripsiyon geç promotor yoluyla gerçekleştirilir. Tam virüs oluşumu enfeksiyondan sonra 20-24 saat içinde gerçekleşir, ve hücre 2 ila 3 gün içinde lizise uğrar [79].

Yardımcı virüse bağımlı olmayan adenoviral vektörlerin üretiminde, hücrelere aktarılmak istenen DNA, genomda en az üç bölge içine yerleştirilebilir. Bunlar E1 ve E3'te birer bölge, ve E4 ile genomun sonu arasında kalan bir bölgedir. Birinci jenerasyon adenoviral vektörlerde, terapötik gene yer açılması ve viral replikasyonun önlenmesi amacıyla E1 bölgesi çıkarılmıştır [75] (Şekil 2.11).

Adenovirüsleri hem *in vitro* hem de *in vivo* gen tedavi çalışmaları için uygun yapan birçok farklı özellikleri vardır. Öncelikle, doğada yaygın olarak bulunurlar. Çok farklı tipte insan hücresini enfekte edebilir, ve diğer mevcut vektörlere oranla daha yüksek seviyelerde gen transferi gerçekleştirebilirler. Bunun yanında, insanlarda patojeniteleri düşüktür; genellikle nezle ile ilgili hafif semptomlara neden olurlar. Ayrıca insanlarda onkojenik etkileri bildirilmemiştir. Adenovirüsler oldukça geniş DNA segmentlerini taşıyabilir ve bölünmeyen hücreleri de transdüksiyona uğratabilirler. Adenoviral vektörlerin bir başka avantajı, genomlarında yeniden düzenlenme oranının düşük olması, ve takılan yabancı genlerin viral replikasyon süresince genellikle değişikliğe uğramadan muhafaza edilmesidir. Ayrıca adenoviral vektör genomlarının, rekombinant DNA teknikleri ile manipülasyonu kolaydır [75].



Şekil 2.10. Adenovirüslerin hayat döngüsü [75]



Şekil 2.11. Adenoviral DNA'nın yapısı Yaklaşık 8 kb'lik yabancı DNA adenoviral vektörlere yerleştirilebilir Protein IX paketlenme için gereklidir, ve vektör üretiminde E1 bölgesine gen yerleştirilmesini kısıtlayıcı bir faktördür

2.7.2. Kanser Gen Tedavisinde Ölüm Ligantlarının Kullanımı

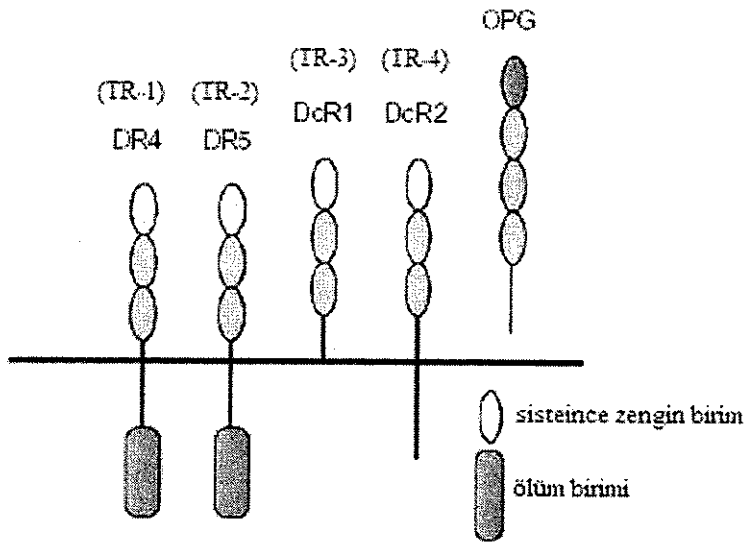
Kanserde ölüm ligantları ile gen tedavisi yaklaşımları, potansiyel tedavi edici stratejiler olarak değerlendirilmektedir [80]. Radyoterapi ve kemoterapiden farklı olarak, ölüm ligantlarının apoptozisi p53'ten bağımsız olarak indükledikleri gösterilmiştir [81]. Apoptozis, kompleks hücre içi yolların aktivasyonu ile gerçekleşen programlı hücre ölümü olarak bilinir [82]. Embriyonik dönemde ve sonrasında, homeostasinin anahtar bir mekanizmasıdır. Programlı hücre ölümünü bloke eden genetik aberasyonlar, tümör oluşumuna ve ilaca dirençliliğe neden olur. Bu nedenle, tümör hücrelerinde apoptozisin spesifik olarak aktivasyonu, prostat kanserinin tedavisinde oldukça etkili olabilir.

TNF (Tumor Necrosis Factor) ailesi üyesi ölüm ligantları kullanılarak prostat kanser hücrelerinde apoptozisin indüklenmesinin amaçlandığı birçok çalışma yapılmıştır. Bu çalışmalar arasında, dışarıdan verilen Fas agonistleri, anti-Fas antikorları, ve membrana bağımlı FasL'ların kullanıldığı çalışmalarda başarı sağlanamamıştır. Öte yandan, antisense oligonükleotitler kullanılarak hücre içi anti-apoptotik c-FLIP molekülünün sentezinin azaltıldığı bir çalışmada, metastatik ileri evre prostat kanser hücre hattı DU145'ın anti-Fas monoklonal antikoruna karşı duyarlı hale geldiği gösterilmiştir [83]. Ancak bu yaklaşımla etkin hücre ölümü sağlanamamıştır. Adenovirüsler aracılığıyla FasL'ın hücre içi sentezinin artırıldığı çalışmalar sonucunda ise, kullanılan birçok farklı insan prostat kanser hücre hattının %70 ila %90'ının apoptozise uğradığı bildirilmiştir [84]. Ayrıca, hücrelerdeki bu yüksek ölüm oranı, kısmen apoptotik cisimcikler içinde ve hücresel artıklar içinde taşınan FasL aracılığıyla gerçekleşen by-stander etkiye bağlanmıştır [85]. Ancak LNCaP gibi bazı ileri evre hücre hatlarının ise, FasL aracılı hücre ölümüne dirençli olduğu gözlenmiştir. Buna rağmen, dirençli hücrelerde dahi, önceden IFNγ uygulamasının ortotropik prostat primer tümörlerini rekombinant adenovirüs aracılı FasL aktarımına duyarlı hale getirdiği gösterilmiştir [86].

FasL [87] ve bunun yanında TNF [88] ile birçok çalışma yapılmış olmasına ve bu moleküllerin kanser hücrelerinde apoptozisi etkin bir şekilde indükledikleri gösterilmiş olmasına rağmen, bu moleküllerin kanser gen tedavisinde sistemik kullanımları, yol açtıkları sistemik toksisite nedeniyle önerilmemektedir. Örneğin hayvanlara rekombinant Fas ligand veya anti-Fas mAb'ın enjeksiyonu, hepatosit dejenerasyonuna, nekroza ve kanamaya yol açmıştır [89, 90]. Fas kaynaklı hasar, yüksek seviyede Fas sentez eden hepatositlerde Fas-bağımlı apoptozisin indüksiyonundan kaynaklanır [87]. TNF ise, 1985 yılından bu yana medikal onkolojide kullanılabilir durumdadır. Ancak TNF'in ileri evre kanser hastalarının tedavisinde sistemik kullanımı, yüksek sistemik toksisiteye neden olmuştur. Yüksek sistemik toksisite, maksimum tolere edilebilir dozun azalmasına, tümörde nadiren gerileme sağlanabilmesine, ve organ bozukluklarına yol açmıştır [91, 92]. TNF infüzyonunun organizmada septik şoka benzer ölümcül inflamatuar cevaba neden olmasının temel nedeni, TNF'in vasküler endotelyal hücrelerde ve makrofajlarda proinflamatuar transkripsiyon faktörü NF-kB'yi aktive etmesidir [93]

TRAIL

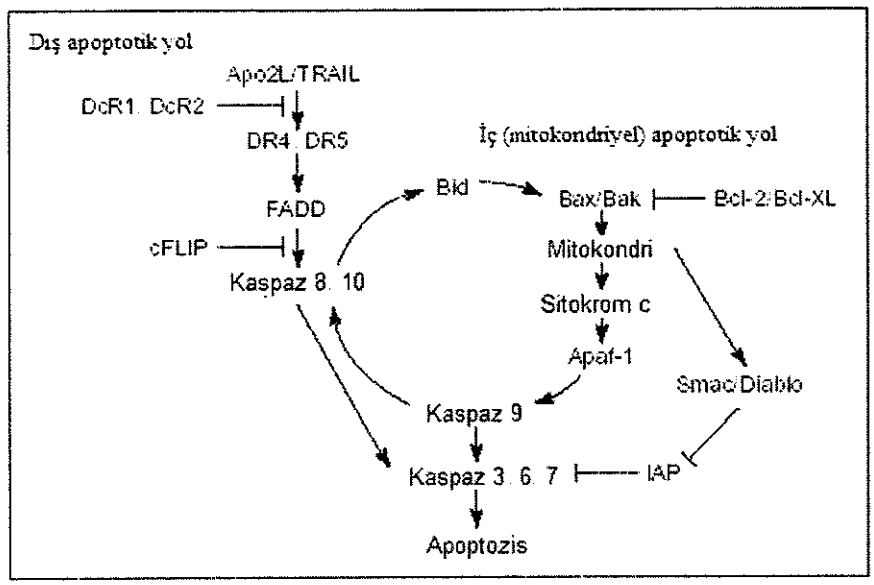
TRAIL, kısa süre önce iki bağımsız grup tarafından tanımlanan bir başka TNF ailesi üyesidir [94, 95]. Amino asit dizisi TNF-α'ya %23, Fas-liganda ise %28 benzerlik gösteren TRAIL'ın keşfi, ölüm ligantlarının kanser gen tedavisinde kullanımında yeni bir devrin başlangıcı olarak kabul edilmektedir [96], çünkü TRAIL'ın tümör hücrelerinde apoptozisi indüklediği, ancak normal hücrelerde apoptotik etki göstermediği bildirilmiştir [97]. TRAIL'ın bağlanabildiği beş farklı reseptör bulunmaktadır; TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4 ve osteoprotegrin [98, 99]. TRAIL-R1 ve TRAIL-R2 ölüm reseptörleri olarak görev yapar. TRAIL bu reseptörlere bağlandığında apoptotik hücre ölümü indüklenir. TRAIL-R3, TRAIL-R4 ve osteoprotegrin ise yalancı reseptör olarak tanımlanır. TRAIL bu reseptörlere bağlanabilmesine rağmen apoptotik hücre yolu indüklenmez [100] (Şekil 2.12).



Şekil 2.12. TRAIL reseptörlerinin şematik gösterimi. Hücre yüzeyindeki sisteince zengin birimler renkli oval şekillerle gösterilmiştir [101]

TRAIL, TR-1 veya TR-2 ölüm reseptörlerine bağlandığında, hücrede dış apoptotik yol aktive olur [102]. TRAIL'ın bağlanması ile TR-1 ve TR-2, ölüm birimi içeren adaptör molekül Fas-Associated Death Domain-containing adaptor molecule (FADD) aracılığıyla, apoptozisi başlatıcı kaspazlar olan kaspaz 8 veya kaspaz 10'u reseptöre çekerek aktive eder. Kaspaz 8 ve kaspaz 10 ise, hücrenin apoptotik ölümünü sağlayan efektör kaspazları, yani kaspaz 3, 6, ve 7'yi aktive eder. Bazı kanser hücre hatlarında, kaspaz 3'ün TRAIL aracılı aktivasyonu, iç apoptotik yolun da indüklenmesi ile artar [103-105]. Bu durumda, kaspaz 8 veya kaspaz 10, bir pro-apoptotik Bcl-2 ailesi üyesi olan Bid molekülünü kırarak aktive eder. Aktive olan Bid molekülü, Bax ve Bak molekülleri ile etkileşime girerek, mitokondriden sitokrom c ve Smac/Diablo'nun salınmasını sağlar. Sitokrom c, Apaf-1 ile birlikte, başlatıcı proteaz kaspaz 9'u aktive eder. Kaspaz 9, kaspaz 3, 6, ve 7'nin aktivasyonunu sağlayan diğer bir kaspazdır. Smac/Diablo, Inhibitor of Apoptosis Protein (IAP) moleküllerine bağlanır. Bu bağlanma, IAP'lerin kaspaz 3'e

bağlanmasını engeller. Dolayısıyla kaspaz 3'ün aktif durumunun devamı sağlanmış olur (Şekil 2.13) [106].



Şekil 2.13. TRAIL tarafından indüklenen iç ve dış apoptotik yollar [106].

TRAIL'ın tümöre karşı fonksiyon gören bir ajan olarak aktivitesi kabul görmüş olsa da, normal fizyolojik şartlardaki fonksiyonu tam olarak açığa çıkarılamamıştır [101]. TRAIL'ın sitotoksik $CD4^+$ T lenfositleri tarafından gerçekleştirilen hedef hücre eliminasyon mekanizmasında rol aldığına dair bulgular mevcuttur. Ayrıca TRAIL'ın natural killer (NK) hücreler ve makrofajlar tarafından tümör hücrelerinin öldürülmesinde de görev aldığı düşünülmektedir [106]. TRAIL knockout farelerle yapılan çalışmalar, TRAIL'ın NK hücreleri tarafından gerçekleştirilen tümöral immun taramada rolü olduğunu onaylamaktadır [107-109]. Ayrıca, kanser hastalarından alınan tümör hücrelerinde makrofajların aktive olduğu, ve aktive olan makrofajların TRAIL sentezini indükledikleri gösterilmiştir. Bu makrofajların ayrıca tümör hücresi yüzeyinde TR-1 ve TR-2 ölüm reseptörlerinin sentezini artırıcı moleküller salgıladıkları bildirilmiştir [110].

TRAIL'ın birçok transforme hücre hattında apoptozisi indüklemesine rağmen, normal dokularda apoptotik etki göstermemesi, sistemik kullanımını gündeme getirmiştir [97]. TRAIL'ın çözülebilir formu hayvan modellerinde test edilmiş ve sistemik kullanım sonucu herhangi bir toksisite görülmemiştir. Ancak, tümör büyümesini engellemek için yüksek miktarlarda çözülebilir TRAIL kullanılması gerekmektedir. Çözülebilir TRAIL proteinine alternatif olarak ise, TRAIL'ı kodlayan replikasyon defektif bir adenoviral vektör üretilmiştir [111]. Adenoviral vektörlerin düşük titrelerinin dahi hTRAIL geni taşıyan çok sayıda enfeksiyöz adenoviral vektör içerdiği, bu nedenle *in vivo* çalışmalarda lokal uygulamasının da avantajlı olacağı bildirilmiştir [112]. Geliştirilen Ad5hTRAIL vektöründe Cytomegalovirus (CMV) promotoru kullanılmıştır. CMV promotoru, güçlü ve sürekli ekspresyon sağlar. Bu promotorun kullanımı, TRAIL proteininin lokal konsantrasyonunu artırmak için ek bir mekanizma görevi görmektedir [112].

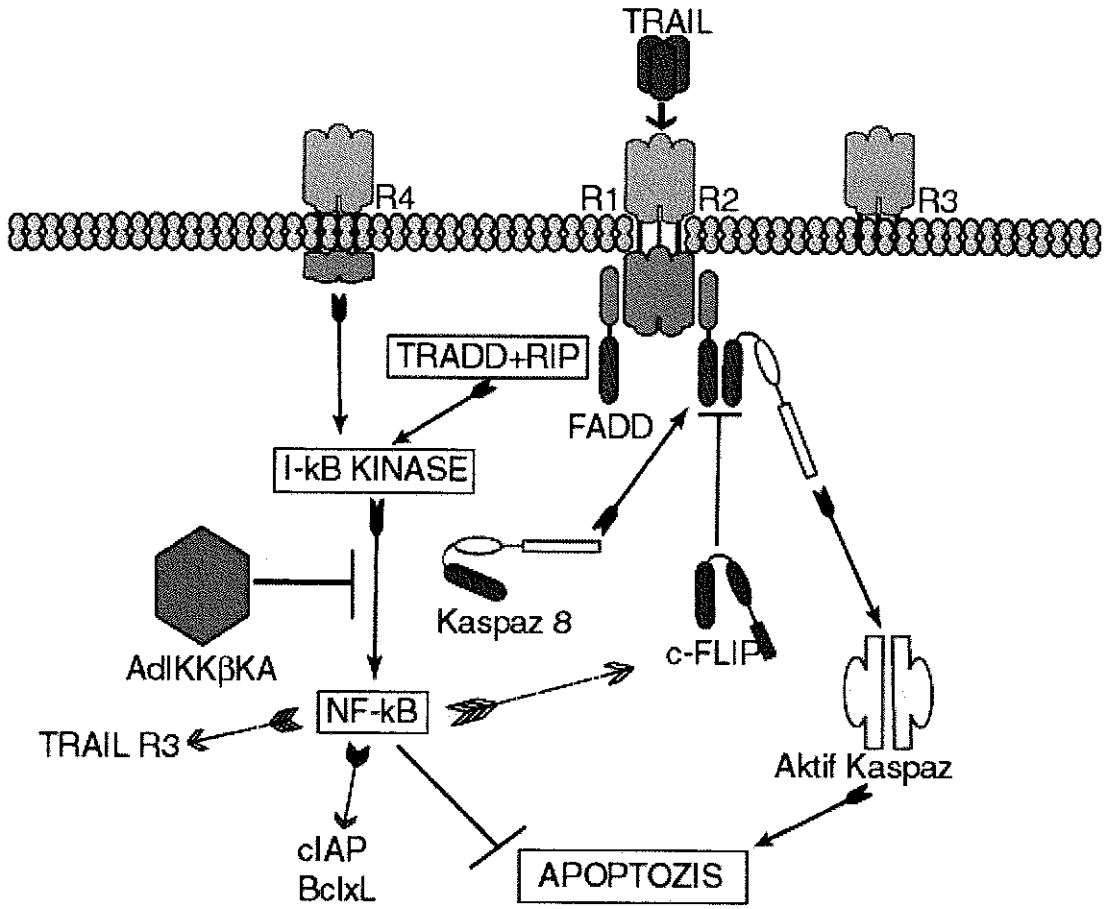
Çözülebilir TRAIL ve Ad5hTRAIL ile çeşitli hücre hatlarında birçok çalışma gerçekleştirilmiştir. Örneğin TRAIL'a duyarlı prostat tümör hücrelerine Ad5hTRAIL enfeksiyonunun, Kaspaz 8 aracılı apoptozisi indüklediği gösterilmiştir [112]. Normal prostat epitel hücreleri ise Ad5hTRAIL uygulaması sonucu apoptotik ölüme gitmemiştir. Bir başka çalışmada, sekiz prostat kanser hücre hattı (CWR22Rv1, DU145, DuPro, JCA1, LNCaP, PC3, PPC1, ve TsuPr1) ve normal prostat epitel hücrelerinin primer kültürlerinde (PrEC), çözülebilir TRAIL'ın etkisi araştırılmıştır [113]. Bu çalışmada 100 ng/ml çözülebilir TRAIL uygulamasının, DU145, DuPro, LNCaP, TsuPr1, ve PrEC hücrelerinde apoptozisi indüklemediği gösterilmiştir. Bunun yanında, kemoterapötik ajan doksorubisin kullanıldığında hemen hemen tüm hücreler TRAIL aracılı apoptozise duyarlı hale gelmiştir. Öte yandan, TRAIL geninin bütününü kodlayan bir adenoviral vektör (AdTRAIL-IRES-GFP), doksorubisin uygulaması olmaksızın prostat kanser hücrelerini ve aynı zamanda beklenmedik bir şekilde PrEC hücrelerini de öldürmüştür [113] Bu çalışma, doku spesifik promotorlarla birlikte kullanıldığında AdTRAIL-IRES-GFP gen tedavi yaklaşımının prostat kanserinin tedavisinde yararlı olacağını göstermiştir. Ancak bazı prostat kanser hücrelerinin TRAIL'a dirençli olduğu gözlenmiştir [114]. Örneğin bir çalışmada, ALVA31, PC3, ve DU145 hücre hatlarının TRAIL aracılı apoptozise yüksek derecede duyarlı olduğu, TSUPr1 ve JCA1 hücre hatlarının ise orta derecede duyarlı olduğu, LNCaP hücrelerinin ise dirençli olduğu bildirilmiştir. [114]. Aktif lipid fosfataz PTEN'in yokluğuna bağlı olarak, LNCaP hücrelerinin sürekli hücre içi Akt aktivitesi gösterdiği bildirilmiştir. Akt, fosfatidilinositol (PI)3 kinaz/Akt hücresel yolunun negatif regülatörüdür. PI3-kinaz inhibitörlerinin, LNCaP prostat kanser hücrelerini TRAIL'a duyarlı hale getirdiği bildirilmiştir. Ayrıca, sürekli olarak aktif Akt kodlayan adenovirüslerle enfeksiyonun, wortmannin ajanının TRAIL aracılı Bid aktivasyonunu güçlendirme yeteneğini engellediği bildirilmiştir. Bu durum, hücre içi sürekli Akt aktivitesinin TRAIL aracılı apoptozisi inhibe ettiğini göstermiştir [114]

TRAIL'ın etkisinin çalışıldığı birçok başarılı *in vitro* çalışmaya dayanarak Walczak ve ark., TRAIL'ın kullanıldığı ilk *in vivo* çalışmayı gerçekleştirmiştir. Çalışmada, MDA-231 meme kanser hücreleri enjekte edilmiş SCID farelere 500 µg insan TRAIL proteini ve 1 mg sıçan TRAIL proteini intravenöz olarak verilmiştir. Deney sonucunda fare canlılığı, doku bütünlüğü ve kan sayımı açısından önemli düzeyde toksisite görülmemiştir. Bunun yanında, enjeksiyondan 9-12 saat sonra tümör taşıyan bölgelerde TRAIL'ın indüklediği apoptotik ölüm açıkça görülebilmiştir [115]. Bir başka çalışmada, maymunlarda tekrarlı TRAIL sistemik uygulamasının, klinik ve histopatolojik testlerde belirlenebilir düzeyde bir değişikliğe yol açmadığı bildirilmiştir [116]

Prostat Kanseri Hücrelerinde Gözlenen TRAIL Dirençliliği

TRAIL mRNA'sının, lenfositler, dalak, prostat, over, kolon, ve plasenta gibi çok çeşitli hücre ve dokularda sürekli olarak sentezlendiği gösterilmiştir [94]. Bu durum, birçok normal hücre tipini özellikle TRAIL aracılı apoptozisten koruyucu fizyolojik mekanizmaların varlığına işaretter. Oysa TNF, lymphotoxin-α, ve FasL gibi diğer TNF ailesi üyelerinin sentezleri, normal hücreler üzerinde toksik etki gösterebileceklerinden dolayı sıkı bir regülasyona tabidir. Normal hücrelerde görülen TRAIL'a dirençlilik mekanizması henüz tam olarak açığa çıkarılamadığı gibi, son

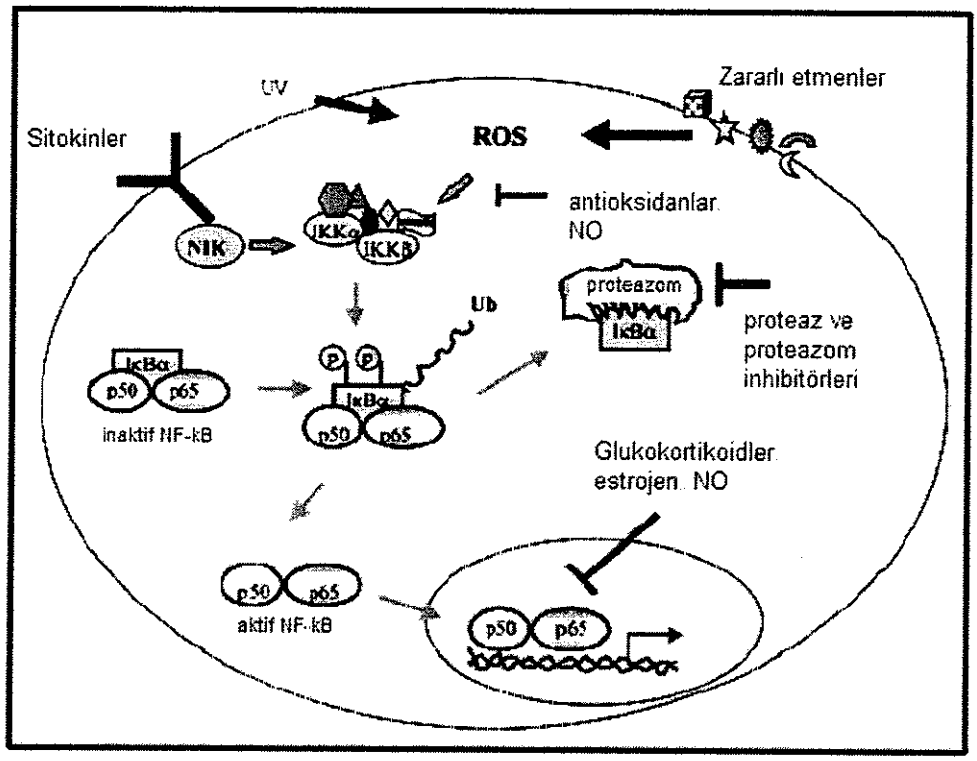
yıllarda yapılan çalışmalar, bazı prostat kanser hücrelerinin TRAIL'a dirençli olduğunu göstermiştir. TRAIL aracılı apoptozisin ve dirençliliğin mekanizması, Şekil 2.14'te gösterilmiştir. Hücrelerde TRAIL'a dirençliliği açıklamak üzere iki farklı hipotez geliştirilmiştir. İlk hipoteze göre, normal hücrelerde ölüm reseptörleri (TRAIL-R1 ve TRAIL-R2) ile rekabete giren yalancı reseptörler (TRAIL-R3 ve TRAIL-R4) bulunur, ve bu reseptörler ölüm reseptörlerine bağlanabilecek TRAIL ligantlarının sayısını düşürerek veya hücre içi anti-apoptotik yolları aktive ederek (TRAIL-R4) TRAIL'a dirençlilik sağlar [117, 118]. TRAIL-R4 bağlanması, anti-apoptotik NF-kB sinyal yolunu aktive eder [119]. Ayrıca, kanser hücrelerinde yalancı reseptörlerin sentezinin promotor hipermetilasyonu sonucu azaldığı gösterilmiştir [120]. Bu durum, kanser hücrelerinde TRAIL'a farklı duyarlılıklara sebep olur. Ancak TRAIL dirençliliği ile yalancı reseptör sentezleri arasında kesin bir ilişki kurulamamıştır [97]. Ayrıca, TRAIL-R1 ve TRAIL-R2 gibi ölüm reseptörlerinin de NF-kB yolunu stimüle edebildiği gösterilmiştir [121, 122]. Bu bulgular ışığında, anti-apoptotik yolların indüksiyonuna rağmen hücrelerin apoptozise gitme nedeni tam olarak anlaşılamamıştır. İkinci hipotez, TRAIL'a dirençli hücrelerde apoptozisi inhibe edici moleküllerin sentezinin arttığını savunmaktadır. Bu hipotez ile ilişkili olarak, bir kaspaz 8 homologu olan c-FLIP'in (cellular **FLICE** Inhibitory **P**rotein) TRAIL ölüm ligantları aracılığıyla gerçekleşen apoptozisi engellediği gösterilmiştir [123]. Ayrıca NF-kB aktive edici ajanların c-FLIP sentezini artırdığı gösterilmiştir [124]. Bunun yanında, NF-kB aktivasyonundaki artışın, TRAIL-R3'ün ve apoptozisi inhibe edici bir protein olan Bcl-xL'in sentezini artırdığı bildirilmiştir [125, 126]. NF-kB yollarının, aynı zamanda cIAP gibi apoptozis inhibitör proteinlerinin sentezini de artırıcı etki gösterdiği bilinmektedir [127]. Bu bulgular ışığında, hücre içi aktif NF-kB sinyal yolunun en az dört farklı yolla hücreleri TRAIL'a dirençli kılabileceği görülmektedir (Şekil 2.14).



Şekil 2.14. Prostat kanser hücrelerinde NF-kB sinyal yolunun bloke edilmesi yoluyla TRAIL'a dirençliliği kırmak için geliştirilmiş bir gen tedavi stratejisi [128].

TRAIL dirençliliğinde rol oynadığı düşünülen NF-kB transkripsiyon faktörleri, Rel protein ailesi üyesi moleküllerden oluşan dimerlerdir. Bu proteinler Rel homoloji birimleri ile karakterizedir. Rel homoloji birimleri, DNA'ya bağlanmada, dimerizasyonda ve IkB gibi inhibitör proteinlerle etkileşimlerde görev alır. IkB proteinleri, NF-kB ile fiziksel etkileşime girererek NF-kB'nin nükleer lokalizasyon sinyallerini bloke eder ve NF-kB proteinlerini sitoplazmada tutar. Bu sayede, NF-kB'nin nukleusta geçici ve kalıcı seviyelerini düzenler (Şekil 2.15) [129-131]. Birçok hücre tipinde, hücre uygun uyarıyı alana dek NF-kB kompleksleri çoğunlukla sitoplazmada tutulur ve transkripsiyonel olarak inaktiftir. IkB proteinleri, TNF gibi inflamasyon indükleyici sitokinlerin veya bakteriyel lipopolisakkaritlerin etkisiyle, N terminal bölgelerinde bulunan iki serin biriminden fosforillenir [129-131]. IkB proteinlerinin fosforilasyonu, hızlı bir şekilde ubiquitinlenmelerine ve sonrasında 26S proteazomlar tarafından parçalanmalarına yol açar. IkB proteinlerinin proteazom bağımlı parçalanması sonucu, NF-kB serbest kalır ve spesifik hedef genlerin sentezini aktive etmek üzere hücre çekirdeğine göç eder [129-131]. IkB'nin fosforilasyonunda, IkB kinaz (IKK) molekülleri görev alır. Üç alt üniteden oluşan bir kompleks şeklinde fonksiyon gören IKK kompleksinde, kinaz aktivitesinin IKKα ve IKKβ'dan oluşan heterodimer tarafından gerçekleştirildiği gösterilmiştir. IKKα ve IKKβ'ya ek olarak, IKK kompleksi ile ilişkilendirilen bir başka protein, IKKγ'dır.

IKKγ'nın, IKK kompleksinin ilgili sinyal yolları ile bağlantısını kuran bir yapısal protein olduğu düşünülmektedir [131-133].



Şekil 2.15. NF-kB aktivasyon ve inhibisyon yolları [134]

Prostat Kanser Hücrelerinde TRAIL Dirençliliğini Kırma Yöntemleri

Kanser hücrelerinde TRAIL dirençliliğini kırmaya yönelik olarak, farklı stratejilerin kullanıldığı birçok çalışma gerçekleştirilmiştir. Örneğin, toksik seviyelerin altında kemoterapötik ajanların kullanımının, *in vitro* ve *in vivo* TRAIL etkinliğini artırdığı bildirilmiştir [103, 135-138]. Bir kemoterapötik ajan olan aktinomisin D'nin (Act D) BclXL anti-apoptotik proteininin sentezini azaltarak Kaposi Sarkoma hücrelerini TRAIL aracılı apoptozise duyarlı hale getirdiği gösterilmiştir [139]. Yine TRAIL ve Act D'nin birlikte kullanıldığı yaklaşımlarla, TRAIL'a dirençli prostat kanser hücrelerinde TRAIL'a duyarlılık sağlanmıştır [140, 141]. Bu hücrelerde Act D'nin XIAP anti-apoptotik molekülünün sentezini azalttığı gösterilmiştir. Bir başka kemoterapötik ajan olan etopozitin, TR-1 ve TR-2 ölüm reseptörlerinin sentezini artırarak meme kanseri hücrelerinde TRAIL aracılı apoptozisin etkinliğini önemli derecede artırdığı gösterilmiştir [142]. TRAIL'ın kanser hücrelerindeki etkinliğini artırmak için kemoterapötik ajanların yanında başka yaklaşımlar da denenmiştir. Örneğin, TRAIL'ın etkinliğinin, IFN-γ [143], γ-radyasyon [144, 145], ve siklooksigenaz inhibitörleri [146] ile birlikte kullanıldığında artış gösterdiği bildirilmiştir. Bu yaklaşımların, genel olarak TRAIL ölüm reseptörlerinin ve kaspazların sentezini artırarak, ve IAP ailesi üyesi moleküllerin veya anti-apoptotik c-FLIP molekülünün inhibisyonunu sağlayarak fonksiyon gördüğü gösterilmiştir [106].

Kanser hücrelerinde TRAIL dirençliliğine sebep olan, ve diğer birçok dirençlilik mekanizmasını da tetiklediği bilinen hücre içi NF-kB aktivitesini engelleyecek yaklaşımların da TRAIL'ın etkinliğini artıracağı ileri sürülmüştür [147]. NF-kB'yi stimüle edici ajanların TRAIL-R3 yalancı reseptörünün [148], bunun yanında Bcl-xL [126], cIAP [127], ve cFLIP [124] gibi apoptozis inhibitörlerinin sentezlerini artırdığı bildirilmiştir. Ayrıca, TRAIL-R4 reseptörünün NF-kB sinyal yolunu indükleyerek TRAIL aracılı hücre ölümünü engellediği bildirilmiştir [119]. Ayrıca TRAIL'ın, ölüm reseptörleri (TRAIL-R1 and TRAIL-R2) aracılığıyla da NF-kB yolunu aktive ettiği gösterilmiştir [121, 122] Tüm bu bulgular, NF-kB'yi inhibe edici yaklaşımların TRAIL'a dirençli prostat kanser hücrelerinde TRAIL duyarlılığı sağlamada oldukça etkin olabileceğini göstermektedir. Prostat kanser hücrelerinde, NF-kB'nin regülatörü olan IKK molekülünün yüksek aktivitesinin yüksek NF-kB aktivitesine yol açtığı bilinmektedir [149]. Dolayısıyla, etkin gen transferi sağlayan adenoviral vektörler aracılığıyla özellikle IKK'in katalitik alt ünitelerini inhibe edici genlerin aktarımı, TRAIL dirençliliğini kırmada oldukça etkili olabilecek bir stratejidir (Şekil 2.12). Bu stratejinin, daha önce akciğer kanser hücrelerinde TNF aracılı apoptozise direnci kırmadaki etkisi araştırılmış, ve IKKβ'nın dominant negatif mutantını kodlayan AdIKKβKA adenoviral vektörünün, akciğer hücrelerini başarılı bir şekilde TNF'e duyarlı hale getirdiği gösterilmiştir [150, 151]. Bizim çalışmamızda, AdIKKβKA'nın TRAIL'a dirençli prostat kanser hücrelerini TRAIL'a duyarlı hale getirme potansiyelini araştırıldı.

MATERYAL ve METODLAR

3.1. Hücre Kültürü

Çalışmada kullanılan DU145, PC3 ve LNCaP ve 293 hücre hatları, American Type Tissue Culture Collection'dan (ATCC) temin edildi. DU145, PC3 ve LNCaP, insan ileri evre prostat kanser hücre hatlarıdır. Hücrelerin özellikleri Tablo 3.1'de verilmiştir. 293 hücreleri, insan embriyonik böbrek hücre hattıdır. 293 hücrelerinin genomuna, replikasyon defektif adenoviral vektörlerin üretilebilmesi amacıyla, adenoviral replikasyon için gerekli olan E1A geni kalıcı olarak eklenmiştir.

Tablo 3.1. Çalışmada kullanılan prostat kanser hücrelerinin özellikleri (ATCC).

Hücre Hattı	**Kökeni**	**Androjen Bağımlılığı**
DU145	İnsan prostat karsinoma, beyin metastazı	(-)
PC3	İnsan prostat adenokarsinoma, 4. derece	(-)
LNCaP	İnsan prostat adenokarsinoma, lenf nodu metastazı	(+)

Kullanılan Solusyonlar:

Tripsin-EDTA (Biological Industries)

RPMI-1640 Besiyeri:

RPMI-1640 Toz Besiyeri (Sigma)
%10 Fetal Bovine Serum (FBS) (Biochrom KG)
2.2 g/l Sodyum Bikarbonat (Sigma)
1 mM L-glutamin (Biological Industries)
%1 penisilin-streptomisin (Biological Industries)

Besiyerinin hazırlanması için, toz RPMI-1640 besiyeri 900 ml bidistile suda çözüldü. Daha sonra çözülmüş besiyeri üzerine 63 ml daha bidistile su eklendi. Besiyerinin pH'sı HCl ile pH4'e ayarlandı ve 121°C'de 15 dakika otoklavlandı. Otoklavlama sonrası besiyeri soğuduktan sonra üzerine 26.7 ml Na Bikarbonat, 10,25 ml L-Glutamin, 11 ml Penisilin-Streptomisin, ve 110 ml FBS eklendi. Hazırlanan besiyeri +4°C'de saklandı.

Hücreler, 100 mm hücre kültürü kaplarında (Sarstedt) RPMI-1640 besiyeri içinde, 37°C sıcaklık ve %5 CO_2'li nemli ortamda üretildi.

3.2. Rekombinant Adenoviral Vektörlerin Üretimi

Kullanılan Solusyonlar:

RPMI-1640:
3.1'de tanımlandığı gibi hazırlandı.

1 M Tris-Cl (Amresco):
24,2 g Tris 160 ml distile H_2O'da (dH_2O) çözüldü. Çözelti dH_2O ile 200 ml'ye tamamlandı. Solusyonun pH'sı HCl ile pH 8'e ayarlandı. Otoklavlanarak steril hale getirildi ve +4°C'de saklandı.

10 mM Tris-Cl (Amresco):
1 M Tris-Cl'den 7,5 ml alınarak solusyon dH_2O ile 750 ml'ye tamamlandı. Otoklavlanarak steril hale getirildi ve +4°C'de saklandı.

Yoğun CsCl Solusyonu (Y-CsCl) (Gibco BRL):
42,3 g CsCl, 57,77 ml 10 mM Tris-Cl içinde çözüldü. Solusyonun pH'sı pH 8'e ayarlandı. Otoklavlanarak steril hale getirildi ve +4°C'de saklandı.

Hafif CsCl Solusyonu (H-CsCl) (Gibco BRL):
22,39 g CsCl, 77,61 ml 10 mM Tris-Cl içinde çözüldü. Solusyonun pH'sı, pH 8'e ayarlandı. Otoklavlanarak steril hale getirildi ve +4°C'de saklandı

Birinci generasyon adenoviral vektörlerde, adenoviral proliferasyonu sağlayan E1A geni çıkarılmıştır. Bu nedenle, adenoviral vektörlerin üretimi için, genomlarına replikasyon için gerekli viral E1A geni kalıcı olarak eklenmiş olan 293 sayılı insan embriyonik böbrek hücre hattı kullanıldı

3.2.1. İnsan 293 Hücrelerinin Enfeksiyonu

İnsan 293 hücreleri, RPMI-1640 besiyerinde toplam 40 adet 150 mm'lik doku kültürü kabını kaplayacak şekilde üretildi, ve hücreler %80 yoğunluğa ulaştığında serumsuz besiyeri ortamında virüs enfeksiyonu gerçekleştirildi. Enfeksiyondan iki saat sonra hücrelere mevcut besiyeri miktarı kadar (10 ml) %20 FBS içeren besiyeri eklenerek, besiyeri içindeki serum konsantrasyonu %10'a çıkarıldı. Hücrelerde sitopatik etki belirgin hale geldiğinde (enfeksiyondan yaklaşık 30 saat sonra), adenoviral vektörleri içeren hücreler toplandı ve 250 ml'lik steril tüplere aktarıldı. Hücreler 4^0C'de 4000 rpm'de 10 dakika santrifuj edildi. Santrifüj sonrası dökelti atıldı ve hücreler 10 mM Tris-Cl içinde, her 150 mm'lik kap için 0,5 ml olacak şekilde çözüldü.

3.2.2. Adenovirüs Pürifikasyonu

Adenovirüsleri hücrelerden çıkarabilmek için örnekler 3 kez etil alkol-kuru buz aşamasından geçirilip hızlı donma-çözünme işlemine tabi tutuldu. Lizatlar 3000 rpm'de 10 dakika santifüj edildi ve dökeltiler toplandı. Ultrasantrifüj tüplerine 10 ml

Y-CsCl koyuldu ve üzerine 10 ml H-CsCl eklendi. En üste viral dökelti konuldu ve örnekler Beckman SW28 rotoru ile 20,000 rpm'de, 4^0C'de, 12 saat santifüj edildi. Viral bantlar bir tüpe şırınga yardımı ile toplandı ve miktarı, 10 mM'lık Tris-Cl, pH 8.1 ile 4 ml'ye çıkarıldı. Temiz bir ultrasantrifüj tüpüne 4ml Y-CsCl ve 4 ml H-CsCl konup üzerine sulandırılmış virüs bandı yüklendi. Bu tüpler SW41 rotoruna yüklenerek 20,000 rpm'de 4°C'de, 4 saat santrifüj edildi. Santifüj sonrası viral bantlar izole edildi ve konsantrasyonları spektrofotometrik A_{260} okuma ile belirlendi. Viral vektörlerin ortalama titreleri, 10^{13} DNA partikülü/ml idi. Fonksiyonel titreler ise 293 hücrelerinde plak titreleme yoluyla ölçüldü. Partikül/plak oluşturan birim oranı tipik olarak 50'ye eşitti. Ad5hTRAIL [112], AdIKKβKA [151], AdEGFP [150], AdCMVLacZ [152] ve AdNFkBLuc [151] adenoviral stokları, yukarıda tanımlandığı şekilde hazırlandı ve 10mM Tris, %20 gliserol içinde -80°C'de saklandı.

3.3. Prostat Kanser Hücrelerinin Rekombinant Adenoviral Vektörler ile Transdüksiyonu

Çalışmada kullanılan adenoviral vektörlerin özellikleri Tablo 3.2.'de verilmiştir.

Tablo 3.2. Çalışmada kullanılan adenoviral vektörlerin özellikleri

Adenoviral Vektör	Özelliği
Ad5hTRAIL	İnsan TRAIL genini kodlayan vektör
AdIKKβKA	IKKβ'nın dominant negatif mutantını kodlayan vektör
AdEGFP	Yeşil flöresan veren 'Enhanced Green Fluorescence Protein' kodlayan vektör
AdCMVLacZ	Beta galaktosidaz geni taşıyan, kontrol amaçlı kullanılan adenoviral vektör
AdLucNFkB	NF-kB aracılı transkripsiyonel indüksiyonu ölçmek için lusiferaz belirteç genini kodlayan vektör

Kullanılan Solusyonlar:

Tripsin-EDTA (Biological Industries)
Fötal Bovine Serumlu (FBS) ve FBS'siz RPMI-1640 besiyeri:

3.1'de tanımlandığı gibi, %10 serumlu, %20 serumlu, ve serumsuz olarak hazırlandı.

Prostat kanser hücre hatları DU145, PC3, ve LNCaP, RPMI 1640 besiyerinde üretildi. Hücreler, Enhanced Green Fluorescent Protein (EGFP) reporter geni (AdEGFP) sentez eden adenoviral vektörlerin artan konsantrasyonları ile (0, 100, 500, 1000, 5000, ve 10000 MOI), 37°C'de FBS'siz RPMI 1640 besi ortamı içinde enfekte edildi. Doku kültürü besi ortamındaki serum konsantrasyonu, enfeksiyondan iki saat sonra %20 FBS konularak %10'a çıkarıldı. Transdüksiyon seviyesi, enfeksiyondan 48 saat sonra flöresan mikroskop altında ve sonrasında akış sitometrisi ile EGFP(+) hücrelerin oranına bakılarak belirlendi. Prostat kanser

hücrelerinde hTRAIL proteininin sentezini artırmak için Ad5hTRAIL vektörü kullanıldı. IKK aktivitesini ve bu yolla NF-kB aktivasyonunu bloke etmek için, kinaz inaktif IKKβ kodlayan adenoviral vektörlerden (AdIKKβKA), tek başına ve Ad5hTRAIL ile birlikte enfeksiyon deneylerinde yararlanıldı. Dominant negatif IKKβ (IKKβKA) sentezi, diğer IKK alt üniteleri ile etkileşime girerek fonksiyonel olarak inaktif IKK kompleksleri oluşturur. Transkripsiyon aktivasyon deneyleri, lusiferaz reporter genine takılı NF-kB regülatör bölgelerini taşıyan AdNFkBLuc vektörleri aracılığıyla gerçekleştirildi. Deneylerde kontrol amaçlı olarak hücreler AdCMVLacZ virüsü ile enfekte edildi.

3.4. NF-kB Transkripsiyon Aktivasyon Deneyleri

Kullanılan Kit:

Luciferase Assay System with Reporter Lysis Buffer (Promega)

Kullanılan Solusyonlar:

Radioimmunoprecipitation (RIPA) Tamponu:

100 ml 1XPBS
1 ml NP-40 (%1)
1 ml %10 SDS (%0.01)

Prostat kanser hücrelerinin NF-kB aktivasyon durumlarını belirlemek için AdNFkBLuc vektörleri kullanıldı. AdNFkBLuc vektöründe, HSV virüsüne ait timidin kinaz geninin TATA-benzeri promotoru ve bu promotora bitişik olarak, 4 kopya ortak NF-kB bağlanma dizisini bulunmaktadır. Bu promotor, bir ateş böceği (*Photinus pyralis*) lusiferaz belirteç geninin sentezini sağlamaktadır. Endojen NF-kB proteinlerinin kappa enhancer birimine bağlanması ile transkripsiyon indüklenir. Deneysel aşamalarda ilk olarak, 35 mm'lik doku kültürü kaplarında (Corning) üretilen hücreler 5000 MOI AdNFkBLuc virüsü ile enfekte edildi. Viral enfeksiyondan 24 saat sonra, RIPA tamponu kullanılarak hücre lizatları hazırlandı. Bunun için, hücreler önce 1XPBS ile yıkandı. Üzerlerine 0,5 ml RIPA tamponu eklendi. Doku kültürü kabı, +4°C'de 15 dakika sallanarak inkübe edildi. Kalkan hücreler 1,5 ml'lik mikro santrifüj tüpüne aktarıldı ve 45 dakika buzda tutuldu. Daha sonra 15000 rpm'de 10 dakika santrifüj edildi. Bu işlem sonunda toplam hücre lizatını içeren dökelti, temiz bir tüpe aktarıldı. Hücre lizatlarındaki protein konsantrasyonları, kolorimetrik Bradford Deneyi ile belirlendi [153]. Lusiferaz deney sistemi kullanılarak, bu lizatlarda TRAIL varlığında ve yokluğunda NF-kB aracılı transkripsiyonel indüksiyon ölçüldü. Ölçümlerde Perkin-Elmer LSB50 luminometre cihazından yararlanıldı. Analiz aşamasında standart eğri oluşturulmasında BSA protein miktarları kullanıldı. Lusiferaz aktivitesine yönelik tüm ölçümler (RLU, Relative Light Unit = Rölatif Işık Ünitesi), protein konsantrasyonlarına normalize edildi.

3.5. Hücre Canlılık Oranlarının Belirlenmesi

Kullanılan Kit:

Cellular Viability and Cytotoxicity Kit (Eugene, OR)

Farklı işlemler sonrasında ölü hücrelerin canlı hücrelerden ayırt edilebilmesi için hücre canlılık deneyi uygulandı. Bu deneyde, hücre içi kalsein esteraz için bir florojenik substrat olan Calsein AM, yalnızca canlı hücrelerde, yeşil flöresan veren bir bileşiğe (kalsein) dönüşür. Sadece membran bütünlüğü bozulmamış olan canlı hücreler aktif esteraz içerdiğinden, kalseinin florometrik metodlarla belirlenmesi canlı hücreler için bir belirteç görevi görür. Etidium homodimer-1 (EthD-1), bütünlüğü bozulmamış hücre membranlarından geçemeyen bir kırmızı flöresan nükleik asit boyasıdır. Membran bütünlüğü bozulmamış hücreler EthD-1'i dışarı verirken, hasarlı membranı olan hücreler boyayı alır ve pozitif boyanır. Deneyde, hücreler PBS içinde EthD-1 ve Calsein AM solusyonları ile 50 dakika inkübasyona bırakıldı ve flöresan mikroskop altında canlı/ölü hücre oranına bakıldı.

3.6. İnsan TRAIL Reseptörleri için Kantitatif Gerçek Zamanlı RT-PCR

3.6.1. Total RNA İzolasyonu

Kullanılan Solusyonlar:

TRIzol Reagent (Life Technologies, Gaithersburg, MD)
Kloroform (Sigma)
İzopropanol (Sigma)
%75 Etanol
steril dH_2O

100 mm çaplı doku kültürü kaplarında yaklaşık 5-6 milyon hücre olacak şekilde üretilen prostat kanser hücreleri üzerine, 1 ml TRIzol Reagent eklendi. TRIzol ajanının eklenmesinden sonra kendiliğinden doku kültürü kabının yüzeyinden kalkan hücreler, lizat halinde kültür kaplarından toplandı. Toplanan hücreler homojen hale getirilip oda ısısında 5 dakika bekletildi ve üzerine 0,2 ml kloroform eklendi. Örnekler 13,000 rpm, 4°C'de 15 dakika santrifüj edildikten sonra üç farklı faza ayrıldı. En üstteki RNA fazı temiz bir tüpe aktarıldı, ve üzerine 0,5 ml izopropanol eklendi. On dakika oda ısısında inkübasyon sonrasında, 13,000 rpm, 4°C'de 10 dakika santrifüj edildi. Dökelti uzaklaştırıldı ve 1 ml %75 etanol eklendikten sonra tekrar 13,000 rpm, 4°C'de 5 dakika santrifüj edildi. Oluşan RNA çökeltisi 10 dakika oda ısısında kurutuldu. Çökeltiye uygun miktarda steril dH_2O eklendi ve çözülmesi için 15 dakika 56°C su banyosunda tutuldu. Spektrofotometrede OD_{260}/OD_{280} değeri ölçüldü ve konsantrasyonu belirlendi.

3.6.2. cDNA Eldesi

Kullanılan Kit:

TaqMan Reverse Transcription Reagents (Applied Biosystems)

Kit içeriği

10X Reverse Transkripsiyon Tamponu, $MgCl_2$ (25 mM), 10X Random Hexamer (50 mM, 5nmol), Multiscribe Reverse Transkriptaz (50 U/µl), RNaz İnhibitörü, nükleaz içermeyen dH_2O

cDNA reaksiyonu:

10X Reverse Transkripsiyon Tamponu	5 µl
$MgCl_2$	11 µl
dNTP'ler	2.5 µl
10X Random Hexamer	2.5 µl
RNaz İnhibitorü	3 µl
MultiScribe Reverse Transkriptaz	1.25 µl
Nükleaz içermeyen dH_2O	14.75 µl
RNA	2 µg

Yukarıda belirtilen oranlarda karıştırılan içerikler, Applied Biosystems marka Gerçek Zamanlı RT-PCR aletinde 25°C'de 10 dakika, ve 48°C'de 60 dakika RT-PCR reaksiyonuna tabi tutuldu.

3.6.3. TaqMan PCR Reaksiyonu

Kullanılan Solusyonlar:

Universal Master Mix (UMM) (Applied Biosystems)

TRAILR1 ve TRAILR2 ölüm reseptörleri için Gerçek Zamanlı Kantitatif RT-PCR primer ve problarının tasarımı, bir intron içerecek şekilde, daha önceden yayımlanmış TRAIL sekanslarına bakılarak (Human Genome Project BAC clone RP11-1149023 ve RPRP11-875011) yapılmıştı [154]. Ancak TRAIL-R3 ve TRAIL-R4 yalancı reseptörlerinin Gerçek Zamanlı Kantitatif PCR için primer ve prob dizileri henüz yayınlanmadığından, bu reseptörlere ait primer ve problar da yukarıda belirtildiği şekilde tasarlandı. TRAIL reseptörlerine ait tüm primer ve probların dizileri, aşağıda verilmiştir. Reaksiyonda iç kontrol olarak, ikinci bir flöresan boya bağlı olan rRNA primer ve probları kullanıldı. Böylece TRAIL reseptörlerinin ve rRNA'nın aynı reaksiyon içinde analizi sağlandı. TaqMan PCR reaksiyonunda, 250 ng cDNA kalıbı, 12,5 µl 2XUMM, ve 0,5'er µl primer ve prob kullanılarak hem TRAIL reseptörlerinin hem de rRNA'nın mesaj miktarı aynı anda belirlendi. rRNA primer ve probları PE Applied Biosystems firmasından satın alındı.

TRAIL reseptörlerine ait primer ve prob dizileri:

TRAILR1-5' TGT-ACG-CCC-TGG-AGT-GAC-AT,
TRAILR1-3' CAC-CAA-CAG-CAA-CGG-AAC-AA,
TRAIL R1 Probe: 5'-6FAM-TGTCCACAAAGAATCAGGCAATGGACATAAT-TAMRA-3';
TRAILR2-5' CAC-TCA-CTG-GAA-TGA-CCT-CCT-TT,
TRAILR2-3' GTG-CAG-GGA-CTT-AGC-TCC-ACT-T,
TRAIL R2 Probe: 5'-6FAM-TCACACCTGGTGCAGCGCAAGCAG-TAMRA-3';
TRAILR3-5' CCC-TAA-AGT-TCG-TCGTCG-TCA-T,
TRAILR3-3' GGG-CAG-TGG-TGG-CAG-AGT-A,
TRAIL R3 Probe: 5'6FAM-TCGCGGTCCTGCTGCCAGTCCTAGC-TAMRA 3';
TRAILR4-5' ACA-GAG-GCG-CAG-CCT-CAA,
TRAILR4-3' ACG-GGT-TAC-AGG-CTCCAG-TAT-ATT,
TRAIL R4 Probe: 5' 6FAMAGGAGGAGTGTCCAGCAGGATCTCATAGATC-TAMRA 3'

TaqMan PCR reaksiyonu, yukarıda belirtilen primer ve problar kullanılarak, aşağıda belirtilen şartlarda Applied Biosystems marka Gerçek Zamanlı RT-PCR cihazı ile gerçekleştirildi:

1) 50°C'de 2 dakika (1 döngü)
2) 95°C'de 10 dakika (1 döngü)
3) 95°C'de 15 saniye + 60°C 1 dakika (40 döngü)

Analiz aşamasında kullanılacak standart eğrinin oluşturulması için, ribozomal RNA'dan türeyen klonlanmış cDNA fragmenti kullanıldı. TRAIL reseptörlerinin oranları, ΔΔCt metodu kullanılarak hesaplandı.

3.7. Anneksin V Boyama

Kullanılan Solusyonlar:

1M NaCl (Riedel-de Haen) :

5,844 g NaCl tartılarak, steril dH_2O ile 100 ml'ye tamamlandı. +4°C'de saklandı.

1M $CaCl_2$ (Sigma):

14,702 g $CaCl_2$ tartılarak, steril dH_2O ile 100 ml'ye tamamlandı. +4°C'de saklandı.

HEPES (Amresco):

26,030 g HEPES tartılarak, steril dH_2O ile 100 ml'ye tamamlandı. +4°C'de saklandı.

Anneksin V Bağlanma Tamponu:

Stok solusyonlardan 140 mM NaCl, 2,5 mM $CaCl_2$, ve 10 mM HEPES, 100 ml steril dH_2O içinde karıştırılarak hazırlandı. +4°C'de saklandı.

Propidium Iodide (PI) (Sigma):

1 mg/ml stok solusyondan 20 µl alınıp 980 µl 1XPBS içinde çözülerek son konsantrasyonu 20 µg/ml olacak şekilde hazırlandı +4°C'de saklandı.

Apoptozis olarak bilinen programlı hücre ölümü, bir dizi hücresel morfolojik değişiklik ile karakterizedir. Apoptozisin en erken işaretlerinden biri, plazma membranında meydana gelen değişikliklerdir. Apoptozis süreci sırasında, bir membran fosfolipidi olan fosfatidilserin (FS), plazma membranının iç kısmından dış kısmına dönerek dış ortama sunulur. Anneksin V, Ca^{2+} bağımlı bir fosfolipid bağlayıcı proteindir (35-36 kDa). FS için yüksek afinitesi olması nedeniyle, hücre yüzeyinde FS sunulan erken apoptotik hücrelere bağlanır. FS translokasyonu apoptozisin erken evrelerinde gerçekleştiğinden, Anneksin V boyama, DNA fragmentasyonu gibi daha geç evrede gerçekleşen değişikliklere dayalı olan deneylere göre apoptozisi daha erken bir evrede belirleyebilir. Çalışmamızda, insan Anneksin V proteinine karşı FITC takılı fare monoklonal antikoru (Alexis Biochemicals) kullanıldı. Ölü hücrelerin erken apoptotik hücrelerden ayırt edilebilmesi için, sadece ölü hücreleri boyayan PI boyası kullanıldı. Anneksin V bağlanması ve PI boyama, akış sitometrisi ile izlendi ve apoptotik hücrelerin oranları yazılım programı yardımıyla belirlendi.

Bunun için, daha önce uygun vektörlerle enfekte edilmiş hücreler tripsinizasyon ile kaldırılıp 14 ml'lik Falkon tüplerine toplandı ve 1XPBS ile yıkandı. 1600 rpm'de

5 dakika santrifüj gerçekleştirildi. Çökeltiler üzerine 195 µl Anneksin V bağlanma tamponu ve 5 µl Anneksin V-FITC monoklonal antikoru kondu. 15 dakika karanlıkta inkübasyon sonrası yine 1600 rpm'de 5 dakika santrifüj edildi ve çökelti 190 µl anneksin V bağlanma tamponunda çözüldü. Akış sitometrisi ile analiz öncesi 20 µg/ml PI boyası eklendi ve analiz gerçekleştirildi.

3.8. Akış Sitometrisi

Kullanılan Solusyonlar:

1X Akış Sitometri Tamponu:

0,18 g Na Azid (Sigma), 0,5 g Bovine Serum Albumin (BSA) (Sigma), 0,18 g Na Bikarbonat (Sigma) tartılarak 500 ml 1XPBS'te çözüldü. +4°C'de saklandı.

İleri evre prostat kanseri hücreleri, 24 kuyucuklu doku kültürü kaplarına, ortalama $2,5x10^5$ hücre/kuyucuk olacak şekilde ekildi. Bu hücreler ertesi gün AdEGFP reporter vektörü ile enfekte edildi. Enfeksiyondan 48 saat sonra hücreler tripsinize edildi ve santrifügasyon sonrası 1XPBS'te sulandırıldı. BD FACSCALIBUR cihazı ile akış sitometrisi analizi gerçekleştirildi. Yüzeydeki TRAIL reseptör proteinlerinin sentezini belirlemek için, her bir TRAIL reseptör alt tipi için spesifik olan, işaretlenmemiş monoklonal antikorları içeren anti-TRAIL reseptör akış sitometrisi seti (Cat. ALX-850-273-KI01) kullanıldı. Setin içinde, 100'er µg TRAIL-R1 (clone HS101, Cat. 804-297A), -R2 (clone HS201, Cat.804-298A), -R3 (clone HS301, Cat. 804-344A) ve -R4 (clone HS402, Cat. 804-299A) bulunuyordu. Saflaştırılmış fare IgG1 (MOPC 31C, Cat. ANC-278-010), izotip kontrol olarak kullanıldı. Deneysel aşamalarda, hücreler 35 mm'lik doku kültürü kaplarında, ortalama $1-1,5x10^6$ konsantrasyonda olacak şekilde üretildi. Hücreler doku kültürü kabının yüzeyini %80 civarında kapladığında, tripsinizasyon ile kaldırıldı ve hücre sayımı sonrasında 1500 rpm'de 5 dakika santrifüj edildi. Hücreler akış sitometri tüplerine, 100 µl'de $1x10^6$ hücre olacak şekilde 100'er µl dağıtıldı. Bir tüp boya almayan kontrol, bir tüp izotip kontrol, ve 4 tüp TRAIL reseptörleri için ayrıldı. 5 µg/ml konsantrasyonda kullanılan primer antikorlar ile hücreler buz üzerinde 30 dakika inkübe edildi. İnkübasyon sonrası hücreler üzerine 2'şer ml Akış Sitometri Tamponu eklendi ve 2000 rpm'de 10 dakika santrifüj edildi Santrifüj aşaması, her antikor inkübasyonu sonrası gerçekleştirildi. Hücreler biotin bağlı anti-fare IgG1 (Cat. ALX-211-202) sekonder antikoru ile 30 dakika, ve sonrasında Streptavidin-PE (Cat. ANC-253-050) ile 20 dakika inkübe edildi. Antikor bağlanma oranları, akış sitometrisi ile analiz edildi.

3.9. İstatistiksel Analiz

İstatistiksel analiz için, GraphPad Software'den (San Diego, CA) Prism programı kullanıldı. Her bir deney için istatistiksel sonuçlar, şekil açıklamalarında verilmiştir.

BULGULAR

4.1. Prostat Kanser Hücrelerinde Bazal NF-kB Aktivasyon Seviyeleri Farklı Bulundu.

Tümör hücrelerinin apoptozise duyarlılığını etkileyen en önemli faktörlerden birinin hücre içi sürekli NF-kB aktivasyonu olduğu ileri sürülmüştü [128, 155-157]. Bundan yola çıkılarak, çalışmamızda kullanılan üç farklı prostat kanser hücre hattının (DU145, PC3, LNCaP) bazal NF-kB aktivasyon seviyeleri belirlendi. Bu amaçla, hücreler NF-kB Lusiferaz Reporter Geni (AdNF-kBLuc) içeren bir rekombinant adenovirus ile enfekte edildi. Enfeksiyondan 24 saat sonra, Reporter Lizis Tamponlu Lusiferaz Deney Sistemi kullanılarak NF-kB Transkripsiyon Aktivasyon Deneyleri gerçekleştirildi. Deneyler sonucunda en yüksek bazal NF-kB aktivasyon seviyesinin DU145 hücrelerinde olduğu görüldü (Şekil 4.1). İkinci en yüksek seviye PC3 hücrelerinde görülürken, en düşük seviye LNCaP hücrelerinde görüldü. Enfeksiyondan 48 saat sonra gerçekleştirilen lusiferaz deneylerinde, çok daha yüksek oranda NF-kB aktivasyonu belirlenmesine rağmen, hücreler arasındaki NF-kB aktivasyon seviyesi sıralaması değişmedi. Sonuçlarımız, DU145, PC3, ve LNCaP hücre hatlarında hücre içi sürekli NF-kB aktivasyon seviyelerinin farklı olduğunu gösterdi.

4.2. Prostat Kanser Hücreleri, Adenoviral Vektörler Tarafından Eşit Olarak Transdüksiyona Uğratıldı.

Çalışmamızın ilk basamağında prostat kanser hücrelerinde bazal NF-kB aktivasyon seviyelerinin farklı olduğu gösterilmişti. Gözlenen bu farklılıkların hücrelerin adenoviruslar tarafından farklı transdüksiyonuna bağlı olma ihtimalini ortadan kaldırmak için, hücrelerin adenoviral vektörlerle transdüksiyon etkinliğine bakıldı. Hücreler, Enhanced Green Fluorescent Proteini (EGFP) kodlayan adenovirüslerle artan dozlarda enfekte edildi. Enfeksiyondan 48 saat sonra, EGFP pozitif hücrelerin yüzdesi floresan mikroskopi ile belirlendi (Şekil 4.2). Akış sitometrisi analizi sonucu, her üç prostat kanseri hücre hattında da eşit seviyede viral transdüksiyon görüldü (Şekil 4.3). Bu deneyle aynı zamanda prostat kanser hücrelerinin adenoviral vektörlerle etkin transdüksiyonu için gerekli optimum enfeksiyon dozu da belirlendi. 5000 DNA partikülü/hücre MOI (Multiplicity of Infection), hücrelerin %90'ının transdüksiyona uğratılması için yeterli oldu. 10000 DNA partikülü/hücre dozda ise, hücrelerin hemen hemen %100'ü enfekte oldu. Bu sonuçlar, her üç hücre hattının da adenovirüslerle eşit oranda ve etkin bir şekilde transdüksiyona uğratıldığını gösterdi. Dolayısıyla, prostat kanser hücrelerinde bazal NF-kB aktivasyon seviyelerinin farklı bulunmasının, hücrelerin adenovirüsler tarafından farklı transdüksiyonuna bağlı olmadığı görüldü.

4.3. Prostat Kanser Hücreleri Değişken Derecede Adenovirüs Aracılı TRAIL Sitotoksisitesi Gösterdi.

Prostat kanser hücreleri, TRAIL proteinini kodlayan Ad5hTRAIL veya kontrol olarak AdCMVLacZ vektörleri ile artan dozlarda enfekte edildi. Enfeksiyondan 48 saat sonra, hücre canlılık deneyleri gerçekleştirildi. Flöresan mikroskopi ile analiz sonucu, prostat kanser hücreleri arasında LNCaP ve DU145'ın, hücre başına 10000 Ad5hTRAIL vektörü verildiğinde dahi ölmedikleri belirlendi (Şekil 4.4). AdCMVLacZ enfeksiyonundan sonra da hücre ölümü gözlenmedi (Şekil 4.5). Öte yandan, PC3 hücrelerinde, 5000 DNA partikülü/hücre MOI Ad5hTRAIL virüsü ile enfeksiyon sonucu %63 oranında hücre ölümü gözlendi. Viral vektör dozu 10000 DNA partikülü/hücre MOI'a çıkarıldığında ise, %90 oranında hücre ölümü gözlendi (Şekil 4.5). Bu sonuçlara göre, DU145 ve LNCaP hücre hatları TRAIL'a yüksek derece dirençli bulundu. PC3 hücre hattı, DU145 ve LNCaP hücrelerine oranla önemli seviyede TRAIL duyarlılığı gösterdi.

4.4. Prostat Kanser Hücrelerinde Hücre içi NF-kB Aktivitesi, hTRAIL Sentezi ile Artarken, IKKβKA Sentezi ile Azaldı.

TRAIL yalancı reseptörü TRAIL-R4'ün ve TRAIL ölüm reseptörlerinin (TRAIL R1 ve TRAIL-R2), NF-kB yollarını aktive ettiği daha önce gösterilmişti [121, 122]. Bu nedenle, hem TRAIL ile indüklenen NF-kB aktivasyonu, hem de hücrenin endojen NF-kB durumu, prostat kanserinin TRAIL aracılı tedavisinde dikkate alınması gereken önemli faktörlerdendir. Çalışmamızda, prostat kanser hücrelerinde TRAIL sentezi ile aktive olan NFkB aktivitesi araştırıldı. Bu amaçla, DU145, PC3 ve LNCaP hücreleri AdNF-kBLuc ve Ad5hTRAIL vektörleri birlikte kullanılarak enfekte edildi. Hücre ölümünün en az seviyede tutulabilmesi için, hücre başına verilen Ad5hTRAIL dozu 1000 MOI'da sabit tutuldu. Enfeksiyondan 24 saat sonra NFkB transkripsiyon aktivasyon deneyleri yapıldı. Sonuçta NFkB aktivitesinin, sadece TRAIL'ın sentezinin arttığı hücrelerde yükseldiği görüldü (Şekil 4.6). IKK inhibe edici strateji aracılığıyla NFkB inhibisyonunun derecesini görebilmek amacıyla, prostat kanser hücre hatları AdIKKβKA vektörünün artan dozları ile ve ayrıca AdNF-kBLuc ve Ad5hTRAIL vektörleri ile birlikte enfekte edildi. TRAIL ile indüklenen NFkB aktivitesinin her üç hücre hattında da azaldığı, hatta endojen NF-kB aktivitesinin de altına düştüğü belirlendi (Şekil 4.6, A-C Panelleri). Öte yandan, hücreler kontrol olarak AdCMVLacZ virüsü ile enfekte edildiğinde benzer bir NFkB inhibe edici etki gözlenmedi. Sonuç olarak, prostat kanser hücrelerinde TRAIL sentezinin artışı, hücre içi NF-kB aktivasyon seviyelerinde artışa yol açtı. Bunun yanında IKK inhibe edici strateji, prostat kanser hücre hatlarında hem TRAIL aracılığıyla indüklenen NF-kB aktivitesini, hem de endojen NF-kB aktivitesini düşürücü etki gösterdi.

4.5. IKKβKA Sentezi Yoluyla Fonksiyonel IKK İnhibisyonu, Prostat Kanser Hücrelerini TRAIL Aracılı Hücre Ölümüne Karşı Duyarlı Hale Getirdi.

IKKβ'nın (AdIKKβKA) [128, 157] veya IkBα'nın (AdIkBαSR) [152] dominant negatif mutantlarını kodlayan adenovirüslerle enfeksiyonun, akciğer kanser hücre hatlarını TNF ölüm ligandına karşı duyarlılaştırmada başarılı olduğu daha önce gösterilmişti. Bazı kanser hücrelerinde daha yüksek bazal NFkB aktivitesi olduğundan, NFkB bloke edici ajanlar, bu hücreleri TRAIL'ın apoptozisi indükleyici

etkilerine karşı duyarlılaştırmada etkin bir strateji olabilir. Prostat kanser hücre hatlarında IKK inhibisyonunun TRAIL'a dirençliliğe karşı etkisini araştırmak amacıyla, bu hücre hatları hem Ad5hTRAIL vektörünün 5000 MOI sabit dozu ile, hem de AdIKKβKA virüsünün artan dozları ile enfekte edildi. Deney sonucunda, her üç hücre hattının da, hücrenin başlangıçtaki endojen NFkB aktivasyonuna bağlı olmaksızın TRAIL'a duyarlı hale geldiği görüldü (Şekil 4.7). DU145 ve LNCaP hücrelerinde, TRAIL sentezi varlığında hücre başına 1000 MOI AdIKKβKA vektörü verildiğinde dahi %50 civarında hücre ölümü gözlendi. PC3 hücreleri TRAIL'a zaten duyarlı olduğundan, DU145 ve LNCaP hücrelerinin IKKβKA ile enfeksiyonu sonucu TRAIL'a duyarlılaşma derecesi, PC3 hücrelerine oranla çok daha fazla idi (Şekil 4.8). Öte yandan, prostat kanser hücreleri AdIKKβKA yerine AdCMVLacZ virüsü ile enfekte edildiğinde, hücre ölümü gözlenmedi. Bu sonuçlar, IKK inhibisyonu yoluyla NFkB'nin bloke edilmesinin, TRAIL dirençliliği gösteren prostat kanser hücrelerini TRAIL'a duyarlı hale getirmede etkin olduğunu göstermektedir.

4.6. Prostat Kanser Hücrelerinin Yalnızca AdIKKβKA Vektörleri ile Enfeksiyonu Hücre Ölümüne Yol Açmadı.

Daha önceki çalışmalarda, prostat kanser hücre hatlarında gözlenen yüksek NF-kB aktivitesinin, IKK aktivitesindeki bir artıştan kaynaklandığı öne sürülmüştü [149]. Bu nedenle, bu çalışmada IKK aktivitesi inhibisyonunun, tek başına prostat kanser hücrelerinin canlılık oranı üzerine etkisi araştırıldı. Bu amaçla, prostat kanser hücreleri, IKKβ'nın dominant negatif mutantını kodlayan adenoviral vektörlerin (AdIKKβKA) artan dozlarıyla enfekte edildi. Enfeksiyondan 48 saat sonra, hücre canlılık oranları floresan mikroskop altında belirlendi. PC3 hücrelerinde gözlenen TRAIL sitotoksisitesinin tersine, prostat kanser hücre hatlarının hiçbirinde, hücre başına 10000 AdIKKβKA virüsü verildiğinde dahi, hücre canlılık seviyesinde azalma görülmedi (Şekil 4.9). Bu sonuçlar, prostat kanser hücrelerinin AdIKKβKA virüsleri ile tek başına enfeksiyonu sonucunda önemli derecede hücre ölümü gerçekleşmediğini gösterdi.

4.7. Ad5hTRAIL ve AdIKKβKA'nın Birlikte Enfeksiyonu Sonucu, Prostat Kanser Hücre Hatlarında Apoptotik Ölüm Gerçekleşti.

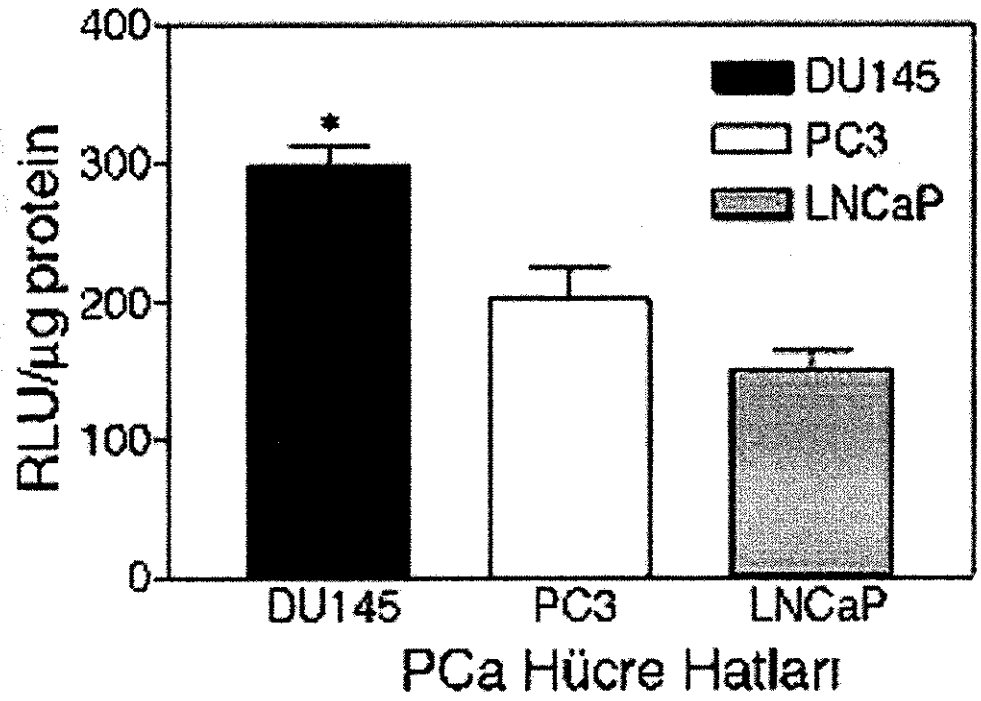
Prostat kanser hücrelerinde IKK inhibisyonu altında TRAIL'ın indüklediği hücre ölümünün moleküler mekanizmasının açığa çıkarılması için, DU145 hücrelerinde Anneksin V boyama yoluyla apoptotik ölüm oranı ölçüldü. Bu amaçla, Ad5hTRAIL ve AdIKKβKA vektörleri ayrı olarak ya da birarada DU145 hücrelerine enfekte edildi. Enfeksiyondan 35 saat sonra Anneksin V bağlanma deneyleri gerçekleştirildi, ve bağlanma oranları akış sitometrisi ile analiz edildi. Sonuçta Ad5hTRAIL veya AdIKKβKA enfeksiyonu tek başına önemli derecede Anneksin V bağlanma göstermedi. Ancak, hücreler Ad5hTRAIL ve AdIKKβKA ile birlikte enfekte edildiğinde, önemli seviyede Anneksin V bağlanması görüldü (Şekil 4.10). Bu durum, AdIKKβKA aracılığıyla IKK inhibisyonu sonucu prostat kanser hücrelerinin apoptotik yollu hücre ölümüne gittiğinin göstergesidir. Öte yandan, Ad5hTRAIL ile birlikte AdCMVLacZ enfeksiyonu önemli seviyede Anneksin V bağlanma seviyesi göstermedi. Böylece, IKK inhibisyonu yokluğunda DU145

hücrelerinin TRAIL'a oldukça dirençli olduğu görüldü. Tüm bu sonuçlar, IKK inhibisyonu altında TRAIL'ın indüklediği hücre ölüm mekanizmasının apoptozis olduğunu gösterdi

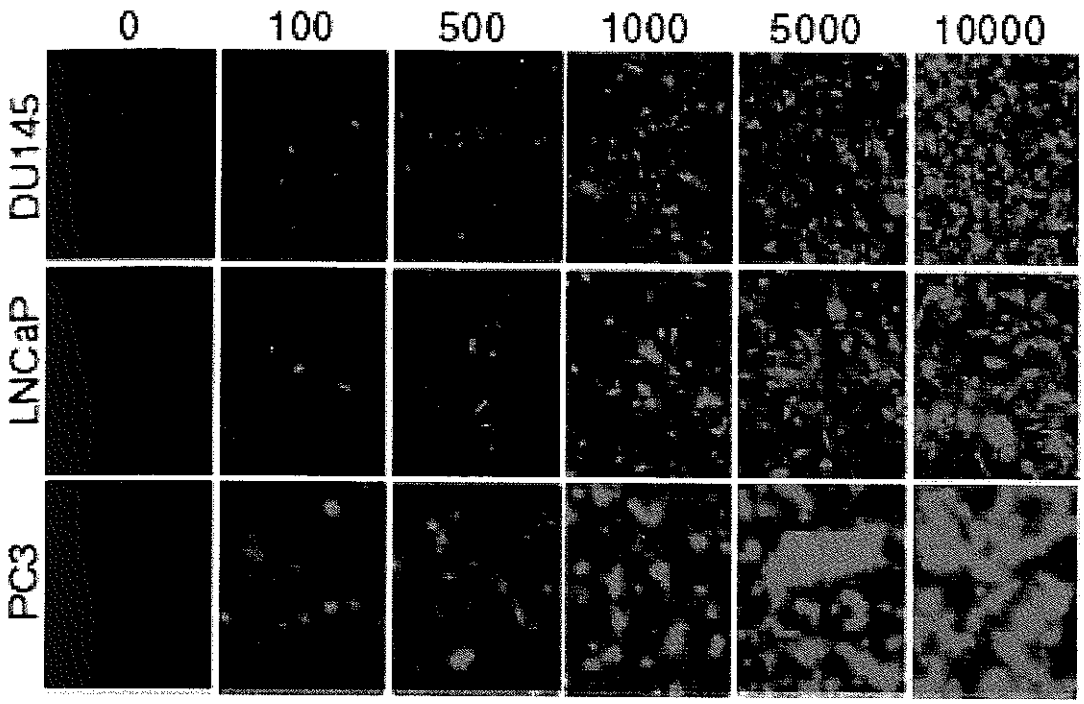
4.8. Prostat Kanser Hücre Hatlarında Birbirinden Farklı TRAIL Reseptör Kompozisyonları Belirlendi.

Prostat kanser hücre hatlarında TRAIL reseptörlerinin sentez profillerinin belirlenmesi için, spesifik primer ve prob setleri kullanılarak Gerçek Zamanlı RT-PCR deneyi gerçekleştirildi. Deneyler sonucunda, çalışılan tüm hücre hatlarında TRAIL-R2 sentez düzeyi, TRAIL-R1 sentez seviyesinden oldukça yüksek bulundu. TRAIL'a duyarlı olan PC3 hücrelerinde, TRAIL'a dirençli olan DU145 ve LNCaP hücre hatlarına göre TRAIL-R2 sentez seviyesi daha yüksek bulundu (Şekil 4.11). PC3 hücrelerinde çok düşük seviyelerde TRAIL-R3 sentezi görülmesine rağmen, TRAIL-R4 sentezi gözlenmedi. Ayrıca, TRAIL'a dirençli DU145 ve LNCaP hücrelerinde, her iki yalancı reseptörün de (TRAIL-R3 ve TRAIL-R4) önemli seviyede sentez edildiği görüldü.

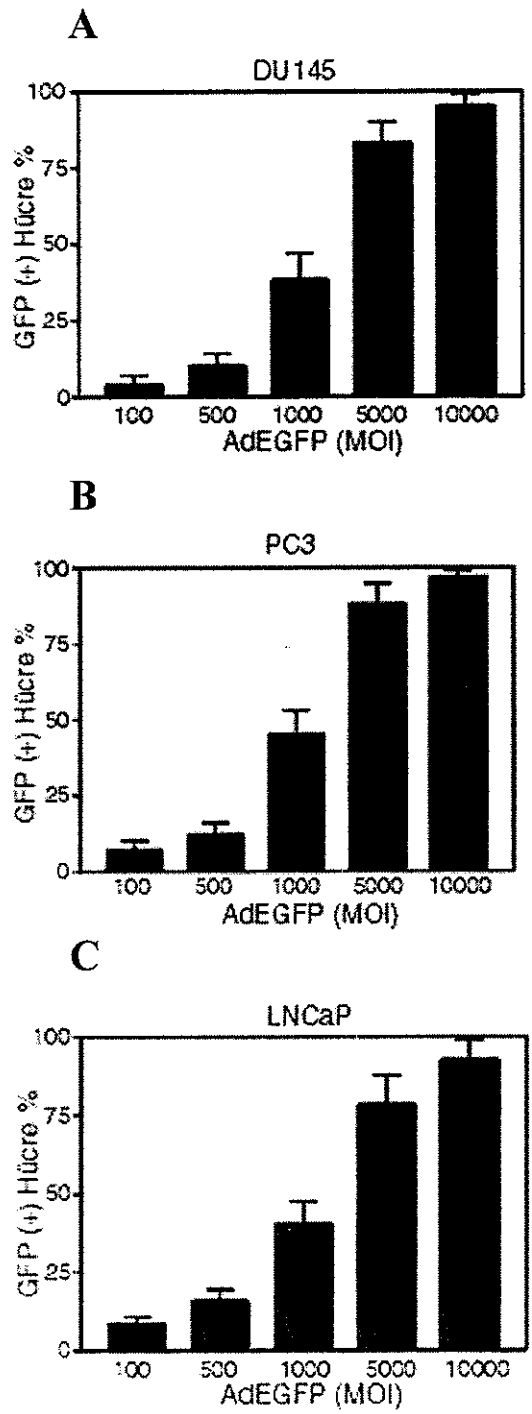
Gerçek Zamanlı RT-PCR, mRNA seviyesinde gen sentezinin miktarı tayininde yararlı olmasına rağmen, hücre içindeki gen sentezi, protein bazında hücre yüzeyindeki reseptör sentezi ile mutlak uyumlu olmayabilir. Bu nedenle, prostat kanser hücrelerinin yüzeyinde hangi TRAIL reseptörlerinin sentez edildiği ve sentez seviyeleri akış sitometrisi aracılığıyla belirlendi. Analiz sonucunda, PC3 hücrelerinin yüzeyinde TRAIL ölüm reseptörlerinin (TRAIL-R1 ve TRAIL-R2) sentezi belirlendi, ancak ölçülebilir yalancı reseptör sentezi gözlenmedi (Şekil 4.12). DU145 (Şekil 4.12) ve LNCaP hücrelerinde (Şekil 4.13) her iki tip TRAIL ölüm reseptörünün de sentezinin olduğu, ancak PC3 hücrelerinin tersine, bu hücrelerde önemli miktarda TRAIL-R4 yalancı reseptör sentezi gerçekleştiği gözlendi. Ayrıca, LNCaP hücrelerinin yüzeyinde düşük seviyede TRAIL-R3 yalancı reseptör sentezi belirlenmesine rağmen, DU145 ve PC3 hücrelerinde böyle bir durum gözlenmedi. Sonuç olarak, TRAIL'a dirençli DU145 ve LNCaP hücre hatlarında TRAIL yalancı reseptör sentez seviyelerinin, TRAIL'a duyarlılık gösteren PC3 hücre hattına göre belirgin oranda yüksek olduğu görüldü.



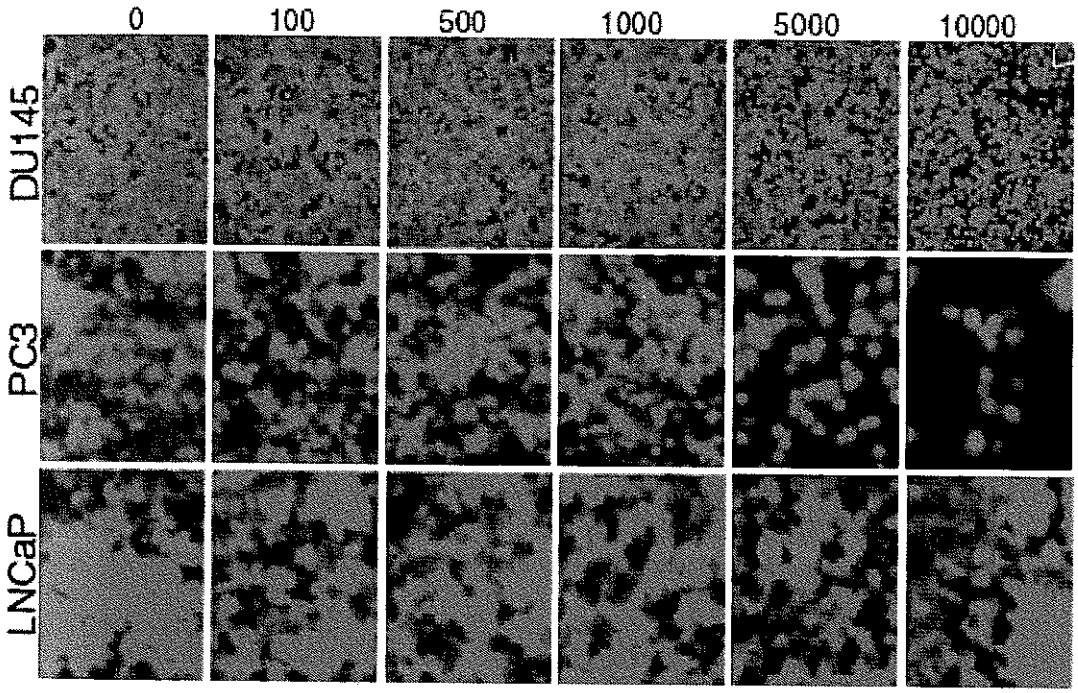
Şekil 4.1. Prostat kanser hücre hatlarında endojen NF-kB seviyeleri Endojen NF-kB seviyeleri, AdNFkBLuc aktivasyonundan 24 saat sonra lusiferaz aktivitelerinin ölçülmesi ile belirlendi. Enfekte edilen hücre hatları, x ekseninde verilmiştir. Mikrogram protein başına düşen Rölatif Işık Birimi (RLU, Relative Light Units) olarak ifade edilen lusiferaz aktivitesi, y ekseninde verilmiştir Veriler, altı bağımsız verinin ortalamasını (± SEM) temsil etmektedir. ANOVA ve Tukey çoklu karşılaştırma testi kullanılarak DU145 ile PC3 ve LNCaP hücreleri arasındaki istatistiksel farklılık belirlenmiştir *$p < 0.01$



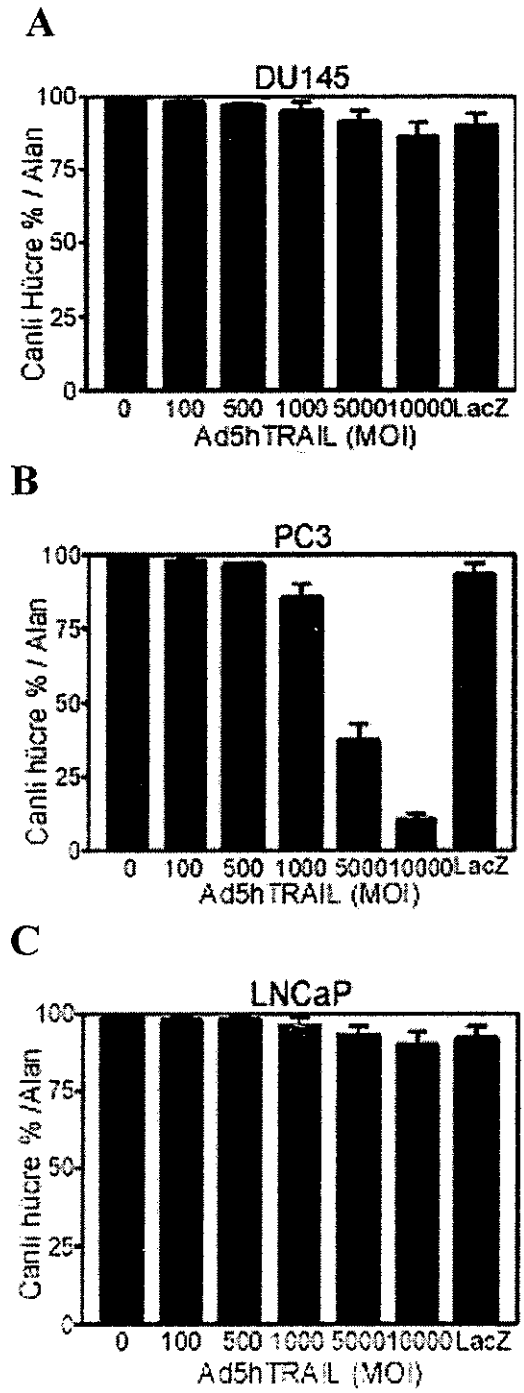
Şekil 4.2. Prostat karsinoma hücrelerinin birinci generasyon rekombinant adenoviral vektörler ile transdüksiyonu Prostat kanser hücreleri EGFP reporter geni içeren adenovirüslerle enfekte edildikten 48 saat sonra viral transdüksiyon oranlarına bakıldı Şekilde, adenoviral transdüksiyonların flöresan fotoğrafları görülmektedir. Enfeksiyonda kullanılan virüslerin MOI değerleri (DNA partikülü/hücre), resmin üst kısmında verilmiştir.



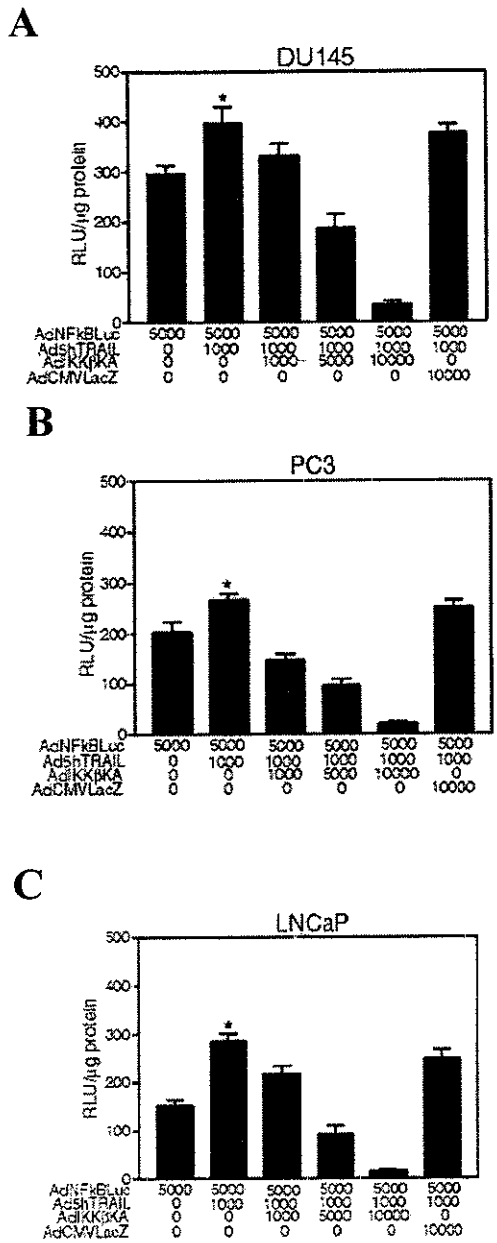
Şekil 4.3. Prostat kanser hücrelerinin adenoviral vektörlerle transdüksiyon etkinliklerinin akış sitometri ile değerlendirmesi Panel A'da DU145 hücrelerinin, Panel B'de PC3 hücrelerinin, Panel C'de ise LNCaP hücrelerinin AdEGFP ile transdüksiyonlarının akış sitometrik analiz sonuçları gösterilmiştir



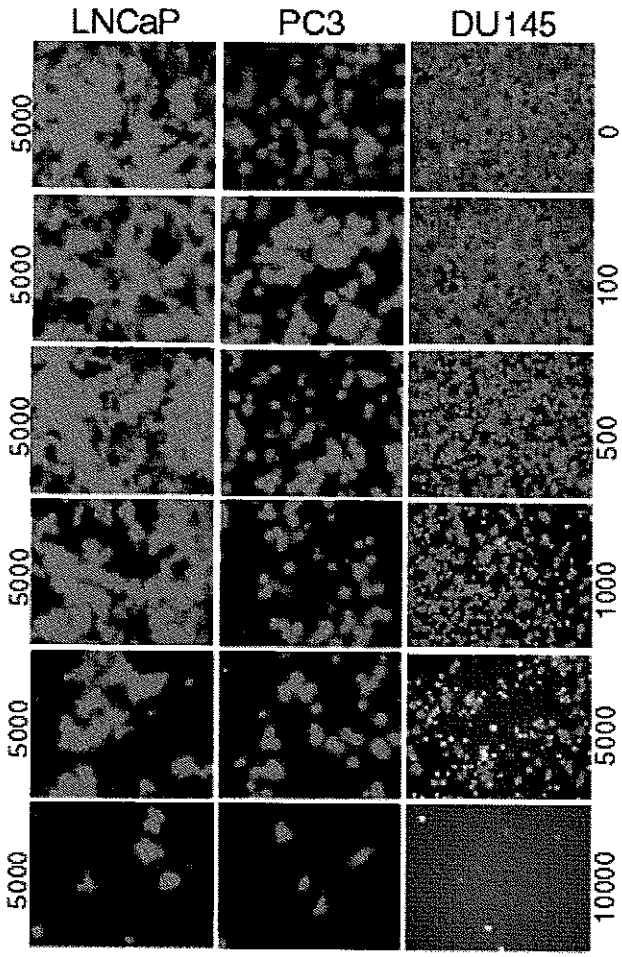
Şekil 4.4. Prostat kanser hücrelerinin TRAIL duyarlılık seviyeleri. DU145, PC3 ve LNCaP hücreleri, artan konsantrasyonlarda Ad5hTRAIL ve kontrol olarak AdCMVLacZ adenoviral vektörleri ile enfekte edildi. Enfeksiyondan 48 saat sonra hücre canlılık deneyi gerçekleştirildi. Şekilde, Ad5hTRAIL ile enfeksiyon sonuçları görülmektedir. Fotoğraflar FITC kanalında çekilmiştir. Şeklin üst kısmında, uygulanan vektör dozları MOI olarak (DNA partikülü/hücre) verilmiştir



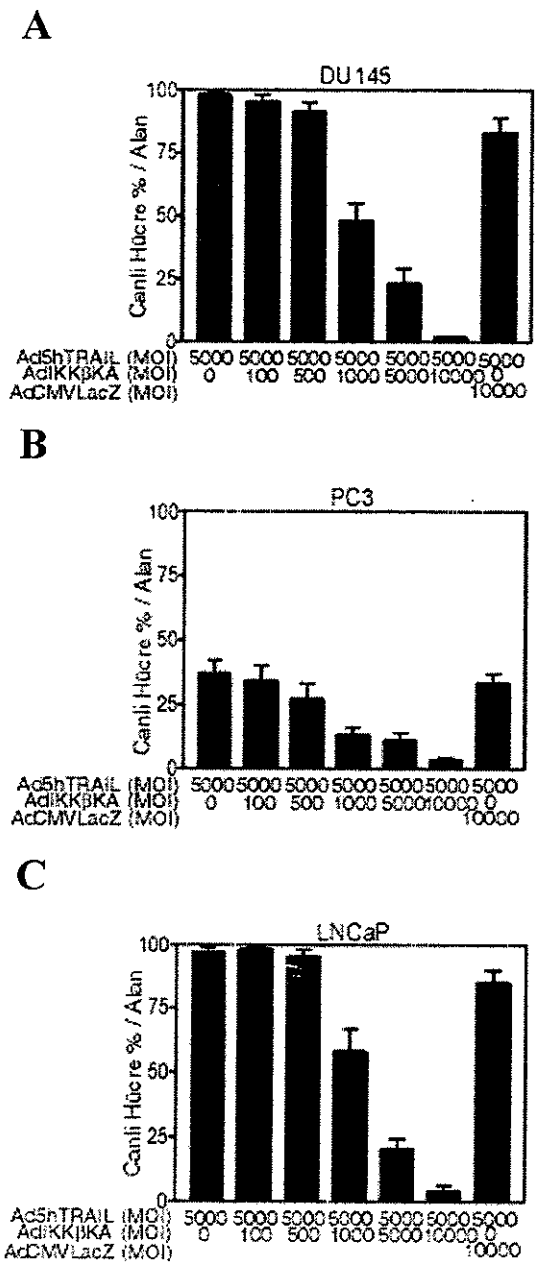
Şekil 4.5. Hücre canlılık deneylerinin kantitatif sonuçları. LacZ sütunu, 10000 DNA partikülü/hücre MOI konsantrasyonda AdCMVLacZ vektörü ile enfeksiyon sonuçlarını göstermektedir. Hücre canlılık deneyleri üçer set halinde gerçekleştirildi, ve ikişer kez tekrar edildi. Değerler, altı bağımsız verinin ortalamasını (±S.E.M) temsil etmektedir.



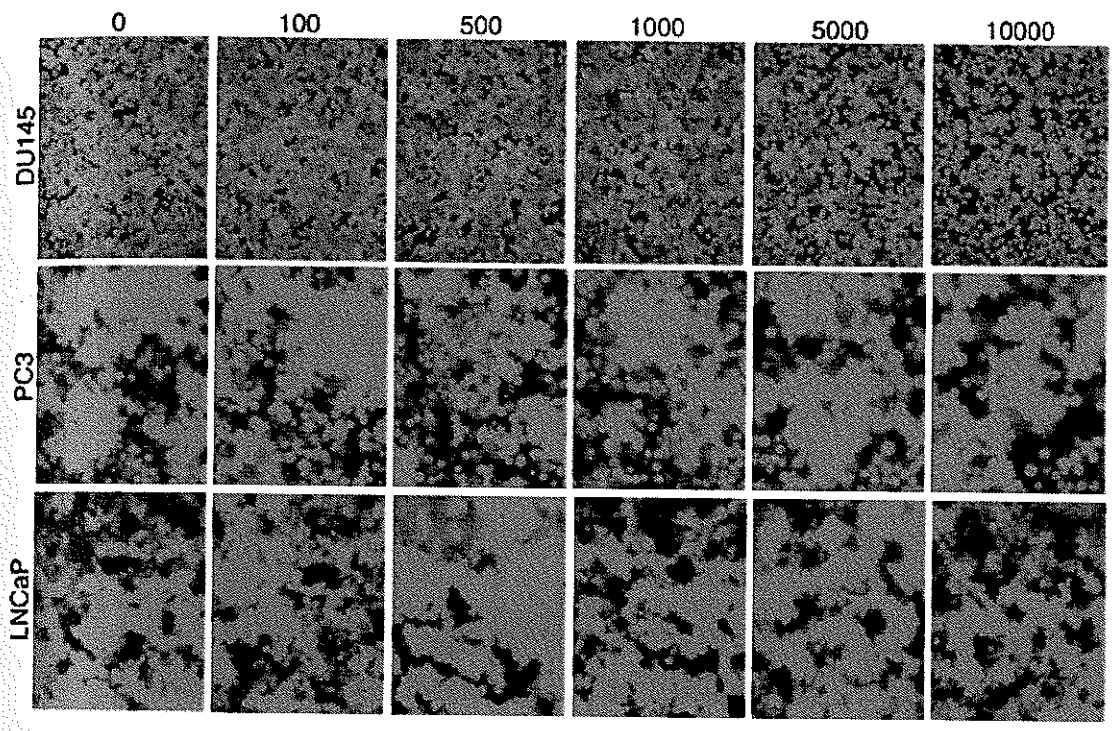
Şekil 4.6. Prostat kanser hücrelerinde hTRAIL ve IKKβKA sentezinin NF-kB aktivasyonu üzerine etkisi. DU145 (Panel A), PC3 (Panel B) ve LNCaP (Panel C) hücre hatları, AdNFkBLuc, Ad5hTRAIL, ve/veya AdIKKβKA'nın artan dozları ile enfekte edildi. Kontrol olarak AdCMVLacZ virüsleri kullanıldı. Enfeksiyondan 24 saat sonra hücrelerde NF-kB aktivitesine bakıldı. Kullanılan viral vektörler ve viral MOI değerleri (DNA partikülü/hücre), x ekseninde verilmiştir. y ekseninde lusiferaz aktivitesi (RLU/µg), gösterilmektedir. Değerler, altı bağımsız verinin ortalamasını (±S.E.M) temsil etmektedir. Hücrelerin AdNFkBLuc ile ve AdNFkBLuc ve Ad5hTRAIL ile enfeksiyon sonuçları arasındaki farklılıkların açığa çıkarılması için "student's t test" kullanılmıştır. *$p<0.05$



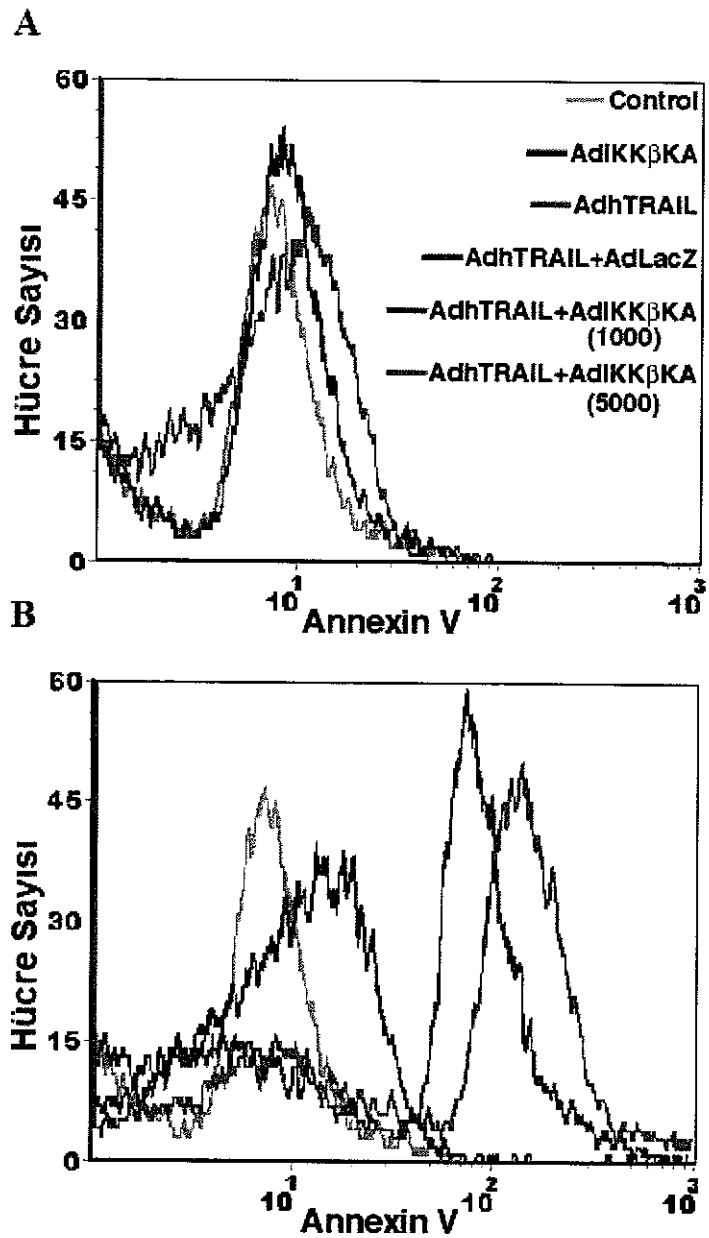
Şekil 4.7. Adenoviral vektörler yoluyla IKKβKA sentezinin, prostat kanser hücre hatlarında TRAIL dirençliliği üzerine etkisi. DU145, PC3 ve LNCaP hücre hatları, 5000 DNA partikülü/hücre sabit MOI'da Ad5hTRAIL ile birlikte, IKKβ'nın dominant negatif mutantını (AdIKKβKA) veya AdIKKβKA yerine kontrol olarak LacZ'yi (AdCMVLacZ) kodlayan adenoviral vektörlerlerin artan dozları ile enfekte edildi. Şekilde, Ad5hTRAIL ve AdIKKβKA'nın birlikte kullanıldığı enfeksiyondan 48 saat sonra gerçekleştirilen hücre canlılık deneyi sonuçları görülmektedir



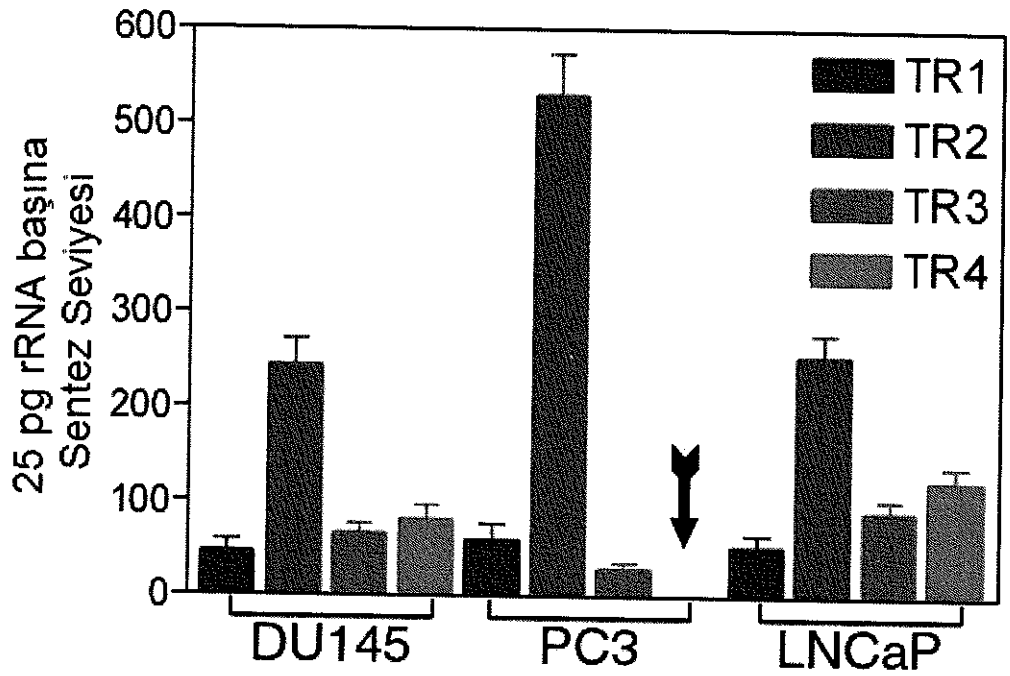
Şekil 4.8. IKKβKA sentezinin TRAIL dirençliliği üzerine etkisinin kantitatif olarak değerlendirilmesi. DU145, PC3 ve LNCaP hücreleri, Ad5hTRAIL ile birlikte AdIKKβKA ile, veya kontrol olarak AdIKKβKA yerine AdCMVLacZ adenoviral vektörleri ile enfekte edildikten 48 saat sonra canlı hücre oranına bakıldı. Şekilde, flöresan mikroskop altında 20x büyütmede canlı hücre sayımı sonuçları görülmektedir. Uygulanan viral dozlar, x ekseninde MOI olarak (DNA partikülü/hücre) verilmiştir. Değerler, altı bağımsız verinin ortalamasını (±S.E.M) temsil etmektedir.



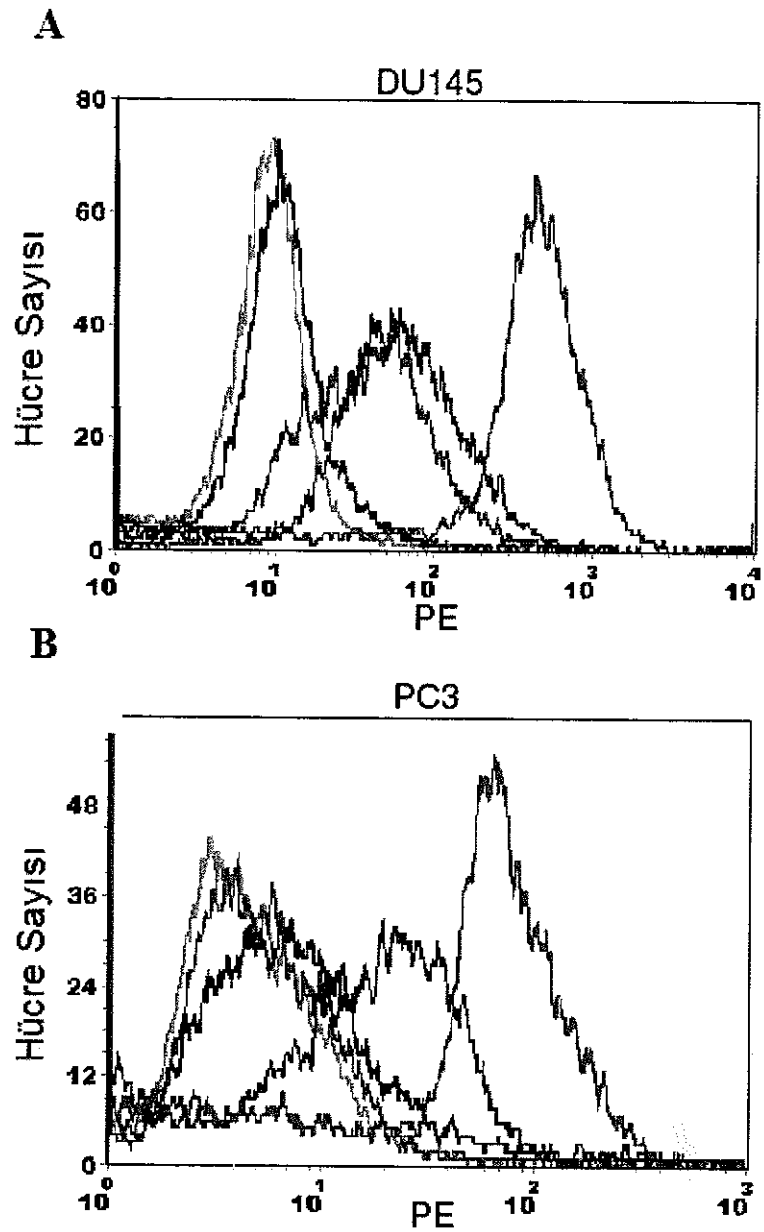
Şekil 4.9. Tek başına AdIKKβKA enfeksiyonunun prostat kanser hücreleri üzerindeki etkisi Prostat kanser hücre hatları, artan dozlarda AdIKKβKA veya kontrol olarak AdCMVLacZ adenoviral vektörleri ile enfekte edildi Enfeksiyondan 48 saat sonra hücre canlılık deneyi yapıldı Flöresan fotoğrafların üst kısmında, kullanılan viral dozlar MOI olarak (DNA partikülü/hücre) verilmiştir. Deneyler, üçlü setler halinde ikişer kez tekrar edilmiştir.



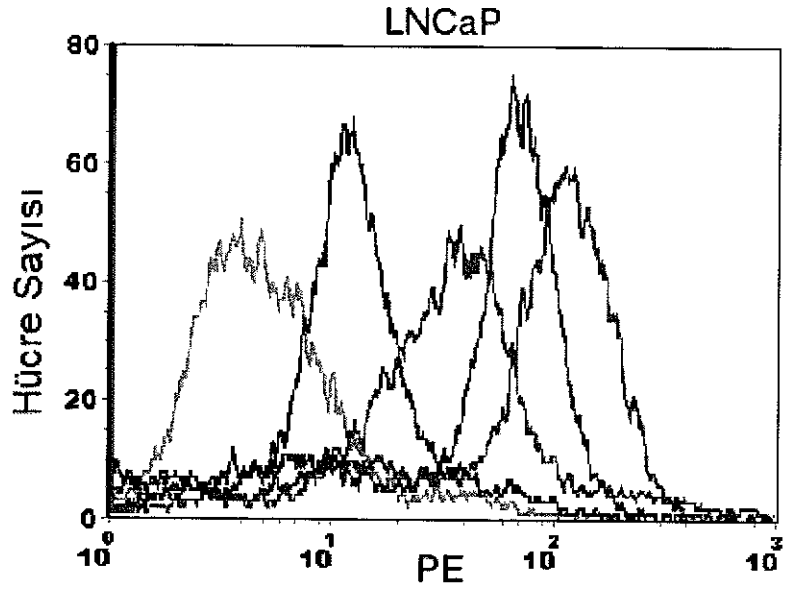
Şekil 4.10. DU145 prostat kanser hücre hattında TRAIL ve IKKβKA aracılı apoptozisin gösterilmesi. DU145 hücreleri, Ad5hTRAIL, AdIKKβKA, ve negatif kontrol olarak AdCMVLacZ virüsleri ile belirtilen şekillerde enfekte edildi. Şekilde aksi belirtilmedikçe viral vektörler 5000 DNA partikülü/hücre MOI'da kullanılmıştır. Enfekte edilen DU145 hücreleri, FITC ile konjuge Anneksin V ve Propidium Iodide (PI) ile boyandı, ve akış sitometrisi ile analiz edildi. Enfekte edilmeyen ancak FITC-Anneksin V ve PI boyalı hücreler kontrol olarak kullanıldı. Her bir histogramda, 10^4 DU145 hücresi analize dahil edilmiştir. Histogramlar, açıklık amacıyla iki panel halinde gösterilmiştir. Uygulama şartları, Panel A'da gösterilmiştir. Anneksin V bağlanma deneyi, üç kez tekrar edilmiştir.



Şekil 4.11. Prostat karsinoma hücre hatlarında TRAIL reseptör mRNA düzeyleri. Prostat kanser hücrelerinde TRAIL reseptörlerinin sentez seviyelerini belirlemek için total RNA'dan kantitatif Gerçek Zamanlı RT-PCR deneyleri gerçekleştirildi. Sentez oranlarının karşılaştırılması için standart eğri yaratmak amacıyla klonlanmış bir ribozomal cDNA fragmenti kullanıldı. Şekilde, 25 pg ribozomal cDNA'ya karşılık gelen TRAIL reseptör seviyeleri gösterilmektedir. Her TaqMan deneyine iç kontrol olarak ribozomal RNA primerleri ve probları eklenmiştir. Şekildeki ok, PC3 hücrelerinde TRAIL-R4 sentezinin yokluğuna işaret etmektedir.



Şekil 4.12. DU145 ve PC3 hücre yüzeylerinde TRAIL reseptörlerinin sentez seviyeleri. TRAIL reseptörlerinin DU145 (Panel A) ve PC3 hücre (Panel B) yüzeylerindeki sentez seviyeleri, her bir TRAIL reseptörü için spesifik monoklonal antikor kullanılarak belirlendi. Renkli çizgiler, deneysel parametreleri göstermektedir. Gri: İzotip spesifik kontrol, Mavi: TRAIL-R1, Kırmızı: TRAIL-R2, Kestane rengi: TRAIL-R3, Yeşil: TRAIL-R4. Her histogram için 10^4 hücre analize dahil edilmiştir. Herbir deney üç kez tekrar edilmiştir.



Şekil 4.13. LNCaP hücre hattında TRAIL reseptörlerinin hücre yüzeyindeki sentez seviyeleri LNCaP hücrelerinde TRAIL reseptörlerinin hücre yüzeyindeki sentez seviyeleri, her bir reseptör için spesifik monoklonal antikor kullanılarak belirlendi. Renkli çizgiler, deneysel parametreleri göstermektedir. Gri: İzotip spesifik kontrol, Mavi: TRAIL-R1, Kırmızı: TRAIL-R2, Kestane rengi: TRAIL-R3, Yeşil: TRAIL-R4. Her histogram için 10^4 hücre analize dahil edilmiştir. Herbir deney üç kez tekrar edilmiştir

TARTIŞMA ve SONUÇLAR

Prostat kanseri, batı ülkelerinde erkekler arasında en yaygın olarak görülen kanserdir, ve erkeklerde kanser sebebiyle ölümlerde ikinci sırada yer almaktadır [2]. İleri evre prostat kanserinin tedavisinde mevcut tedavi yöntemleri hastaların yaşam süresini uzatmada yeterli olmadığından, ölüm ligantlarının adenoviral yollarla prostat kanser hücrelerine aktarımı, mevcut yöntemleri tamamlayıcı bir tedavi metodu olarak düşünülmektedir [128]. TRAIL ölüm ligandı prostat kanserinin tedavisi için ümit verici bir moleküldür. Ancak bazı prostat kanser hücre hatlarının TRAIL'a dirençli olduğu gösterilmiştir. Bu dirençlilikten ise, hücrelerde belirlenen sürekli Akt kinaz aktivitesinin sorumlu olduğu bildirilmiştir [114]. Demarchi ve arkadaşları tarafından, NF-kB'nin Akt aracılığıyla aktivasyonunda IkB Kinaz'ın (IKK) rol aldığı ileri sürülmüştür [159]. Ayrıca prostat kanser hücrelerinde yüksek IKK aktivitesinin sürekli NF-kB aktivasyonu ile sonuçlanabileceği bildirilmiştir [149]. Çalışmamızda, bu bulgular ışığında prostat kanser hücre hatlarının IKKβ'nın dominant negatif mutantını (AdIKKβKA) ve insan fonksiyonel TRAIL molekülünü kodlayan (Ad5hTRAIL) adenovirüslerle birlikte enfekte edilmesinin prostat kanser hücrelerini TRAIL'a duyarlı hale getirip getiremeyeceğini test etmek istedik. Hipotezimizin doğruluğunu araştırmak için, üç farklı ileri evre prostat kanser hücre hattı kullandık (DU145, PC3, LNCaP).

İleri evre prostat kanseri, tedavide en çok zorluk yaşanan hastalık evresidir. Bu evrede tümör hücrelerinin androjen bağımlılıklarını kaybetmeleri de, tedaviyi zorlaştırıcı önemli faktörlerdendir [60, 61]. Bu nedenle, mevcut tedavi yöntemlerini tamamlayıcı gen tedavi yaklaşımları geliştirmeyi amaçladığımız çalışmamızda, ileri evre prostat kanser hücre hatları kullanıldı. Bu hücrelerden DU145 ve PC3 androjen bağımsız, ve LNCaP androjen bağımlı hücre hatlarıdır. Androjen bağımlı ve bağımsız hücre hatlarının birarada kullanımı, androjen bağımlılık durumunun TRAIL dirençliliği ve TRAIL dirençliliğini kırma metodlarının etkinliği üzerindeki etkisine bakmamızı sağlamıştır. Bahsedilen durumlar arasında bir ilişki gözlenmemiştir.

NF-kB transkripsiyon faktörlerinin, farklı hücrelerde çoğunlukla apoptozisi bloke edici düzenleyici moleküller olarak rol aldıkları bilinmektedir. Prostat kanser hücrelerinde TRAIL dirençliliğinden sorumlu tutulan Akt kinaz enzimi, NF-kB transkripsiyon faktörünün regülatörüdür [160, 161]. Dolayısıyla Akt aktivasyonunda artış, prostat kanser hücrelerinde NF-kB aktivitesinde de artışa yol açmaktadır. Örneğin Oya M ve arkadaşları, TRAIL'a dirençli renal karsinoma hücre hatlarında sürekli NF-kB aktivasyonu olduğunu göstermiş, ve NF-kB'nin, TRAIL dirençlilik mekanizması altında yatan anahtar molekül olduğunu bildirmiştir. Çalışmada, TRAIL'a duyarlı renal karsinoma hücre hatlarında NF-kB aktivasyonu düşük bulunurken, TRAIL'a dirençli renal karsinoma hücre hatlarında ise yüksek NF-kB aktivasyonu gösterilmiştir [155]. Bu nedenle çalışmamızda birinci basamak olarak, DU145, PC3, ve LNCaP prostat kanser hücre hatlarında TRAIL dirençliliğinden sorumlu olabilecek endojen NF-kB aktivasyon düzeyleri NF-kB transkripsiyon

aktivasyon deneyleri ile belirlendi (Şekil 4.1). Deneysel şartlarımızda, en yüksek endojen NF-kB aktivitesi DU145 hücrelerinde görülürken, bu hücreleri PC3 ve LNCaP hücre hatlarının izlediği görüldü. Hücreler arasında hücre içi sürekli NF-kB aktivasyon seviyeleri açısından farklılık bulunmasının yanında, her üç hücre hattında da yüksek NF-kB seviyesinin bulunması, TRAIL dirençliliği açısından önemli olabilir. Palayoor ve ark. ile Gasparian ve arkadaşları da, Mobility Shift deneyleri kullandıkları çalışmalarında bizim bulgularımızla uyumlu olarak PC3 ve DU145 hücrelerinde endojen NF-kB aktivitesini yüksek bulurken, LNCaP hücrelerinde NF-kB aktivasyonunun daha düşük olduğunu bildirmişlerdir [149, 162].

Çalışmamızın bir sonraki aşamasında, prostat kanser hücre hatları, TRAIL'a dirençlilikleri açısından incelendi. Hücre canlılık deneyleri sonucunda, DU145 ve LNCaP hücrelerinin Ad5hTRAIL ile indüklenen apoptozise dirençli olduğu, PC3 hücrelerinin ise önemli seviyede TRAIL duyarlılığı gösterdiği görüldü. Bizim bulgularımızın aksine, Nesterov ve arkadaşları, DU145 hücre hattının çözülebilir TRAIL'a yüksek oranda duyarlı olduğunu bildirmiştir [114]. Bu çalışmada PC3 hücre hattı da TRAIL'a yüksek oranda duyarlı bulunmuş, ancak başka bir çalışmada PC3 hücrelerinde orta derecede TRAIL duyarlılığı gözlenmiştir [163]. Bu çalışmada da çözülebilir TRAIL proteini kullanılmıştır. Nesterov ve arkadaşlarının çalışmasında LNCaP hücre hattının ise, bizim bulgularımızla uyumlu olarak TRAIL'a dirençli olduğu bulunmuştur. Bunun yanında, Voelkel-Johnson ve arkadaşları, çalışmalarında DU145 ve LNCaP hücrelerinin Ad-IRES-TRAIL ile indüklenen apoptozise duyarlı olduğunu göstermiştir [113]. Ayrıca, Beresford ve ark.'nın yaptığı bir çalışmada PC3 hücrelerinin çözülebilir TRAIL'a dirençli olduğu bildirilmiştir [164]. Nesterov ve arkadaşları ile Thakkar ve arkadaşlarının çalışmaları ile bizim bulgularımız arasındaki farklılıkların, fonksiyonel çalışmalarda kullanılan farklı TRAIL formundan kaynaklandığı düşünülebilir. Bahsedilen her iki çalışmada da *in vitro* olarak saflaştırılmış çözülebilir TRAIL kullanılmıştır. Ayrıca kullanılan TRAIL moleküllerinin biri mayadan [114], diğeri de E.coli'den saflaştırılmıştır [163]. *In vitro* çalışmalarda kullanılan vektörlerin, *in vivo* çalışmalar için de uygun olması gerektiği açıktır. Ancak çözülebilir TRAIL proteininin kan dolaşımındaki yarı ömrü sınırlıdır. İntravenöz olarak verilen çözülebilir TRAIL proteinin büyük bir kısmı, 5 saat içinde atılmaktadır, dolayısıyla tümör oluşumunu engellemek için yüksek miktarlarda kullanılması gerekir. Voelkel-Johnson ve arkadaşları ile Beresford ve arkadaşlarının çalışmalarında elde edilen bulgularla bizim bulgularımız arasındaki farklılıkların ise, enfeksiyonda kullanılan virüsün fonksiyonel titresinden kaynaklandığı düşünülebilir.

Çalışmamızda, TRAIL'a dirençlilik gösteren DU145 ve LNCaP hücrelerinde gözlenen yüksek hücre içi NF-kB aktivasyonu, TRAIL dirençliliği ile uyumlu görülmektedir. Ancak TRAIL'a dirençli LNCaP'te hücre içi NF-kB aktivasyonu, DU145'a göre düşük bulunmuştur. PC3 hücreleri ise, LNCaP hücrelerine göre yüksek hücre içi NF-kB aktivasyonuna sahip olmasına rağmen çalışmamızda TRAIL'a önemli oranda duyarlılık göstermiştir. Bu bulgular, hücrelerde TRAIL dirençliliğinden tamamen hücre içi NF-kB aktivasyon seviyelerinin sorumlu tutulamayacağını, bilinmeyen başka faktörlerin de bu dirençlilikte etkili olduğunu göstermektedir.

Önceki çalışmalarda, TRAIL'a dirençli tümör hücre hatlarında TRAIL-R1 ve TRAIL-R2 ölüm reseptörlerinin ve TRAIL-R4 yalancı reseptör aktivasyonunun da NF-kB sinyal yolunu stimüle ederek hücre içi NF-kB aktivasyonunu artırabileceği bildirilmiştir [121, 122, 156]. TRAIL indüksiyonunun hem apoptotik hem de anti-apoptotik yolları aktive edebilmesi, TRAIL'ın da Tumor Necrosis Factor (TNF) gibi kendi apoptotik etkisini anti-apoptotik yolları indükleyerek nötralize ettiğine işarettir. Bu bulgular ışığında çalışmamızda TRAIL'ın indüklediği NF-kB aktivasyon seviyeleri araştırıldığında, hücrelere TRAIL aktarımının, her üç hücre hattında da endojen NF-kB aktivasyonunda artış meydana getirdiği görüldü (Şekil 4.6)

Son yıllarda, kanser hücrelerindeki apoptotik ligand dirençliliğini kırmak için, iyonize radyasyon ve kemoterapötik ajanların kullanımı gibi yöntemler üzerinde çalışılmıştır. Örneğin Mitsiades ve ark., multiple myeloma (MM) hücre hatlarının, TRAIL uygulaması öncesinde, TRAIL-R2 sentezini artıran Doxorubicin ile muamelesinin MM hücrelerinde TRAIL duyarlılığını önemli oranda artırdığını göstermiştir [165]. Ayrıca, genotoksik ajan etopozitin epitel hücre kökenli kanser hücre hatlarında TRAIL-R1 ve TRAIL-R2 sentezini artırdığı, ve TRAIL'a duyarlılık sağladığı gösterilmiştir [142]. Bunun yanında, bir başka apoptotik ligand olan TNF tarafından indüklenen apoptozisin, NF-kB aktivasyonu ile engellenebileceği bildirilmiş, ve farklı stratejilerle bu dirençlilik kırılmaya çalışılmıştır. Örneğin akciğer kanser hücrelerinde adenovirüsler aracılığıyla IKKβKA [150, 151] veya IkBαSR [158] aktarımı gibi NF-kB aktivitesini inhibe edici yaklaşımların etkisi araştırılmıştır. Bu çalışmalarda, akciğer hücreleri başarılı bir şekilde TNF'e duyarlı hale getirilmiştir. Prostat kanser hücrelerinde sürekli hücre içi NF-kB aktivitesinden IKK aktivitesindeki artışın sorumlu olduğu bildirildiğinden [149], çalışmamızda IKK inhibisyonunu içeren bir gen tedavi yaklaşımı kullanarak prostat kanseri hücrelerini TRAIL'a duyarlı hale getirebileceğimizi düşündük. Şekil 4.7'de görüldüğü gibi, TRAIL'a dirençli olan DU145 ve LNCaP hücre hatları, IKKβ'nın dominant negatif mutantını sentez eden bir NF-kB inhibe edici vektör (AdIKKβKA) ile birlikte enfekte edildiklerinde Ad5hTRAIL aracılı apoptozise duyarlı hale geldi. TRAIL'a orta derecede duyarlılık gösteren PC3 hücreleri ise, bahsedilen strateji ile TRAIL'a tam duyarlı hale geldi. Prostat kanser hücrelerinin AdIKKβKA virüsü ile tek başına enfeksiyonu ise hücre ölümüne yol açmadı (Şekil 4.9).

Önceleri yapılan çalışmalarda, TRAIL-R3 ve TRAIL-R4 yalancı reseptörlerine ait mRNA'larının çoğunlukla normal hücrelerde bulunduğu, ancak tümör hücrelerinde bulunmadığı bildirilmişti [117, 118]. Bu nedenle, TRAIL reseptörlerinin hücrelerde dağılımının, TRAIL aracılığıyla indüklenen apoptozisi regüle eden faktör olduğu düşünülmüştü. Bu hipotezle uyumlu olarak, endojen TRAIL-R4 yalancı reseptörleri olmayan melanoma hücrelerinde TRAIL-R4 sentezinin transfeksiyon deneyleri ile geri kazandırılmasının, hücreleri TRAIL'a duyarlı fenotipten TRAIL'a dirençli fenotipe geçirdiği gösterilmiştir [166]. Ancak, farklı orijinli kanser hücre hatlarında yapılan çalışmalar, hücre yüzeyindeki yalancı reseptörlerin sentezinin artması veya azalması ile TRAIL duyarlılığı arasında bir ilişki gösterememiştir. Örneğin insan melanoma hücre hatlarında TRAIL reseptör mRNA'larının seviyeleri araştırıldığında, TRAIL yalancı reseptörleri ile TRAIL'a dirençlilik ya da duyarlılık arasında bir ilişki gösterilememiştir [167]. Bunun yanında, melanoma, kolon

karsinoma, meme adenokarsinoma, ve akciğer adenokarsinoma gibi tümör hücre hatlarında uygulanan regüler RT-PCR deneyleri, TRAIL dirençliliği ile TRAIL yalancı reseptör gen sentezleri arasında herhangi bir ilişki açığa çıkarmamıştır [97]. Çalışmamızda, TRAIL dirençliliği ile ilgili olarak NF-kB dışında diğer olasılıkları araştırmak için, ileri evre prostat kanser hücre hatları TRAIL reseptör sentez düzeyleri açısından incelendi. TRAIL reseptör gen sentezinin miktarını belirlemek için, her TRAIL reseptörüne spesifik prob setleri kullanılarak bir kantitatif Gerçek Zamanlı RT-PCR deneyi gerçekleştirildi (Şekil 4.11). Deneyler sonucunda, sadece TRAIL'a dirençli hücre hatları olan DU145 ve LNCaP hücrelerinde önemli seviyede TRAIL-R3 ve TRAIL-R4 sentezi belirlendi. PC3 hücrelerinde TRAIL-R4 reseptör sentezinin olmamasının nedeni henüz bilinmemektedir. Ancak, yakın tarihli bir çalışmada, TRAIL yalancı reseptörlerinin susturulmasına neden olan anormal promotor metilasyonunun prostat kanseri de dahil olmak üzere birçok kanser tipinde sık olarak görüldüğü (%60) bildirilmiştir [120] Griffith ve arkadaşlarının çalışmalarında TRAIL reseptör sentez düzeyleri, regüler RT-PCR ile araştırılmıştır. Regüler RT-PCR deneyi her ne kadar bilgi verici olsa da, hücre yüzeyinde sunulan reseptör miktarları hakkında kantitatif bilgi sağlayamaz. Ancak kanser hücrelerinde sentezlenen TRAIL ölüm reseptörlerinin ve yalancı reseptörlerin oranı hücrelerin TRAIL'a dirençliliğini etkileyebilecek önemli bir faktördür. Bu nedenle, reseptör sentez seviyelerinin Gerçek Zamanlı RT-PCR ile araştırılması gerekmektedir.

mRNA seviyeleri, her zaman hücre yüzeyindeki protein sentez seviyelerini yansıtmayacağı için, akış sitometrisi analizi ile hücre yüzeyindeki TRAIL reseptör sentez seviyeleri belirlendi (Şekil 4.12, 4.13). Sadece TRAIL'a dirençli olan DU145 (Şekil 4.12, Panel A) ve LNCaP (Şekil 4.13) hücrelerinde önemli miktarda TRAIL-R4 sentezi olduğu görüldü. TRAIL'a duyarlı PC3 hücrelerinde ise benzer bir durum gözlenmedi (Şekil 4.12, Panel B). Ayrıca, DU145 ve PC3 hücrelerindekinin tersine, TRAIL-R3 yalancı reseptörünün sadece LNCaP hücrelerinde sentez edildiği belirlendi (Şekil 4.13).

Yakın tarihli bir çalışmada, Perlman ve arkadaşları, römatoid artrit sinovial fibroblastlarında ölüm reseptörlerinin yokluğunun TRAIL dirençliliğine sebep olduğunu bildirmiştir [168] Bizim çalışmamızda ise, ölüm reseptörleri yokluğundan ziyade yalancı reseptörlerin varlığının prostat kanser hücrelerinde TRAIL dirençliliğine sebep olduğu sonucuna varıldı. Bu bulgularla uyumlu olarak, TRAIL-R3 veya TRAIL-R4 yalancı reseptörleri ile transfekte edilen TRAIL'a duyarlı hücrelerde apoptotik hücre ölümünde azalma gözlenmiştir [117, 118]. Bunun yanında, TRAIL-R4 yalancı reseptörünün, hücreleri TRAIL-aracılı apoptozisten korumada TRAIL-R3'e göre daha etkili olduğu bildirilmiştir [117] Bizim çalışmamızda da, TRAIL'a dirençli hücre hatları olan DU145 ile LNCaP'te TRAIL-R4 reseptör sentez seviyesi, TRAIL-R3 reseptör seviyesine göre oldukça yüksek bulunmuştur (Şekil 4.12 ve Şekil 4.13).

Sonuçlarımıza göre, hem TRAIL yalancı reseptör kompozisyonu, hem de hücre içi NF-kB aktivitesi, prostat kanser hücrelerinde TRAIL dirençliliğine katkıda bulunan iki önemli faktördür Ayrıca, hücrelerdeki NF-kB aktivasyon düzeylerine, reseptör sentez profillerine veya androjen bağımlılık durumlarına bakılmaksızın, IKK

inhibe edici stratejilerin, TRAIL dirençliliğini kırmada oldukça etkili olduğu görülmüştür. Çalışmamız, prostat kanser hücrelerinde TRAIL reseptör kompozisyonu ile TRAIL dirençliliği arasında anlamlı bir ilişki açığa çıkarmıştır. Bunun yanında, çalışmamız, Ad5hTRAIL ve AdIKKβKA ikili vektör sistemi ile prostat kanser hücrelerinde TRAIL duyarlılığı kazandıran ilk çalışma olması açısından da önemlidir. Bu bulgular ışığında, IKK inhibisyonu altında adenoviral yolla TRAIL gen aktarımı, prostat kanserli hastalarda TRAIL'ın terapötik indeksini genişletmede değerli bir yaklaşım olacaktır.

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ÖZGEÇMİŞ

1971 yılında Ankara'da doğan Ahter D. (TORAMAN) ŞANLIOĞLU, ilköğrenimini 1977-1981 yılları arasında babasının bayındırlık müdürlüğü görevi nedeniyle farklı ilkokullarda yapmış, ve Ankara Namık Kemal İlkokulu'nda tamamlamıştır. 1981-1988 yılları arasında Ankara Özel Yükseliş Lisesi ve İstanbul Özel Dost Lisesi'nde orta ve lise öğrenimi görmüştür. 1988-1990 yılları arasında Amerika Birleşik Devletleri'nde Kuzey Carolina eyaletinde Fayetteville Technical Institute'ta burslu olarak biyoloji ağırlıklı dersler almıştır. 1990-1993 yılları arasında turizm alanında çalışmıştır. 1993 yılında öğrenimine başladığı Akdeniz Üniversitesi Fen Fakültesi Biyoloji Bölümü'nden, 1997 yılında bölüm dördüncüsü olarak mezun olmuştur. 1998 yılında Akdeniz Üniversitesi Tıp Fakültesi Tıbbi Biyoloji ve Genetik Anabilim Dalı'nda araştırma görevlisi olarak göreve başlamış, bunun yanında Akdeniz Üniversitesi Sağlık Bilimleri Enstitüsü'ne bağlı olarak, Tıbbi Biyoloji ve Genetik Anabilim Dalı'nda Temel Genetik yüksek lisans programına kayıt olmuştur. 2001 yılında yüksek lisans programından mezun olmuş, ve aynı Enstitü ve aynı Anabilim Dalı'nda Tıbbi Genetik doktora programına başlamıştır.

2001 yılında 10. ICHG (International Congress of Human Genetics) Kongresi'ne katılım için genç bilim adamı katılım bursu, 2003 yılında 11. ESGT (European Society of Gene Therapy) Kongresi'ne katılım için ise genç bilim adamı seyahat ödülü almıştır. 2001 yılında Eskişehir'de düzenlenen 7. Ulusal Tıbbi Biyoloji Kongresi'nde poster birincilik ödülü, 2003 yılında Adana'da düzenlenen 8. Ulusal Tıbbi Biyoloji Kongresi'nde Altan Günalp Araştırma Birincilik Ödülü almıştır. 2004 yılında Evry, Fransa'da düzenlenen "Endüstri Kalitesinde Gen Vektörlerinin İleri Düzeyde Üretimi, Saflaştırılması ve Karakterizasyonu" kurs ve kongresine tamamen burslu olarak katılmıştır. 2004 yılında TÜBİTAK aracılığıyla verilen NATO-A2 Yurtdışı Doktora Araştırma Bursu'nu (Amerika Birleşik Devletleri) almaya hak kazanmıştır. Science Citation Index'e kayıtlı bilimsel dergilerde 7 makalesi yayınlanmış, 1 makalesi yayına kabul edilmiştir. Ulusal ve uluslararası kongrelerde 23 bildirisi bulunmaktadır.

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ORIGINAL ARTICLE

Adenovirus-mediated IKKβKA expression sensitizes prostate carcinoma cells to TRAIL-induced apoptosis

AD Sanlioglu[1,2], IT Koksal[1,3], B Karacay[1,4], M Baykara[1,3], G Luleci[1,2] and S Sanlioglu[1,2]

[1]*Human Gene Therapy Unit, Akdeniz University, Faculty of Medicine, Antalya, Turkey,* [2]*Department of Medical Biology and Genetics, Akdeniz University, Faculty of Medicine, Antalya, Turkey,* [3]*Department of Urology, Akdeniz University, Faculty of Medicine, Antalya, Turkey and* [4]*Center for Gene Therapy at the University of Iowa, Iowa City, IA, USA*

Despite the fact that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) can selectively induce apoptosis in cancer cells, TRAIL resistance in cancer cells has challenged the use of TRAIL as a therapeutic agent. First, prostate carcinoma cell lines (DU145, LNCaP and PC3) were screened for sensitivity to adenovirus delivery of TRAIL (Ad5hTRAIL). As amplified Ikappa B kinase (IKK) activity is responsible for the constitutive nuclear factor-κB (NF-κB) activation leading to uncontrolled cell growth and metastasis, a dual vector approach using both an adenovirus vector (Ad) expressing the dominant-negative mutant of IKKβ (AdIKKβKA) and Ad5hTRAIL was employed to determine if prostate cancer cells were sensitized to TRAIL in the setting of IKK inhibition. Inhibition of the NF-κB pathway through IKK blockade sensitized all three prostate cancer cell lines to TRAIL, regardless of NF-κB activation or decoy receptor gene expression. Moreover, a novel quantitative real-time RT-PCR assay and conventional flow cytometry analysis indicated that TRAIL-resistant DU145 and LNCaP cells, but not TRAIL-sensitive PC3 cells, expressed substantial amounts of TRAIL Decoy Receptor 4. In conclusion, TRAIL decoy receptor expression appeared to be the chief determinant of TRAIL resistance encountered in prostate carcinoma cell lines.

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Keywords: prostate carcinoma; TRAIL resistance; decoy receptors; IKK

Introduction

Chemotherapy and radiotherapy are among the most commonly used treatment modalities for cancer. Tumor suppressor gene p53 is required for both of these treatment methods to function as antitumor agents.[1] But, more than 50% of human tumors acquire p53 mutations in the course of tumorigenesis.[2] As a result, tumors without a functional p53 gene are resistant to both chemotherapy and radiotherapy.[3] However, death ligands induce apoptosis independent of the cells' p53 status.[4] Thus, direct induction of cell death through the activation of death receptors is a viable complementary approach to conventional treatment modalities.[5,6] Among the death ligands tested, tumor necrosis factor (TNF)[7] and FasL[8] have been demonstrated to efficiently induce apoptosis in cancer cells. But their systemic use in cancer gene therapy is not feasible due to their systemic toxicity. On the other hand, TNF-related apoptosis-inducing ligand (TRAIL)[9] is not toxic for normal cells, but selectively induces apoptosis in cancer cells.[10] Despite these properties, TRAIL resistance observed in some cancer cell lines represents a handicap for any proposed gene therapy approach utilizing TRAIL as a death ligand.[11]

Two different hypotheses have been asserted to explain the molecular mechanisms of TRAIL resistance. The first hypothesis suggests that normal cells carry decoy receptors (TRAIL-R3, TRAIL-R4) that compete against death receptors (TRAIL-R1, TRAIL-R2) for binding to TRAIL.[12] These receptors either dilute TRAIL ligands (like TRAIL-R3) or supply antiapoptotic signals (like TRAIL-R4) to cells. While the presence of decoy receptor gene expression can account for TRAIL resistance in normal cells, the lack of decoy receptor gene expression in tumor cells would be expected to lead to TRAIL sensitivity.[13] The second hypothesis advocates the presence of apoptosis inhibitory substances, such as cFLIP (FLICE inhibitory protein), in cancer cells.[14] Intriguingly, chemotherapeutic agents augmented TRAIL-induced apoptosis in prostate cancer cells through upregulation of death receptors[15] and/or downregulation of cFLIP expression.[16] In addition, engagement of the TRAIL-R1 receptor, using an antibody in combination with doxorubicin, selectively killed prostate cancer cells.[17] Despite these studies investigating TRAIL receptors in prostate cancer, a direct link between TRAIL sensitivity and the

Correspondence: Professor S Sanlioglu, Akdeniz University, Faculty of Medicine, B-Block, 1st floor, Campus, Antalya 07070, Turkey
E-mail: sanlioglu@akdeniz.edu.tr
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pattern of TRAIL receptor gene expression is yet to be proven for prostate cancer cells

Both TRAIL-induced and the endogenous nuclear factor-κB (NF-κB) activities present in cancer cells have recently been under heavy investigation due to NF-κB's role in the constitution of TRAIL resistance.[18] The fact that NF-κB-stimulating agents upregulated the synthesis of TRAIL-R3 decoy receptor,[19] and apoptosis inhibitors such as Bcl-xL,[20] cIAP[21] and cFLIP,[22] suggested that NF-κB activation may contribute to TRAIL resistance in at least four different ways. Interestingly, TRAIL-R4 receptor engagement stimulated the NF-κB signaling pathway and blocked TRAIL-induced cell death.[23] Furthermore, TRAIL binding to death receptors (TRAIL-R1 and TRAIL-R2) also activated the NF-κB pathway.[24,25] Thus, why some cancer cells still undergo apoptosis, despite the activation of the antiapoptotic NF-κB pathway, is not known.

To examine the contribution of the NF-κB pathway to TRAIL resistance, prostate carcinoma cell lines were analyzed in terms of basal NF-κB activation using NF-κB-mediated transcription activation assay.[26] Following the screening for TRAIL resistance, complementary gene therapy modalities targeting the NF-κB pathway through the inhibition of Ikappa B kinase (IKK)[27,28] were also utilized to determine whether this approach is useful in breaking down TRAIL resistance in prostate cancer cell lines. Lastly, the TRAIL receptor composition of prostate cancer cells and its connection to TRAIL resistance were studied in detail using both conventional flow cytometry and novel quantitative real-time RT-PCR techniques.

Materials and methods

Production of recombinant adenovirus vectors

Recombinant adenoviral stocks Ad5hTRAIL,[29] AdIKKβKA,[26] Ad enhanced green fluorescent protein (EGFP),[27] AdCMVLacZ[30] and AdNFkBLuc[26] were amplified as described previously,[31] and were stored in 10 mM Tris with 20% glycerol at −80°C. AdIKKβKA is a first-generation adenovirus vector expressing kinase inactive IKKβ. The expression of the dominant-negative IKKβ (IKKβKA) generates functionally inactive IKK complexes by interacting with other IKK subunits. The particle titers of adenoviral stocks were obtained by A_{260} readings, and were in the range of 10^{13} DNA particles/ml, whereas functional titers were measured by plaque titering on 293 cells and expression assays for encoded proteins. Typically, the particle/plaque-forming unit ratio was 50.

Transduction of prostate cancer cells with first-generation recombinant adenovirus vectors

Prostate cancer cell lines LNCaP, PC3 and DU145 (ATCC) were grown in RPMI 1640 medium supplemented with 10% FBS, 2.2 g/l sodium bicarbonate, 1 mM L-glutamine and 1% penicillin–streptomycin mixture. All cells were maintained at 37°C in a humidified 5% CO_2 environment. Prostate cancer cell lines were transduced with adenoviral vectors expressing the EGFP reporter gene (AdEGFP). Briefly, prostate cancer cells were infected with an increasing multiplicity of infection (MOI) of AdEGFP vectors at 37°C in RPMI 1640 without FBS. The serum concentration in the tissue culture media was increased to 10% by adding an equal volume of RPMI 1640 supplemented with 20% FBS 2 h described after the infection. The level of transduction was determined by assessing percent GFP (+) cells 48 h after the infection under fluorescent microscopy than using flow cytometry. Cell viability was determined by propidium iodide (PI) exclusion. The Ad5hTRAIL construct was used to overexpress hTRAIL in prostate cancer cells. To block IKK activity, and thereby NF-κB activation, an adenoviral vector encoding the IKKβ dominant-negative mutant (AdIKKβKA) was utilized in coinfection experiments in conjunction with Ad5h-TRAIL. An AdNFkBLuc construct carrying NF-κB regulatory sites hooked up to a Luciferase reporter gene was deployed to conduct NF-κB-mediated transcription activation assays. This construct possessed a Luciferase reporter gene hooked up to the herpes simplex virus thymidine kinase gene promoter with four tandem copies of the NF-κB-binding consensus sequence.[27]

NF-κB-mediated transcription activation assay

AdNFkBLuc was employed to provide information on the prostate cancer cell's NF-κB activation status. The Luciferase assay system with Reporter Lysis Buffer (Promega, Inc.) was used to measure NF-κB-mediated transcriptional induction in the presence or absence of TRAIL expression according to the manufacturer's protocol. All measurements of Luciferase activity (relative light units) were normalized to the protein concentration.

Assessment of cell viability

Live/dead Cellular Viability/Cytotoxicity Kit from Molecular Probes (Eugene, OR) was used to discriminate the live cells from the dead cells. In this assay, Calsein AM, a fluorogenic substrate for intracellular calsein esterase, is modified to a green fluorescent compound (calsein), which is demonstrable only in live cells. Since only live cells with intact membranes contain active esterase, detection of calsein by fluorometric methods serves as a marker for viable cells. Ethidium homodimer-1 (EthD-1) is a red fluorescent nucleic acid stain that cannot disseminate across unharmed cell membranes. While intact cells exclude EthD-1, cells with damaged membranes take up the dye and stain positive.

Regular and quantitative real-time RT-PCR for human TRAIL receptors

Total RNA was extracted from LNCaP, DU-145 and PC-3 human prostate cancer cell lines using TRIzol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. In all, 2 μg of total RNA was reverse-transcribed into cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems Cat. N8080234). Regular RT-PCR reactions were carried out as described previously.[14] Our group has recently

published the sequences of TRAIL-R1 and TRAIL-R2 primers and probes in an article on neuroblastoma [32] However, since TRAIL-R3 and TRAIL-R4 decoy receptor probes have not been published, we designed new probe sets for the TRAIL decoy receptors The sequence of TRAIL decoy receptor sets is as follows: **TRAILR3 – 5′** CCC-TAA-AGT-TCG-TCG-TCG-TCA-T, **TRAILR3 – 3′** GGG-CAG-TGG-TGG-CAG-AGT-A, **TRAILR3 Probe – 5′** 6FAM-TCGCGGTCCTGCTGCCAGTCC-TAGC-TAMRA 3′; **TRAILR4 – 5′** ACA-GAG-GCG-CAG-CCT-CAA, **TRAILR4 – 3′** ACG-GGT-TAC-AGG-CTC-CAG-TAT-ATT, **TRAILR4 Probe – 5′** 6FAM-AGGAGGAGTGTCCAGCAGGATCTCATA-GATC-TAMRA 3′ The rRNA probes were labeled with a second dye to analyze TRAIL receptors and rRNA as an internal control in the same reaction The rRNA primers and probes were purchased from PE Applied Biosystems (Cat 4308329). A cloned cDNA fragment derived from ribosomal RNA was used to construct a standard curve Relative quantities of TRAIL receptors were calculated using the ΔΔCt method as described by Applied Biosystems The TaqMan PCR reaction was carried out as described in the manufacturer's protocols (Applied Biosystems Cat N8080228)

Annexin V binding

An FITC-conjugated mouse monoclonal antibody to human Annexin V (ALX-804-100F-T100) was employed for Annexin V binding using flow cytometry. Annexin V binding assay was performed according to the manufacturer's instructions (Alexis Biochemicals)

Flow cytometry analysis for adenovirus transduction and the detection of surface TRAIL receptor expression

Prostate cancer cells were seeded at approximately 2.5×10^5 cells per well in 24-well plates and then infected with AdEGFP reporter construct At 48 h postinfection, the cells were trypsinized and resuspended in PBS, following centrifugation. FACS analysis was carried out using BD FACSCALIBUR at the Akdeniz University Hospitals. To assess TRAIL receptor protein expression on the cell surface, unlabeled monoclonal antibodies specific for each TRAIL receptor subtype were employed using the anti-TRAIL receptor flow cytometry set (Cat. ALX-850-273-KI01) from Alexis Biochemicals. The set contained 100 μg each of MAb to TRAIL-R1 (clone HS101, Cat. 804-297A), -R2 (clone HS201, Cat 804-298A), -R3 (clone HS301, Cat 804-344A) and-R4 (clone HS402, Cat. 804-299A) All primary antibodies were used at 5 μg/ml concentration, followed by biotinylated goat anti-mouse IgG1 (Cat. ALX-211-202) and streptavidin-PE (Cat. ANC-253-050) Flow analysis was performed according to manufacturer's protocols. Purified mouse IgG1 (MOPC 31C, Cat ANC-278-010) was used as an isotype control.

Statistical analysis

The Prism program from GraphPad Software (San Diego, CA) was used for statistical analyses The statistical results for each experiment are provided in the figure legends

Results

Prostate carcinoma cell lines exhibited differential basal NF-κB activation levels

As constitutive NF-κB activation is claimed to be a prominent factor influencing the sensitivity of tumor cells to the apoptosis-inducing effects of death ligands,[28 33–35] unstimulated (basal) NF-κB activation levels were analyzed using three different prostate cancer cell lines (DU145, PC3 and LNCaP) Prostate cancer cell lines were infected with a recombinant adenovirus vector carrying the NF-κB-driven Luciferase reporter gene (AdNFkBLuc) for 24 h prior to harvesting NF-κB-mediated transcription activation assays were performed using the Luciferase assay system with reporter lysis buffer As shown in Figure 1, the highest constitutive NF-κB activation level was detected in DU145 cells, followed by PC3 The lowest NF-κB activation was observed in LNCaP cells Luciferase assays conducted 48 h following the infection yielded a much higher magnitude of NF-κB activation, but this did not change the order of activation, DU145 leading PC3, followed by LNCaP (data not shown) AdCMVLacZ infection did not generate any readable Luciferase activity compared to uninfected controls (data not shown). Thus, the prostate cancer cell lines PC3, LNCaP and DU145 exhibited substantially different levels of constitutive NF-κB activation

Differences in constitutive NF-κB activation levels detected in prostate cancer cells were not caused by differential adenovirus transduction

A reporter adenovirus vector encoding the enhanced green fluorescent gene (AdEGFP) was infected into

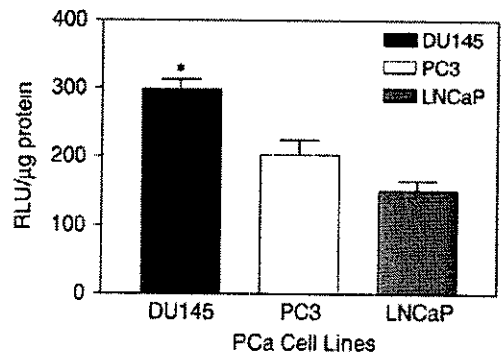

Figure 1 Prostate cancer cell lines display diverse NF-κB activation levels Prostate cancer cell lines were infected with an MOI of 5000 DNA particles/cell of AdNFkBLuc construct for 24 h Luciferase activity was measured as described in Materials and methods Cell types used in the infection are provided on the *x*-axis Luciferase activity expressed as relative light units (RLU) per microgram protein is given on the *y*-axis Data represent the mean (±s e m) of six independent data points ANOVA followed by Tukey's multiple comparison tests indicated the existence of a statistically significant difference between DU145 versus PC3 and LNCaP *$P<0.01$

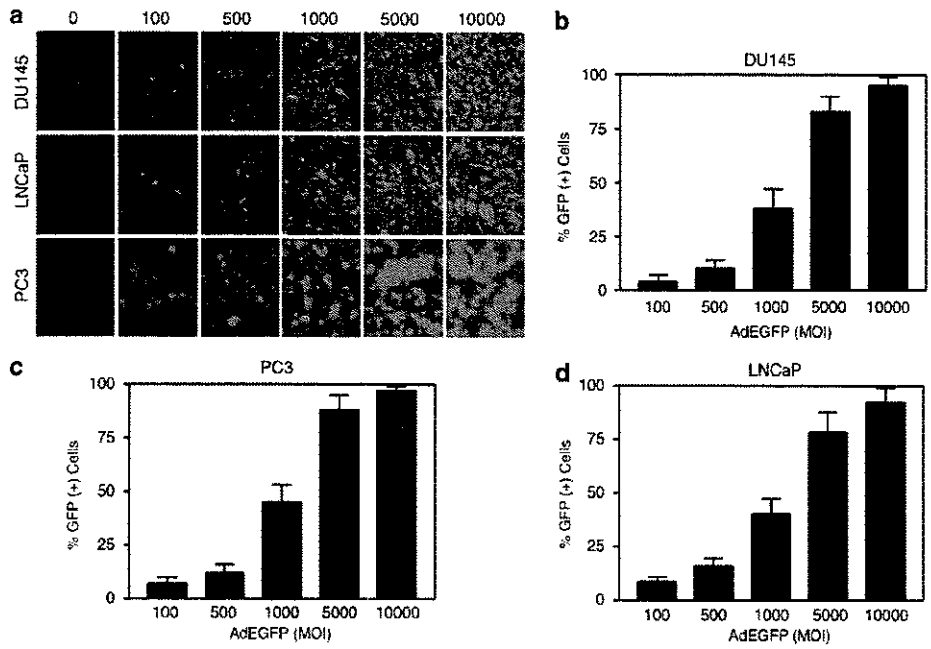

Figure 2 Transduction of prostate carcinoma cell lines by first-generation recombinant adenovirus vectors Prostate carcinoma cell lines were infected with an adenovirus encoding the EGFP reporter gene (AdEGFP) at increasing MOIs for 48 h as described in Materials and methods. Panel a displays fluorescent micrographs of such adenovirus transductions. MOI values (DNA particles/cell) of viruses used in the infection are given above each fluorescent micrograph Corresponding results from flow cytometry are illustrated in panel b (DU145), panel c (PC3) and panel d (LNCaP) Numbers displayed on the *x*-axis represent viral doses applied in MOI values as DNA particles/cell. Values represent the mean (±s.e.m.) of three different experiments

prostate cancer cells at increasing MOIs to determine if differences in NF-κB activation among the prostate carcinoma cell lines were due to differential transduction by adenovirus vectors. Percent EGFP-positive cells were determined by fluorescent microscopy (Figure 2a) and quantified by flow cytometry (Figure 2b–d) 48 h following the infection Flow cytometry indicated that equal levels of adenoviral transduction were observed in all three prostate carcinoma cell lines. These results clearly demonstrated that differences in basal NF-κB activity were not due to differential adenovirus transduction of prostate carcinoma cell lines. This experiment was also pivotal in determining the optimum adenoviral transduction dose of prostate carcinoma cell lines for gene therapy purposes. While an MOI of 5000 DNA particles/cell of AdEGFP was sufficient to transduce more than 90% of prostate carcinoma cell lines, almost 100% transduction efficiency was achieved when cells were infected with an MOI of 10 000 DNA particles/cell Nonetheless, all three prostate carcinoma cell lines were transduced efficiently and equally by AdEGFP. Thus, the differential transduction by adenovirus cannot be accounted for by the disparity in NF-κB activation observed in prostate carcinoma cell lines

Prostate carcinoma cell lines manifested diverse levels of TRAIL resistance

The cytotoxic effects of TRAIL overexpression in prostate cancer cell lines were examined by infecting the cells with increasing doses of Ad5hTRAIL or Ad-CMVLacZ vectors. Cell viability assays were conducted using Molecular Probe's Live/Dead Cellular Viability/Cytotoxicity Kit 48 h following the infection (Figure 3a). Analysis under fluorescent microscopy revealed that prostate carcinoma cell lines LNCaP and DU145 were highly resistant to TRAIL, even when these cells were infected with an MOI of 10 000 DNA particles/cell of Ad5hTRAIL virus. No significant cell death was observed upon AdCMVLacZ infection (Figure 3b). On the other hand, PC3 cells exhibited 63% cell death at an MOI of 5000 DNA particles/cell and 90% cell death at an MOI of 10 000 DNA particles/cell of Ad5hTRAIL virus (Figure 3b). Thus, the PC3 cell line showed substantially greater levels of TRAIL sensitivity than the DU145 or LNCaP cell lines

Recently, elevated NF-κB activation in prostate carcinoma cell lines has been attributed to increased IKK activity [36] Therefore, we sought to determine whether inhibition of IKK activity might decrease the viability of prostate cancer cells. An adenovirus expressing the dominant-negative mutant of IKKβ (AdIKKβKA) was infected into prostate cancer cell lines at increasing MOIs. Cell viability was examined under the fluorescent microscope 48 h following the infection Contrary to the TRAIL cytotoxicity observed in PC3 cells, no significant decrease in cell viability, even at an MOI of 10 000 DNA particles/cell of AdIKKβKA construct, was observed in any of the three prostate carcinoma cell lines (data not

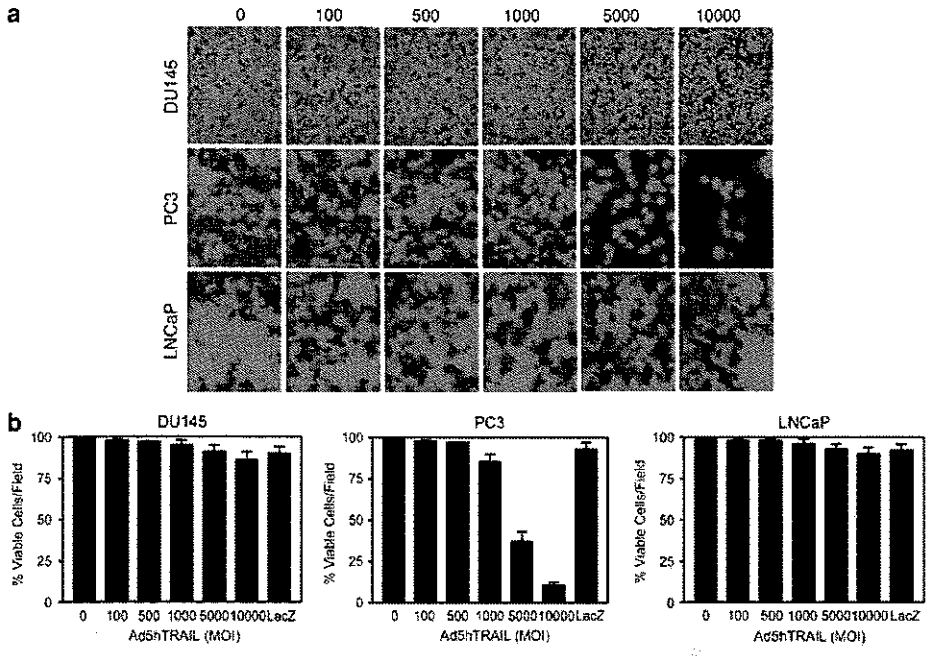

Figure 3 Prostate cancer cells exhibit distinctive patterns of TRAIL sensitivity LNCaP, DU-145 and PC3 cells were infected with increasing MOIs of Ad5hTRAIL or AdCMVLacZ constructs Cell viability was detected using Molecular Probe's Live/Dead Cellular Viability/Cytotoxicity Kit 48 h following the infection Only fluorescent micrographs of FITC channel are shown in panel a Numbers represent viral doses applied in MOI values as DNA particles/cell Quantitative results from the cell viability assays of prostate carcinoma cell lines are provided in panel b LacZ columns in panel b refer to MOIs of 10 000 DNA particles/cell of AdCMVLacZ vector used in the infection. Cell viability assays were performed in triplicates and repeated at least twice to confirm the observation Values represent the mean (±s e m) of six independent data points

shown). Therefore, infection with IKK inhibiting adenovirus vector alone did not induce cell death in prostate cancer cell lines 48 h following infection

While TRAIL overexpression increased intracellular NF-κB activity, IKK inhibiting strategy counteracted both TRAIL-induced and endogenous NF-κB activity

TRAIL decoy receptor TRAIL-R4[23] and TRAIL death receptors (TRAIL-R1 and TRAIL-R2)[24,25] have been shown to activate the NF-κB pathway If TRAIL is to be used as a death ligand to induce apoptosis in cancer cells, then TRAIL-induced NF-κB activation and the cell's endogenous NF-κB status should be considered before the therapy To study the extent of NF-κB activation by TRAIL expression in prostate carcinoma cell lines, PC3, LNCaP and DU145 cells were coinfected with AdNFkBLuc and Ad5hTRAIL vectors To minimize cell death, the Ad5hTRAIL concentration was kept constant at an MOI of 1000 DNA particles/cell and NF-κB Luciferase assays were conducted 24 h following the infection As shown in Figure 4, NF-κB activity was increased only in TRAIL-overexpressing prostate cancer cells

To determine the magnitude of NF-κB inhibition, prostate carcinoma cell lines were superinfected with AdIKKβKA virus at increasing doses in addition to coinfection with AdNFkBLuc and Ad5hTRAIL TRAIL-induced NF-κB activity and basal NF-κB activities were drastically reduced in all three prostate carcinoma cell lines (Figure 4a–c) In contrast, no such NF-κB inhibiting effect was observed when cells were superinfected with AdCMVLacZ virus as a control

Functional IKK inhibition via IKKβKA expression-sensitized prostate cancer cells to TRAIL-induced cell death

NF-κB-inhibiting strategies involving the use of adenovirus delivery of IKKβ (AdIKKβKA)[27,35] or IkBα(AdIkBαSR)[30,37] dominant-negative mutants have been successful in sensitizing lung cancer cells to TNF death ligand As some cancer cells have higher intrinsic NF-κB activity, NF-κB-blocking agents can potentially be very valuable to sensitize these cells to the apoptosis-inducing effects of TRAIL To test whether IKK inhibition sensitizes prostate carcinoma cell lines to TRAIL, the cell lines were coinfected with both Ad5hTRAIL construct at a constant MOI of 5000 and increasing doses of AdIKKβKA virus All three prostate carcinoma cell lines were sensitized to TRAIL regardless of the cell's prior constitutive NF-κB activation status (Figure 5a) Approximately 50% cell death was observed, even at an MOI of 1000 AdIKKβKA virus in the presence of TRAIL expression in DU145 and LNCaP cell lines As PC3 cells were already sensitive to TRAIL, the degree of TRAIL sensitization induced by IKKβKA expression in DU145 and LNCaP cells were much higher than in PC3 cells (Figure 5b) On the other hand, no TRAIL sensitization was observed when prostate carcinoma cell

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lines were infected with AdCMVLacZ virus in place of AdIKKβKA. Therefore, NF-κB blocking through IKK inhibition might be very useful in the treatment of prostate cancer patients displaying TRAIL resistance.

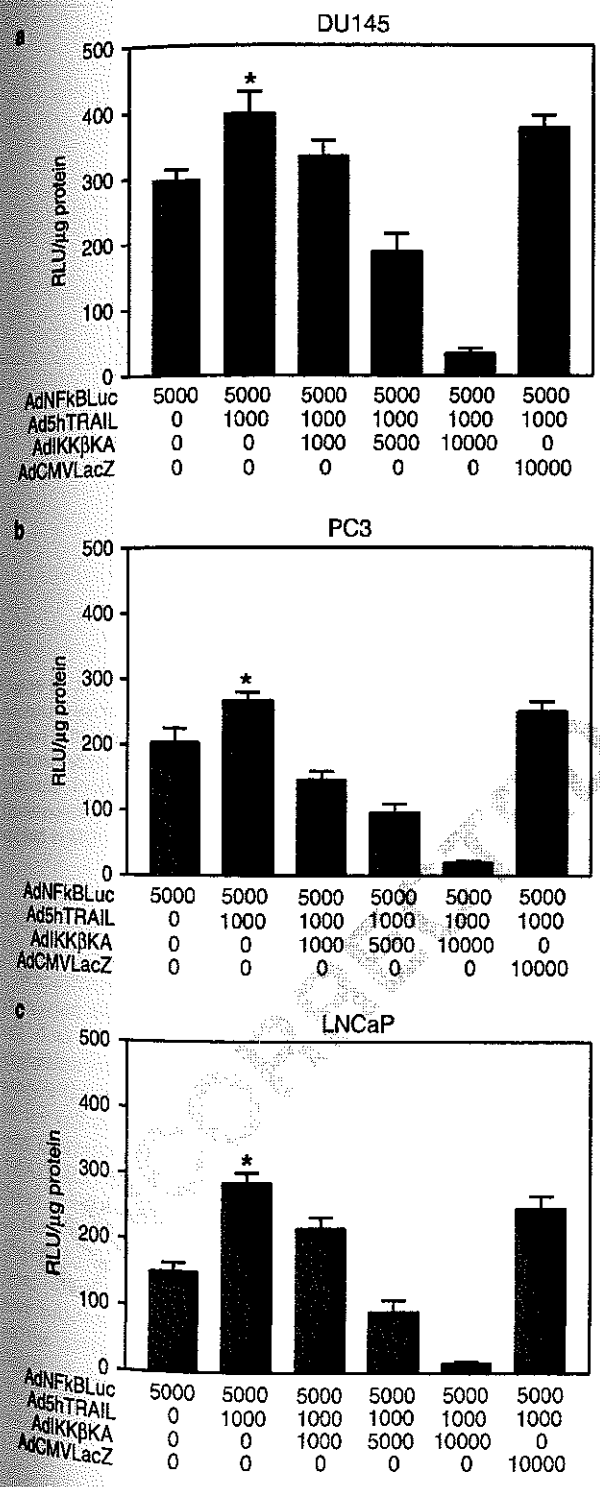

Figure 4 NF-κB activity of prostate cancer cell lines is increased by Ad5hTRAIL infection, but downregulated by AdIKKβKA. DU145 (**a**), PC3 (**b**) and LNCaP (**c**) cell lines were simultaneously infected with AdNFkBLuc, Ad5hTRAIL and/or increasing doses of AdIKKβKA constructs for 24 h. These cell lines were also infected with AdCMVLacZ as a control. Cells were harvested for luciferase activity 24 h after the infection. Both the MOI values provided as DNA particles/cell and the types of constructs used in the infection are given on the *x*-axis. To avoid cell death complicating our assay result, the titer of Ad5hTRAIL was lowered to an MOI of 1000 DNA particles/cell instead of 5000 or 10 000 used in cell viability assays. The *y*-axis shows the luciferase activity expressed in RLU per microgram protein. Data represent the mean (±s.e.m.) of six independent data points. Student's *t*-test was used to reveal the statistical difference between AdNFkBLuc and AdNFkBLuc/Ad5h-TRAIL coinfected cells. $^{*}P<0.05$

Ad5hTRAIL and AdIKKβKA coinfection-induced apoptotic cell death in prostate carcinoma cells

Annexin V staining was performed using flow cytometry to reveal the molecular mechanism of cell death induced by TRAIL under the setting of IKK inhibition. For this purpose, Ad5hTRAIL and AdIKKβKA vectors were infected separately or in combination into the prostate carcinoma cell line DU145. Annexin V binding assays were conducted 35 h following the infection using flow cytometry. As shown in Figure 6a, Ad5hTRAIL or AdIKKβKA infection alone did not generate any significant degree of Annexin V binding. However, when the cells were coinfected with both Ad5hTRAIL and AdIKKβKA, considerable levels of Annexin V binding were observed, indicating that prostate carcinoma cells were undergoing apoptosis (Figure 6b). On the other hand, AdCMVLacZ coinfection (negative control) together with Ad5hTRAIL did not yield any substantial levels of Annexin V binding, suggesting that, in the absence of IKK inhibition, DU145 cells were resistant to TRAIL expression. Taken together, these results suggest that the cell death induced by TRAIL in the setting of IKK inhibition is apoptosis.

Differential TRAIL receptor expression patterns were observed in prostate cancer cell lines

Although RT-PCR is a quick and useful test in assessing whether a relevant gene is expressed in a particular cell line, the technique does not provide quantitative information regarding gene expression. For this reason, a quantitative real-time RT-PCR analysis was conducted using primer-probe sets specifically designed to detect TRAIL receptor gene expression in prostate carcinoma cell lines. As shown in Figure 7, levels of TRAIL-R2 gene expression were higher than levels of TRAIL-R1 in all the cell lines. PC3 cells, which are TRAIL-sensitive, expressed greater levels of the TRAIL-R2 receptor than did the DU145 and LNCaP cell lines, which are TRAIL-resistant (Figure 7). While very low levels of TRAIL-R3 expression were detectable in PC3 cells, no TRAIL-R4 expression was found. Furthermore, TRAIL-resistant DU145 and

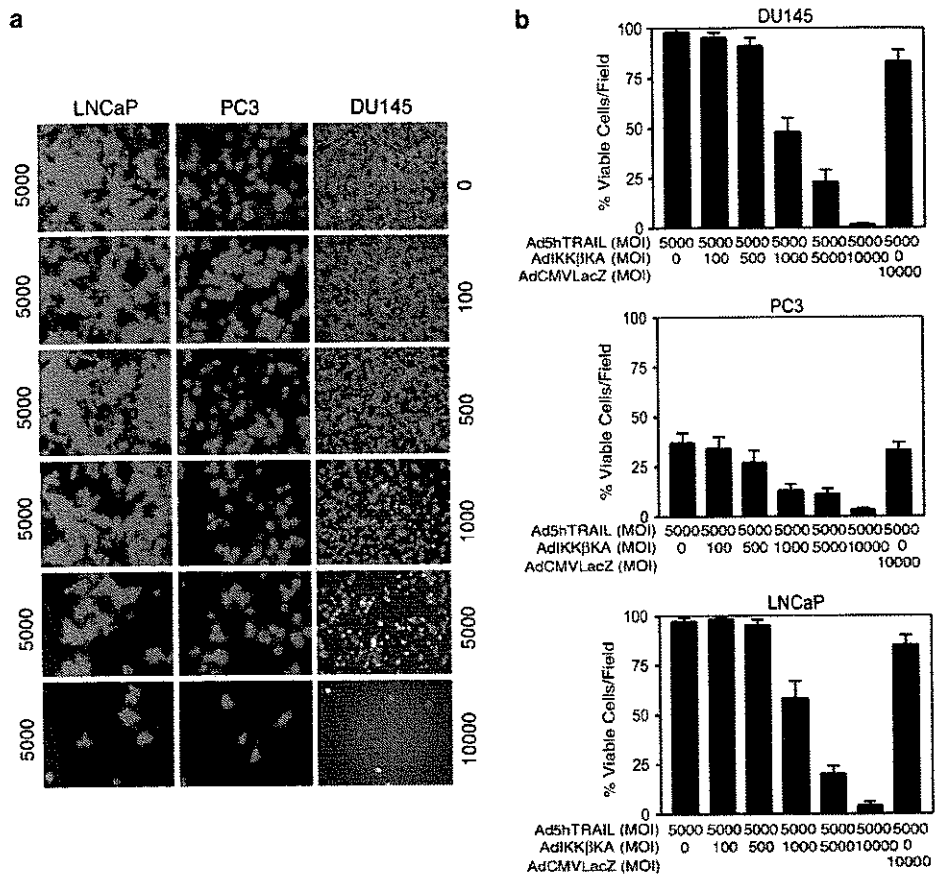

Figure 5 IKKβKA expression via adenoviral vectors defeated the resistance to TRAIL-induced apoptosis in prostate carcinoma cell lines Adenoviral vectors encoding the dominant-negative mutant of IKKβ or LacZ were infected into LNCaP DU145 and PC3 cells at increasing doses (shown to the right of fluorescent micrographs) while simultaneous infection with Ad5hTRAIL was performed at a constant MOI of 5000 DNA particles/cell (as shown to the left of fluorescent micrographs) Cell viability was assessed using Live/Dead Cellular Viability/Cytotoxicity Kit from Molecular Probes 48 h following infection Fluorescent micrographs of cell viability assays (only FITC channel) are provided in panel a Corresponding percent viable cell counts/ × 20 field are shown in panel b for DU145, PC3 and LNCaP cells Numbers represent viral doses applied in MOI values of DNA particles/cell as depicted on the *x*-axis Values represent the mean (±s e m) of six independent data points

LNCaP cell lines expressed both decoy receptors (TRAIL-R3 and TRAIL-R4) at significant levels

While real-time RT-PCR is useful in quantifying gene expression at the mRNA level, gene expression inside the cell does not necessarily correlate with the receptor expression on the cell surface. Therefore, we decided to analyze the level and type of TRAIL receptor expression on the cell surface using flow cytometry. Although PC3 cells expressed TRAIL death receptors (TRAIL-R1 and TRAIL-R2) on the cell surface, no measurable level of decoy receptor gene expression was evident on the surface of these cells (Figure 8b) Despite the fact that DU145 (Figure 8a) and LNCaP cells (Figure 8c) expressed both types of TRAIL death receptors, contrary to PC3, there were substantial amounts of TRAIL-R4 decoy receptors on the surface of both cell types Furthermore, some degree of surface TRAIL-R3 decoy receptor expression was detectable in LNCaP cells, but not in DU145 or PC3 cells.

Discussion

Prostate cancer is one of the leading causes of cancer death in North American men As advanced prostate carcinoma is highly resistant to conventional treatments, adenovirus delivery of death ligands is a viable complementary gene therapy approach [28] Despite the promise of TRAIL death ligand for the treatment of prostate cancer, recent studies have demonstrated that some prostate cancer cell lines are TRAIL-resistant, and this resistance has been attributed to the constitutively active AKT kinase,[11] a key regulator of NF-κB transcription factor [38 39] Demarchi *et al* have demonstrated that AKT-induced NF-κB activation requires IKK activity [40] Since increased IKK activity results in the constitutive NF-κB activation observed in prostate carcinoma cell lines,[36] we hypothesized that coinfection of prostate carcinoma cell lines with adenovirus vectors expressing the dominant-negative mutant form of IKKβAdIKKβKA and 'func-

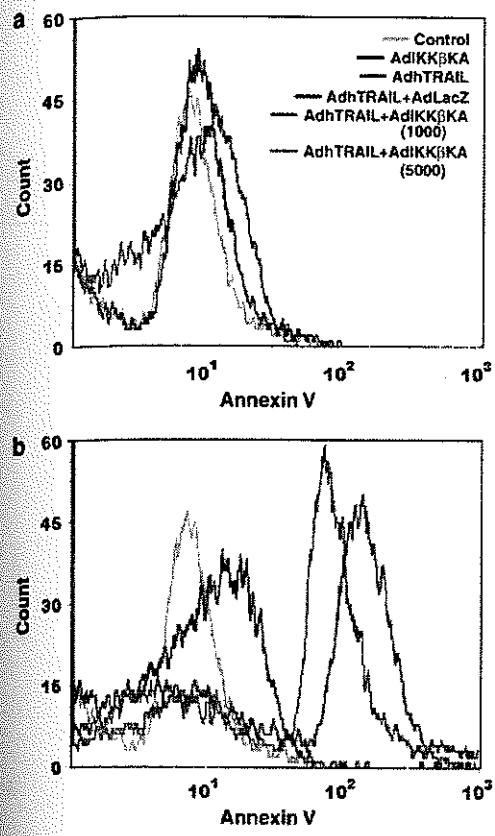

Figure 6 TRAIL and IKKβKA expression induce apoptosis in DU145 prostate cancer cell line Infections with Ad5hTRAIL and AdIKKβKA or AdLacZ (negative control) were performed as described in Materials and methods. All virus constructs were used at an MOI of 5000 DNA particles/cell unless stated otherwise in the figure. Infected DU145 cells were stained with both FITC-conjugated Annexin V and PI, prior to flow analysis. Uninfected, but FITC-Annexin V- and PI-stained, cells served as controls Each histogram represents 10^4 gated DU145 cells Histograms were illustrated in two panels for clarity Treatment conditions were depicted in panel a The Annexin V binding assay was repeated independently three times to confirm the observation, and only one such representative assay was provided in the figure

tional hTRAIL (Ad5hTRAIL) would sensitize advanced prostate cancer cells to TRAIL.

Three different prostate carcinoma cell lines (DU145, PC3 and LNCaP) were used to test this hypothesis As a first step, basal NF-κB activation levels in prostate cancer cell lines were revealed by NF-κB-mediated transcription activation assays using an adenovirus carrying NF-κB regulatory sites fused to a Luciferase reporter gene (Figure 1). In our experimental conditions, DU145 exhibited the highest levels of NF-κB activity, followed by PC3 and LNCaP cells The differences in basal NF-κB activation levels were not due to the differential transduction of prostate cancer cells by adenovirus vectors (Figure 2). Next we screened three prostate cancer cell lines for TRAIL resistance Contrary to some previous reports,[41] cellular viability/toxicity assays indicated that

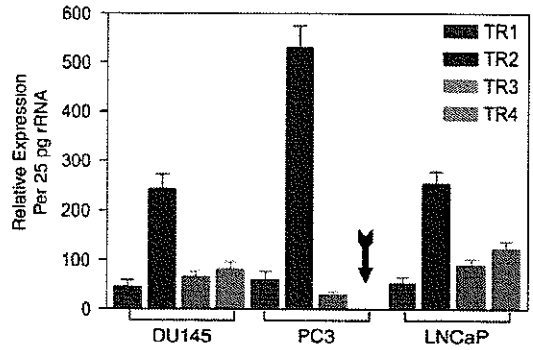

Figure 7 Prostate carcinoma cell lines display distinctive TRAIL receptor expression patterns RT-PCR assays were performed using total RNA isolated from LNCaP, DU145 and PC3 cells to determine the level of expression (quantitative real-time) of TRAIL receptors A cloned ribosomal cDNA fragment was used to generate a standard curve for relative expression. TRAIL receptor levels per 25 pg of ribosomal cDNA are presented in the graph. Ribosomal RNA primers and probes were included in each TaqMan assay as an internal control. Arrow indicates the absence of TRAIL-R4 decoy receptor expression in PC3 cells

DU145 and LNCaP cells were resistant to Ad5hTRAIL-induced apoptosis Furthermore, in contrast to a report from Beresford and coworkers,[42] PC3 cells exhibited considerable levels of TRAIL sensitivity (Figure 3) While some discrepancies can be attributed to the form of TRAIL used for functional studies (purified soluble form versus viral delivery), other inconsistencies might be due to the functional titer of the virus used in infection Recently, several studies have been conducted to overcome TRAIL resistance in cancer cells For instance, ionizing radiation[43] and chemotherapeutic agents[44] have sensitized cancer cells to TRAIL through upregulation of TRAIL death receptors. As increased IKK activity was blamed for the constitutive NF-κB activation responsible for the survival of prostate carcinoma cell lines,[36] we sought to sensitize advanced prostate carcinoma cell lines to TRAIL using a complementary gene therapy modality involving IKK inhibition. As shown in Figure 5, TRAIL-resistant DU145 and LNCaP prostate cancer cell lines were sensitized to TRAIL only when cells were coinfected with AdIKKβKA virus. To rule out possibilities for TRAIL resistance other than NF-κB, we analyzed the pattern of TRAIL receptor gene expression in prostate carcinoma cell lines

Previously, the use of regular RT-PCR assays to screen human tumor cell lines, such as melanoma, colon carcinoma, breast adenocarcinoma and lung adenocarcinoma, did not reveal any connection between TRAIL resistance and TRAIL receptor gene expression.[10] Despite this fact, we wanted to confirm whether or not the expression pattern of TRAIL receptors is connected to TRAIL resistance in prostate cancer cells. A quantitative real-time RT-PCR assay was conducted using specific probe sets for each TRAIL receptor (Figure 7). Substantial levels of TRAIL-R3 and TRAIL-R4 decoy receptor gene expressions were detected only in TRAIL-

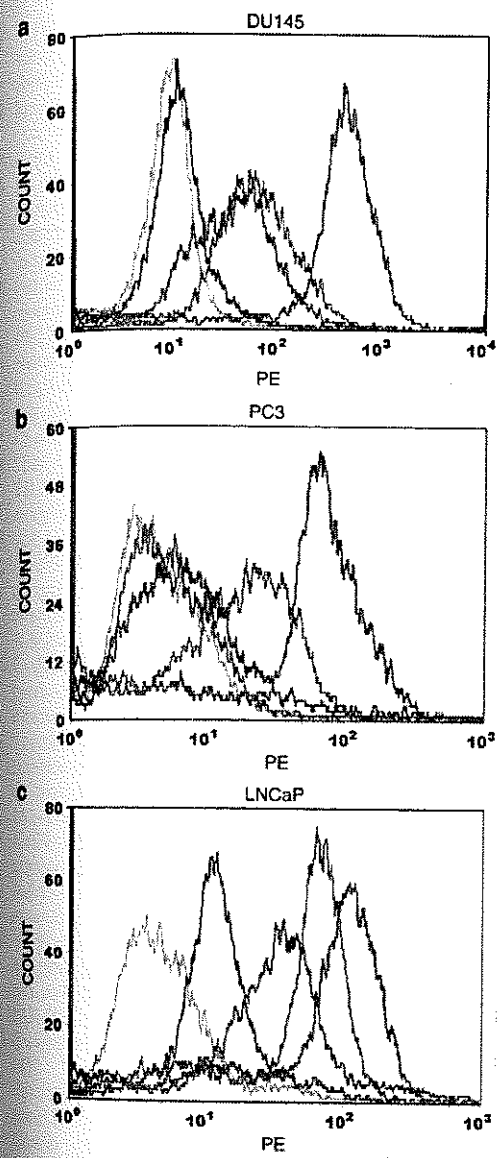

Figure 8 Cell surface expression patterns of TRAIL receptors in prostate carcinoma cell lines. Surface TRAIL receptor expressions of DU145 (**a**), PC3 (**b**) and LNCaP (**c**) cells were detected using monoclonal antibodies specific for each TRAIL receptor according to the manufacturer's protocol using flow cytometry. Colored lines indicate experimental parameters. Gray: isotype-specific control, blue: TRAIL-R1, red: TRAIL-R2, maroon: TRAIL-R3, green: TRAIL-R4. 10^4 cells were gated for each histogram. Each assay was repeated three times to confirm the results. Only one representative assay for each experiment is shown.

resistant DU145 and LNCaP cells. Why PC3 cells do not express TRAIL-R4 is yet to be determined. Intriguingly, aberrant promoter methylation leading to the silencing of TRAIL decoy receptors (TRAIL-R3 and TRAIL-R4) was recently confirmed in multiple tumor types, including prostate cancer (60%).[45] As mRNA levels do not necessarily correlate with protein expression on the cell surface, flow cytometry was employed to determine the level of TRAIL receptor protein on the cell surface (Figure 8). Considerable levels of TRAIL-R4 decoy receptor protein expression were detectable only in the TRAIL-resistant DU145 (Figure 8a) and LNCaP cells (Figure 8c), but not in TRAIL-sensitive PC3 cells (Figure 8b). In addition, unlike the case for DU145 or PC3 cells, surface TRAIL-R3 decoy receptor was expressed on LNCaP cells (Figure 8c). Considering the fact that the absence of death receptors also led to TRAIL resistance as shown recently for rheumatoid arthritis synovial fibroblasts,[46] we have concluded that the presence of decoy receptors, but not the lack of death receptors, correlates with TRAIL resistance in prostate carcinoma. These results are supported by studies indicating that TRAIL-sensitive target cells transfected with either TRAIL-R3 or TRAIL-R4 decoy receptors display a reduction in apoptotic cell death [12,47]. Intriguingly, the TRAIL-R4 decoy receptor was more effective in protecting cells from TRAIL-mediated apoptosis than was TRAIL-R3.[12]

These results demonstrate that both the TRAIL decoy receptor composition and the cell's intracellular NF-κB activity are two major players contributing to TRAIL resistance in prostate cancer cell lines. In addition, IKK inhibiting strategies overwhelmed TRAIL resistance. For this reason, the adenovirus-mediated TRAIL gene delivery approach under the setting of IKK inhibition should be valuable in expanding the therapeutic index of TRAIL for patients with prostate carcinoma.

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Surface TRAIL decoy receptor-4 expression is correlated with TRAIL resistance in MCF7 breast cancer cells

Ahter D Sanlioglu[1,2], Ercument Dirice[1,2], Cigdem Aydin[1,2], Nuray Erin[1], Sadi Koksoy[1] and Salih Sanlioglu*[1,2]

Address: [1]The Human Gene Therapy Unit, Akdeniz University, Faculty of Medicine Antalya, Turkey and [2]Department of Medical Biology and Genetics, Akdeniz University Faculty of Medicine Antalya, Turkey

Email: Ahter D Sanlioglu - ahter@akdeniz.edu.tr; Ercument Dirice - dercument@akdeniz.edu.tr; Cigdem Aydin - cigdemaydin@akdeniz.edu.tr; Nuray Erin - nerin@akdeniz.edu.tr; Sadi Koksoy - sadikoksoy@akdeniz.edu.tr; Salih Sanlioglu* - sanlioglu@akdeniz.edu.tr

* Corresponding author

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Abstract

Background: Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL) selectively induces apoptosis in cancer cells but not in normal cells. Despite this promising feature, TRAIL resistance observed in cancer cells seriously challenged the use of TRAIL as a death ligand in gene therapy. The current dispute concerns whether or not TRAIL receptor expression pattern is the primary determinant of TRAIL sensitivity in cancer cells. This study investigates TRAIL receptor expression pattern and its connection to TRAIL resistance in breast cancer cells. In addition, a DcR2 siRNA approach and a complementary gene therapy modality involving IKK inhibition (AdIKKβKA) were also tested to verify if these approaches could sensitize MCF7 breast cancer cells to adenovirus delivery of TRAIL (Ad5hTRAIL).

Methods: TRAIL sensitivity assays were conducted using Molecular Probe's Live/Dead Cellular Viability/Cytotoxicity Kit following the infection of breast cancer cells with Ad5hTRAIL. The molecular mechanism of TRAIL induced cell death under the setting of IKK inhibition was revealed by Annexin V binding. Novel quantitative Real Time RT-PCR and flow cytometry analysis were performed to disclose TRAIL receptor composition in breast cancer cells.

Results: MCF7 but not MDA-MB-231 breast cancer cells displayed strong resistance to adenovirus delivery of TRAIL. Only the combinatorial use of Ad5hTRAIL and AdIKKβKA infection sensitized MCF7 breast cancer cells to TRAIL induced cell death. Moreover, novel quantitative Real Time RT-PCR assays suggested that while the level of TRAIL Decoy Receptor-4 (TRAIL-R4) expression was the highest in MCF7 cells, it was the lowest TRAIL receptor expressed in MDA-MB-231 cells. In addition, conventional flow cytometry analysis demonstrated that TRAIL resistant MCF7 cells exhibited substantial levels of TRAIL-R4 expression but not TRAIL decoy receptor-3 (TRAIL-R3) on surface. On the contrary, TRAIL sensitive MDA-MB-231 cells displayed very low levels of surface TRAIL-R4 expression. Furthermore, a DcR2 siRNA approach lowered TRAIL-R4 expression on surface and this sensitized MCF7 cells to TRAIL.

Conclusion: The expression of TRAIL-R4 decoy receptor appeared to be well correlated with TRAIL resistance encountered in breast cancer cells. Both adenovirus mediated IKKβKA expression and a DcR2 siRNA approach sensitized MCF7 breast cancer cells to TRAIL.

Background

Cancer still appears to be a challenging disease to treat According to most recent estimates, more than 10 million new cancer cases were reported in the year 2000 killing around 6 million people [1] In addition, 10 % of all cancers appear to be the breast cancer Being the most frequently diagnosed cancer type in women, the breast cancer claims about 370,000 deaths each year around the world [2] Surgery, radiotherapy and chemotherapy are among the most widely used treatment methods for patients with breast cancer [3-5] Still, these conventional treatment modalities did not improve the survival rate of patients with locally advanced or metastatic breast cancer With standard therapy, locally advanced breast cancer has a five year survival rate of 55 % and a ten year survival rate of 35 % [6] There is a 40 % recurrence rate after ten years following the diagnosis and removal of primary tumor in patients with breast cancer [7] For all these reasons, novel treatment methods are needed for the treatment of patients with breast cancer

Induction of programmed cell death known as apoptosis [8], appears to be a viable alternative to currently employed treatment modalities in the fight against cancer [9] In order for chemotherapy and radiotherapy treatment options to work as anticancer agents; tumor suppressor gene, p53, is required [10] Unfortunately, p53 mutations are acquired during the progression of cancer in more than half of the human tumors [11,12] Therefore, the resistance to both chemotherapy and radiotherapy is almost unavoidable in tumors lacking p53 [13] On the other hand, death ligands are capable of inducing apoptosis independently of p53 status of cells [14] Because of this reason, death ligands are currently considered as anticancer agents [15] Among the death ligands tested, Tumor Necrosis Factor (TNF) [16-18] and FasL [19] effectively induced apoptosis in cancer cells However, due to their systemic toxicity, the application of these agents in cancer gene therapy is very limited The discovery of a novel death ligand, TRAIL [20,21], changed this view, since unlike other members of the INF family, TRAIL selectively killed cancer cells without causing any harm to normal cells [22] Thus, treating tumor cells with TRAIL ligand appeared as an invaluable way of inducing apoptosis specifically in tumor cells, as normal cells are protected against the death-inducing effects of TRAIL [23,24] However, the mechanism of TRAIL resistance in normal cells is not understood [25] and significant proportions of cancer cells [26] including those of breast [27,28] appeared to be TRAIL resistant Consequently, TRAIL resistance constitutes a barrier if one wishes to use TRAIL as a death ligand in any breast cancer gene therapy approach

Resistance to TRAIL-induced apoptosis in normal cells was initially considered to be caused by the presence of decoy receptors (TRAIL-R3 and TRAIL-R4), which compete with death receptors (TRAIL-R1 and TRAIL-R2) for binding to TRAIL [29,30] So far, no correlation between TRAIL sensitivity and the expression pattern of TRAIL receptors has been demonstrated in cancer cells yet [31] The presence of intracellular apoptosis inhibitory substances (bcl-xL, c-FLIP, cIAP etc) was also blamed to be responsible for TRAIL resistance [31-33] Intriguingly, the engagement of both TRAIL death receptors and TRAIL-R4 decoy receptor also activated NF-kB pathway [24,34,35] Because NF-kB activation is known to hamper the apoptotic pathways in cells by up-regulating the expression of various apoptosis inhibitory molecules such as cFLIP, bcl-xL, c-IAP and the decoy receptor TRAIL-R3 [34,36,37], high levels of NF-kB activation might be a strong factor responsible for blocking apoptotic processes in order to establish TRAIL resistance For this reason, we analyzed both the TRAIL induced as well as endogenous NF-kB activities using Luciferase reporter gene assays in MCF7 breast cancer cells Because TRAIL-R1, TRAIL-R2 and TRAIL-R4 induced NF-kB activation has been shown to be primarily mediated by TRAF2-NIK-IkappaB kinase alpha/beta signaling cascade [35], MCF7 breast cancer cells were coinfected with adenovirus vectors encoding a dominant negative mutant to IKKβ(AdIKKβKA) [38] and hTRAIL (Ad5hTRAIL) in order to test if TRAIL resistance in breast cancer cells is eliminated through the inhibition of IKK, a leading modulator of NF-kB The molecular mechanism of TRAIL resistance in breast cancer cells (MCF7 and MDA-MB-231) was studied by novel Real Time RT-PCR assays and conventional flow cytometry in order to verify if there is any relationship between TRAIL resistance and the expression pattern of TRAIL receptors Lastly, a DcR2 siRNA approach was utilized to knock down the expression of relevant TRAIL decoy receptor in order to reveal its connection to TRAIL resistance

Methods

Recombinant adenovirus vector production

Amplification of the vectors Ad5hTRAIL [39], AdIKKβKA [17], AdEGFP [18], AdCMVLacZ [40] and AdNFkBLuc [38] was performed as previously described [41] Amplified vectors were stored at -80°C in 10 mM Tris with 20 % glycerol AdIKKβKA expresses a dominant negative mutant of IKKβ, which interacts with other IKK subunits to form inactive IKK complexes The particle titers of adenoviral stocks were in the range of 10^{13} DNA particles/ml, whereas the typical particle/plaque forming unit ratio was equal to 50

Infection of breast cancer cells with first generation recombinant adenovirus vectors

Breast cancer cell lines were cultured in RPMI 1640 medium supplemented with 10 % FBS, 2 2 g/l sodium bicarbonate, 1 mM L-glutamine, and 1 % penicillin-streptomycin mixture, at 37°C in a humidified 5 % CO_2 atmosphere Experimental steps of transduction of breast cancer cells with adenoviral vectors can be summarized as follows: Breast cancer cells were infected with an increasing multiplicity of infection (MOI) of AdEGFP (vector expressing enhanced green fluorescent protein (EGFP) reporter gene) vector at 37°C in RPMI 1640 without FBS. Two hours following infection, equal volume of RPMI 1640 supplemented with 20 % FBS was added to increase the serum concentration in the media to 10 % 48 hours after the infection, the level of transduction was detected by examining of the percentage of GFP (+) cells under a fluorescent microscopy and subsequently by flow cytometry Propidium iodide exclusion technique was used to determine the cell viability Overexpression of hTRAIL was provided by Ad5hTRAIL infection. Cells were coinfected with adenovirus vectors encoding IKKβ dominant negative mutant (AdIKKβKA) and Ad5hTRAIL in order to block IKK activity thereby NF-kB activation. NF-kB promoter based Luciferase assay system was utilized to conduct NF-kB transcription activation assays using AdNFkBLuc construct AdCMVLacZ vector was used as a control.

NF-kB directed transcription activation assays

AdNFkBLuc construct was utilized in order to determine the NF-kB activation status of MCF7 cells AdNFkBLuc vector [38] possesses four tandem copies of the NF-kB consensus sequence fused to a TATA-like promoter from the herpes simplex virus-thymidine kinase gene driving the expression of a Luciferase reporter Transcriptional induction mediated by NF-kB in the presence or absence of TRAIL was measured according to the manufacturer's protocol using the Luciferase assay system with Reporter Lysis Buffer (Promega, Inc) All measurements of Luciferase activity expressed as relative light units were normalized against the protein concentration

Cell viability assays

Discrimination of live cells from dead cells was performed using Live/Dead Cellular Viability/Cytotoxicity Kit from Molecular Probes (Eugene, OR) This assay is based on the use of Calsein AM and Ethidium homodimer-1 (EthD-1) Calsein AM is a fluorogenic substrate for intracellular calsein esterase It is modified to a green fluorescent compound (calsein) by active esterase in live cells with intact membranes, thus serves as a marker for viable cells. Unharmed cell membranes do not allow EthD-1, a red fluorescent nucleic acid stain, to enter inside the cell However, cells with damaged membrane uptake the dye and stain positive

Apoptosis detection by Annexin V binding

Annexin V conjugated to fluorochromes such as FITC has successfully been used as probes to detect cells undergoing apoptosis Annexin V binding assays were carried out according to manufacturer s instructions (Alexis Biochemicals). For this purpose, a FITC conjugated mouse monoclonal antibody to human Annexin V (ALX-804-100F-T100) was employed to detect apoptotic cells via flow cytometry

The detection of TRAIL receptor expression profile by flow cytometry

Anti-TRAIL receptor flow cytometry set (Cat ALX-850-273-KI01) was used to detect TRAIL receptor protein expression on cell surface This kit contains 100μgs of MAb to TRAIL-R1 (clone HS101, Cat 804-297A), -R2 (clone HS201, Cat 804-298A), -R3 (clone HS301, Cat 804-344A) and -R4 (clone HS402, Cat 804-299A) Primary antibodies were used at 5 μg/ml concentration Biotinylated goat anti-mouse IgG1 (Cat ALX-211-202) was used as a secondary antibody followed by streptavidin-PE (Cat ANC-253-050) prior to flow cytometry Flow analysis was performed according to manufacturer's protocols using BD FACSCALIBUR at the Akdeniz University Hospitals Purified mouse IgG1 (MOPC 31C, Cat ANC-278-010) served as an isotype control

Quantitative Real Time RT-PCR assay for human TRAIL receptors

TRIzol reagent (Life Technologies, Gaithersburg, MD) was used to extract total RNA from breast cancer cells, according to the instructions from the manufacturer. Reverse transcription of 2 μg of total RNA was performed using TaqMan Reverse Transcription Reagents (Applied Biosystems Cat N8080234). Despite the fact that the sequences for TRAIL-R1 and TRAIL-R2 primers and probes were recently described by our group [42], we had to design new probe sets for the decoy receptors Following is the sequence information for TRAIL decoy receptor sets: TRAILR3-5' CCC-TAA-AGT-TCG-TCG-TCG-TCA-T, TRAILR3-3' GGG-CAG-TGG-TGG-CAG-AGT-A, TRAILR3 Probe: 5' 6FAM-TCGCGGTCCTGCTGCCAGTCCTAGC-TAMRA 3 ; TRAILR4-5' ACA-GAG-GCG-CAG-CCT-CAA, TRAILR4-3' ACG-GGT-TAC-AGG-CTC-CAG-TAT-ATT, TRAILR4 Probe: 5' 6FAM-AGGAGGAGTGTCCAGCAG-GATCTCATAGATC-TAMRA 3 rRNA was amplified as an internal control in the same reaction Both the rRNA primers and probes were obtained from PE Applied Biosystems (Cat 4308329) ΔΔCt method was used as described by Applied Biosystems to calculate the relative quantities of TRAIL receptors The TaqMan PCR reaction

was performed as described by the manufacturer (Applied Biosystems Cat N8080228)

A DcR2 siRNA approach targeting TRAIL-R4 expression

Posttranscriptional silencing of gene expression became a very useful approach within the last couple of years in research. DcR2 siRNA experiments were performed using DcR2 siRNA (sc-35185), siRNA transfection medium (sc-36868) and siRNA transfection reagent (sc-29528) in MCF7 breast cancer cells as described by the manufacturer (Santa Cruz Biotechnology) Flow cytometry analysis was performed to assess any changes in TRAIL-R4 gene expression. MCF7 cells were infected with Ad5hTRAIL or AdCMVLacZ vectors at increasing doses 35 hours following the transfection Molecular Probe's Live/Dead Cellular Viability/Cytotoxicity Kit was used to assess the amount of live cells 48 hours following the infection.

Results

MCF7 breast carcinoma cells were efficiently transduced with recombinant adenoviruses

In order to find out the efficacy of transduction of breast cancer cells by first generation adenoviral vectors, MCF7 cells were infected with increasing Multiplicity of Infection (MOI) of adenovirus encoding Enhanced Green Fluorescent Protein (AdEGFP) The transduction profiles were followed under fluorescent microscopy and the results were quantitatively analyzed by flow cytometry 48 hours following the infection (Figure 1) While an MOI of 5000 DNA particles/cell was sufficient to transduce more than 90 % of the cells, nearly 100 % of the cells were transduced with AdEGFP at an MOI of 10,000 DNA particles/cell These assays were also pivotal in obtaining the optimum dose of adenovirus required for efficient transduction of MCF7 breast carcinoma cell line without observing deleterious cytotoxic effects These results demonstrated that breast cancer cells were transduced successfully with recombinant adenoviral vectors

MCF7 breast cancer cells displayed complete resistance to TRAIL

Although TRAIL appeared as a promising therapeutic ligand to treat cancer, a variety of tumor types were reported to be resistant to TRAIL-induced cell death For this reason, we wanted to investigate if exogenous TRAIL expression delivered by adenovirus vectors would induce killing of breast cancer cells To test this, MCF7 cells were infected with increasing titers of Ad5hTRAIL or AdCMVLacZ Amount of viable cells were detected using Molecular Probe s Live/Dead Cellular Viability/Cytotoxicity Kit 48 hours following the infections (Figure 2) MCF7 cells displayed complete resistance to TRAIL, as no reduction in the level of viable cells was observed even at an MOI of 10,000 DNA particles/cell, at which almost all cells were infected. Thus, it was concluded that MCF7 breast cancer cells were completely resistant to adenovirus delivery of TRAIL Similarly, AdCMVLacZ infection alone revealed no significant degree of cell death either (data not shown)

Blocking IKK induced NF-kB activation pathway alone did not cause any reduction in the viability of MCF7 breast carcinoma cells

Because increased NF-kB activity was claimed to be responsible for the resistance to death ligand induced cytotoxicity in some tumors [36,37], we wanted to test if the inhibition of IKK activity thereby NF-kB would reduce the viability of breast cancer cells In order to block the intracellular anti-apoptotic NF-kB pathway, MCF7 cells were infected with increasing MOIs of adenoviral vectors encoding a dominant negative mutant of IKKβ(AdIKKβKA), a key molecule involved in the activation of NF-kB Cell viability was examined 48 hours following the infection under fluorescent microscope (Figure 2) Interestingly, AdIKKβKA vector alone proved inefficient in reducing the viability of MCF7 cells, even at an MOI of 10,000 DNA particles/cell

Adenovirus delivery of IKKβKA gene expression sensitized MCF7 breast cancer cells to TRAIL-induced apoptosis

Adenovirus-mediated delivery of IKKβ (Ad IKKβKA) [17,18] or IkBα (Ad IkBαSR) [40,43] dominant negative mutants have previously been demonstrated to sensitize lung cancer cells to TNF death ligand Because most of the breast cancer cell lines tested appeared to be TRAIL resistant [27,28], NF-kB targeting strategies involving IKK inhibition was employed to verify whether MCF7 breast carcinoma cells were sensitized to TRAIL under these circumstances To accomplish this, MCF7 cells were coinfected with a constant MOI of Ad5hTRAIL construct and increasing doses of AdIKKβKA vector In order to better assess the sensitization phenomenon, Ad5hTRAIL was infected at two different MOIs into MCF7 breast cancer cell lines While a constant MOI of 1000 DNA particles/cell of Ad5hTRAIL was used in infection experiments depicted on Figure 3, infection experiments conducted at an MOI of 5000 DNA particles/cell are displayed in Figure 4 The amount of viable cells was detected 48 hours following the infections using Molecular Probe's Live/Dead Cellular Viability/Cytotoxicity Kit Intriguingly, MCF7 cells were sensitized to TRAIL only when Ad5hTRAIL was coinfected with AdIKKβKA vector For instance, nearly 55 % cell death was observed when cells were coinfected with 1000 MOI of Ad5hTRAIL and 5000 MOI of AdIKKβKA constructs (Figure 3). When MOI of Ad5hTRAIL was increased to 5000 as depicted on Figure 4, the death rate went up to 90 % On the other hand, AdCMVLacZ infection instead of AdIKKβKA in breast cancer cells revealed no TRAIL sensitization (data not shown) These results suggested that IKKβKA expression via adenoviral vectors

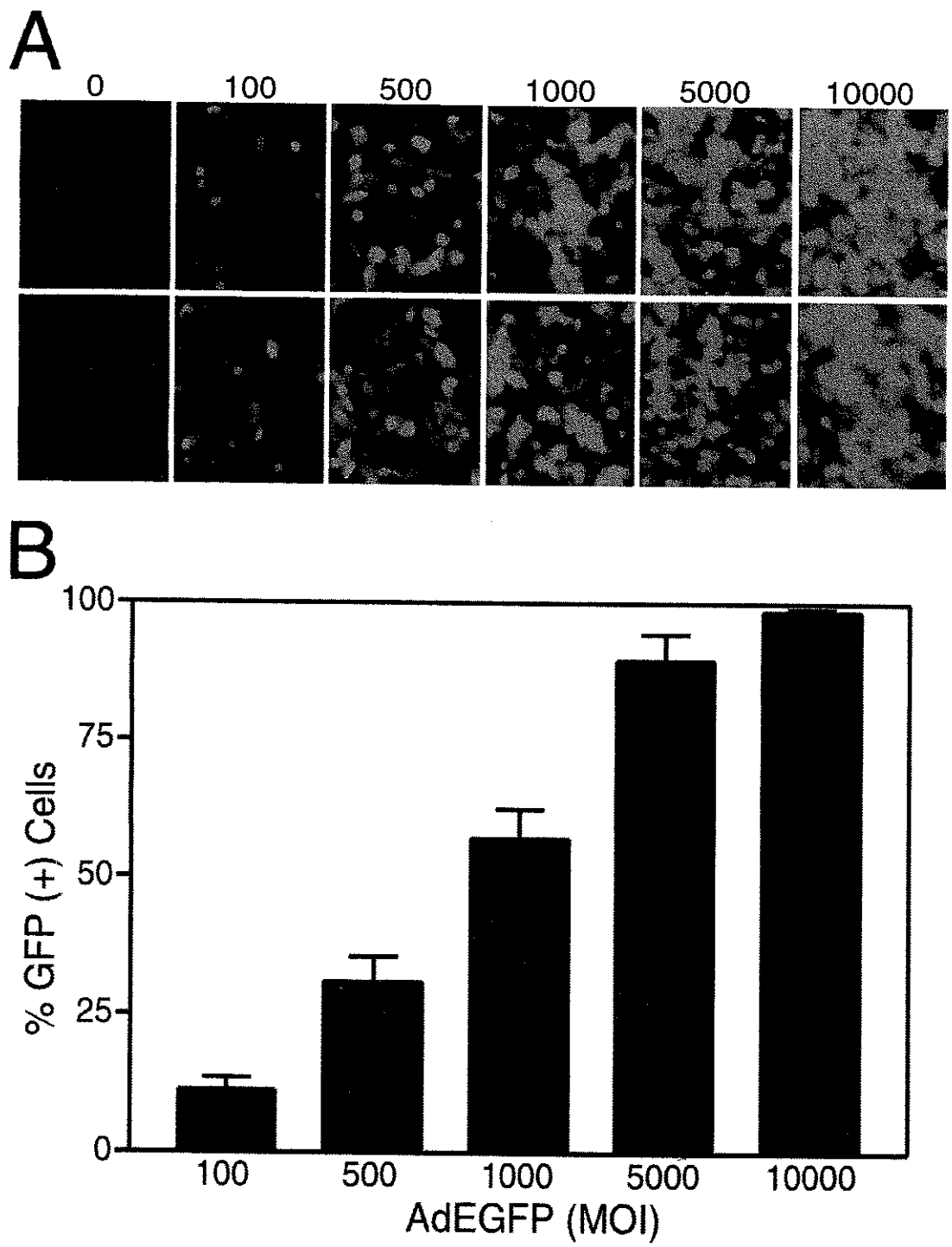

Figure 1
First generation adenoviral vectors efficiently transduced MCF7 breast cancer cells MCF7 cells were infected with increasing MOIs of AdEGFP for 48 hours prior to analysis The number of EGFP expressing cells was detected under fluorescent microscopy (Panel A), and analyzed by flow cytometry (Panel B). Numbers represent viral doses applied in MOI values as DNA particles/cell.

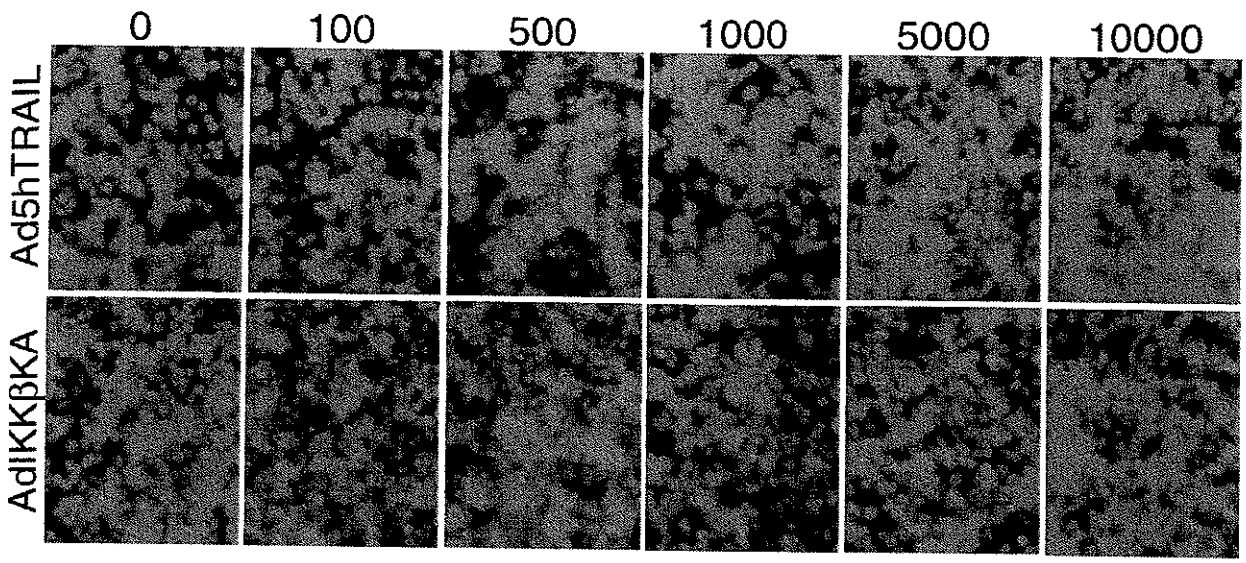

Figure 2
Ad5hTRAIL or AdIKKβKA infection alone did not decrease the viability of MCF7 breast cancer cells. MCF7 cells were infected with increasing MOIs of either Ad5hTRAIL or AdIKKβKA construct. Cell viability was detected using Molecular Probe's Live/Dead Cellular Viability/Cytotoxicity Kit 48 hours following the infection. Numbers represent viral doses applied, in MOI values as DNA particles/cell.

defeated TRAIL resistance observed in MCF7 breast cancer cells

Exogenous TRAIL overexpression elevated the basal NF-kB activity in MCF7 cells, whereas IKKβKA expression blocked both TRAIL-induced and basal NF-kB activities

It is well known that different tumor cells display diverse levels of endogenous NF-kB activities. Furthermore, intracellular NF-kB activity in tumor cells is upregulated by both TRAIL death receptors (TRAIL-R1 and TRAIL-R2) [34,44] as well as TRAIL decoy receptor TRAIL-R4 [45] upon ligand binding. Knowing the endogenous NF-kB status of cancer cells before the therapy is obviously crucial for TRAIL mediated gene therapy targeting to induce apoptosis in cancer cells. A coinfection experiment was performed using a recombinant adenovirus vector carrying NF-kB driven Luciferase reporter gene (AdNFkBLuc) and Ad5hTRAIL vector in order to study the extent of NF-kB activation as a result of TRAIL overexpression in MCF7 breast cancer cell line. NF-kB Luciferase assays were conducted 24 hours following the infection in order to determine cell's NF-kB activation status. As seen in Figure 5, Ad5hTRAIL at an MOI of 5000 DNA particles/cell (Panel B) but not at an MOI of 1000 DNA particles/cell (Panel A) stimulated NF-kB activation. In order to determine the magnitude of NF-kB inhibition, a triple coinfection experiment involving AdNFkBLuc, Ad5hTRAIL and AdIKKβKA or AdCMVLacZ was performed. While IKKβKA overexpression in MCF7 cells gradually reduced both the TRAIL-induced and basal NF-kB activities in MCF7 cells, no such NF-kB inhibiting effect was observed in cells upon superinfection with AdCMVLacZ virus as a control (Figure 5)

Coinfection of Ad5hTRAIL and AdIKKβKA results in apoptotic cell death in MCF7 breast cancer cells

To show that apoptosis is the mechanism of cell death mediated by TRAIL overexpression under the setting of IKK inhibition in MCF7 cells, Annexin V staining was performed using flow cytometry. For this purpose, MCF7 cells were infected with Ad5hTRAIL or AdIKKβKA vectors alone or in combination. Thirty-five hours following the infection, apoptotic cell death was analyzed by Annexin-V-FITC staining. As displayed in Figure 6 Panel A, there was no substantial Annexin V binding generated by the expression of TRAIL or IKKβKA in MCF7 cells. However, considerable levels of Annexin V binding were observed in cells coinfected with Ad5hTRAIL and AdIKKβKA indicating apoptotic cell death (Figure 6, Panel B). As predicted, Ad5hTRAIL and AdCMVLacZ (negative control) coinfection did not yield any significant levels of Annexin V binding as MCF7 cells are resistant to TRAIL in the absence of IKK inhibition. These results suggested that the

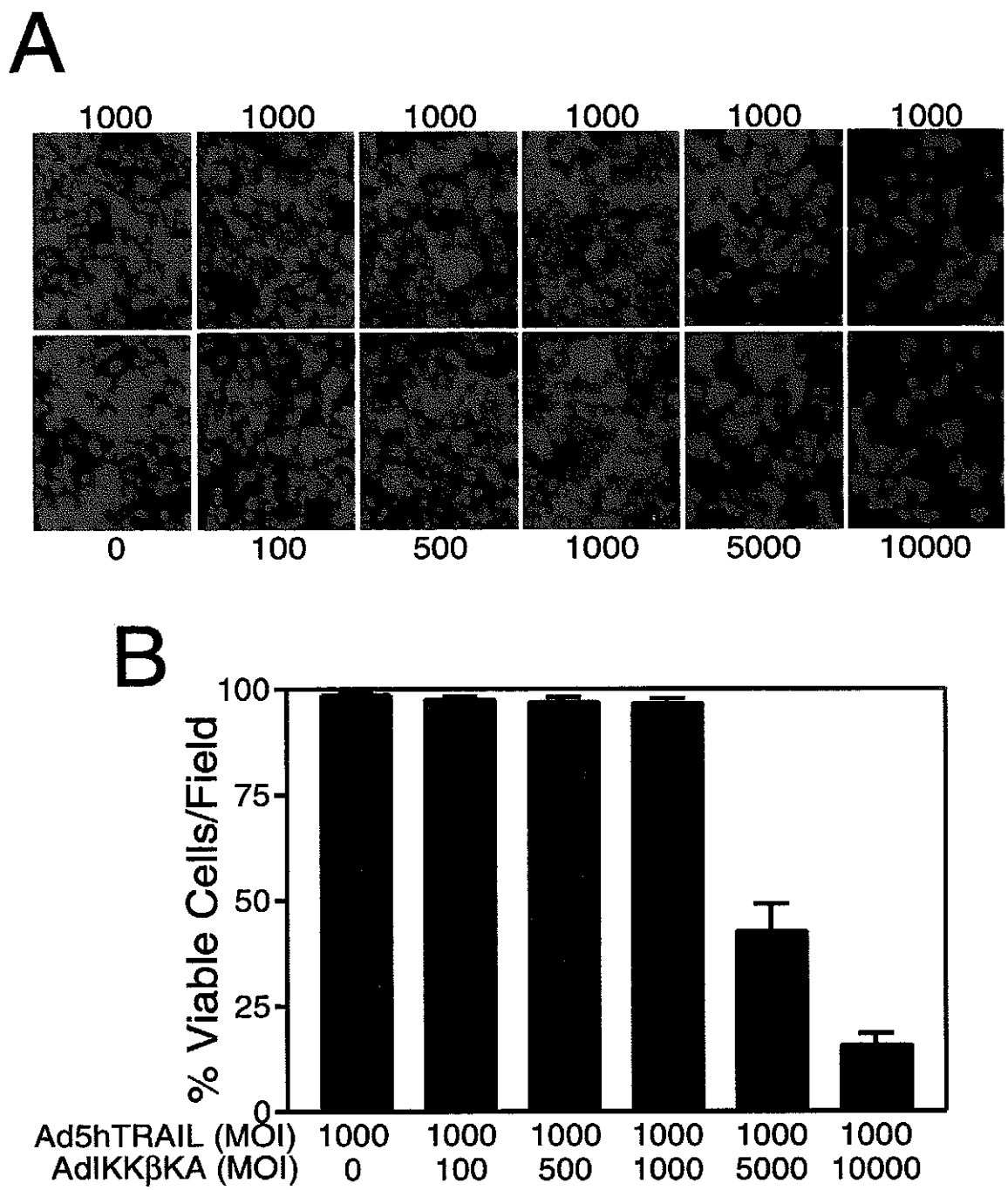

Figure 3
IKKβKA expression via adenoviral vectors sensitized MCF7 cells to TRAIL-mediated apoptosis. MCF7 cells were infected with increasing doses of adenoviral vectors encoding dominant negative mutant of IKKβ (as shown below each panel), while simultaneous infection with Ad5hTRAIL (as shown above each panel) was performed at a constant MOI of 1000. Cell viability was detected using Molecular Probe's Live/Dead Cellular Viability/Cytotoxicity Kit 48 hours following infection. Numbers represent viral doses applied in MOI values as DNA particles/cell Fluorescent micrographs are provided in Panel A; Panel B depicts quantitative analysis of such infections. Values represent the mean (± SEM) of three different experiments.

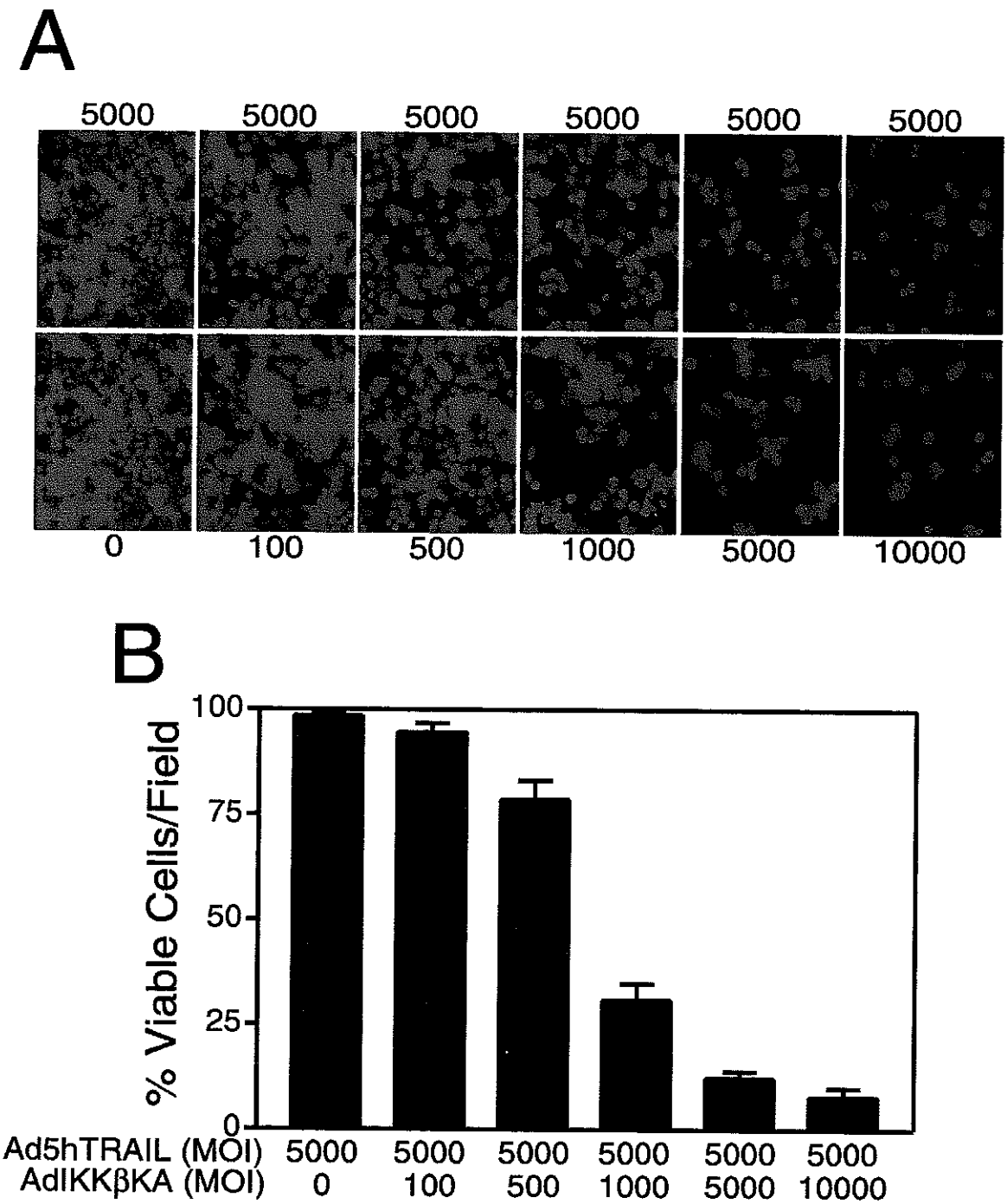

Figure 4

AdIKKβKA infection defeated the resistance to TRAIL-induced apoptosis in MCF7 breast cancer cells. These cells were co-infected with a constant MOI of 5000 DNA particles/cell of Ad5hTRAIL (as shown above each panel) and increasing doses of AdIKKβKA (as shown below each panel). Live/Dead Cellular Viability/Cytotoxicity Kit from Molecular Probe was used to detect TRAIL cytotoxicity 48 hours following infection. Numbers represent viral doses applied, in MOI values as DNA particles/cell. Data represent the mean of (± SEM) six independent data points (n = 6).

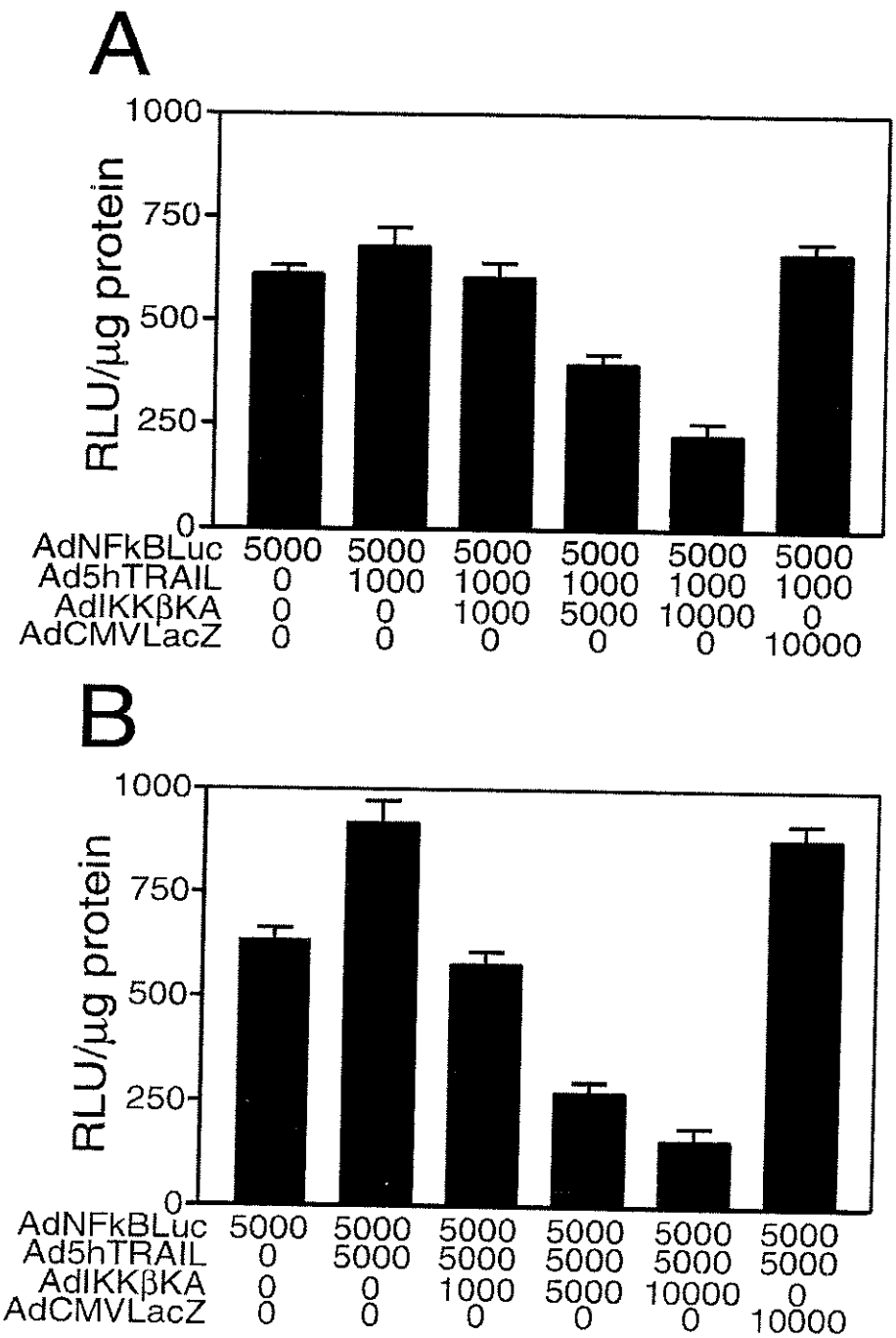

Figure 5

Distinctive regulation of NF-kB activation in MCF7 breast cancer cells by Ad5hTRAIL and/or AdIKKβKA infections. MCF7 cells were simultaneously infected with AdNFkBLuc. Ad5hTRAIL and/or increasing doses of AdIKKβKA construct for 24 hours. AdCMVLacZ infection was also performed as a negative control. The types of constructs used in the infection are shown on the x axis. MOI values represent DNA particles/cell. Ad5hTRAIL vector was used at two different constant MOIs (MOI of 1000 and 5000) in order to avoid cell death complicating our assay result. Luciferase activity expressed in Relative Light Units per microgram protein is shown on y axis. Values represent the mean (± SEM) of six independent data points (n = 6).

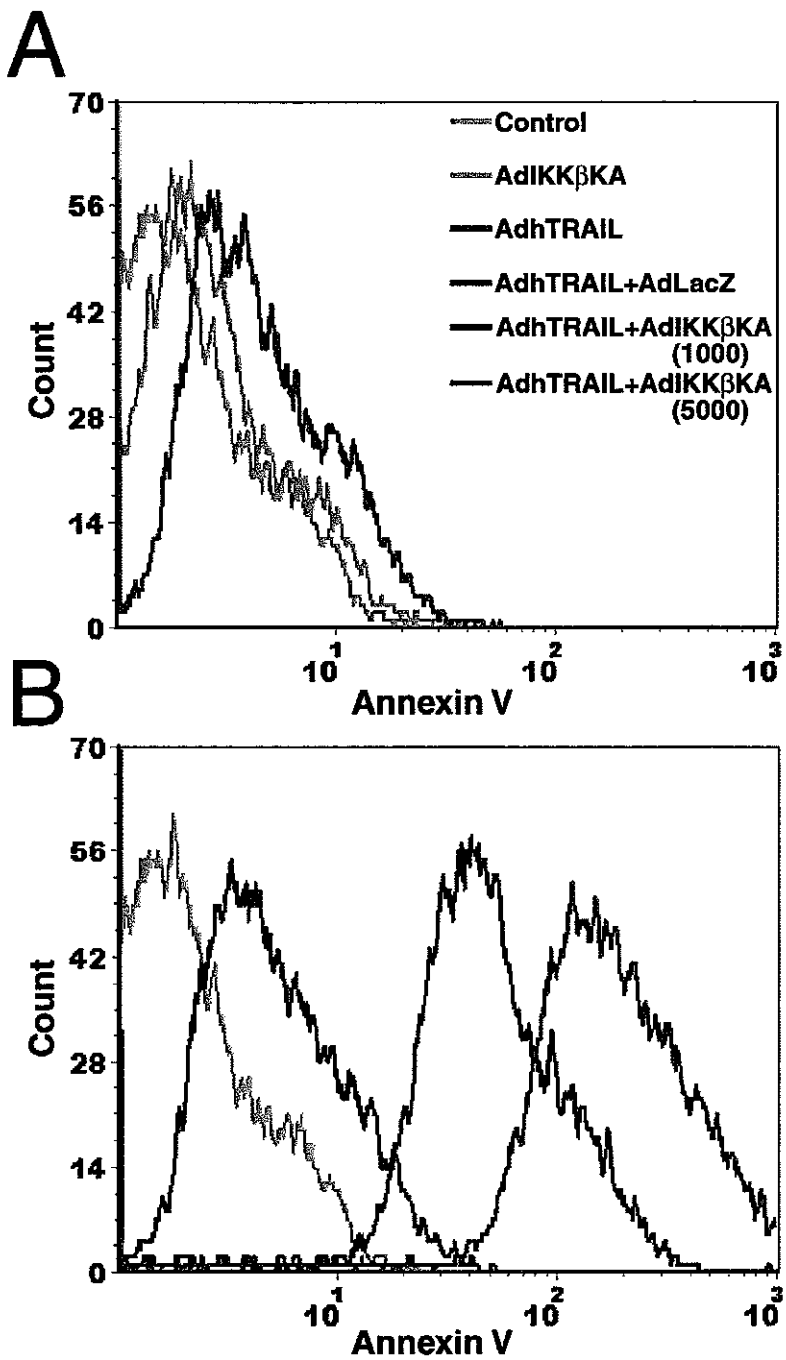

Figure 6

Ad5hTRAIL and AdIKKβKA coinfection induced apoptosis in MCF7 breast carcinoma cells FITC conjugated Annexin V and Propidium Iodide (PI) staining were utilized using MCF7 cells infected with various combinations of adenovirus constructs as described in Methods prior to flow cytometry. Each histogram represents 10^4 gated MCF7 cells. Histograms were illustrated in two panels for clarity. Various treatment settings were provided in Panel A. MOI of 5000 DNA particles/cell was used for each viral construct unless stated otherwise in the Figure. Control line represents uninfected but FITC-Annexin V and PI stained MCF7 cells. Only one representative assay out of three independent assays was provided.

mechanism of cell death experienced by MCF7 cells is apoptosis following TRAIL stimulation under the setting of IKK inhibition

MCF7 breast cancer cell line displayed significant levels of TRAIL decoy receptor-4 expression

So far no evidence of the connection between the expression pattern of TRAIL receptors and TRAIL sensitivity was found in cancer cells [31] Part of the reason might have been the inability to screen all TRAIL receptors at once in breast cancer cells then [28]. In order to compensate this deficiency, quantitative novel Real Time RT-PCR assays were conducted using primer-probe sets specifically designed to detect each TRAIL receptor in MCF7 breast cancer cells (Figure 7, Panel A). According to our results, while all TRAIL receptors were expressed in MCF7 cells, TRAIL-R4 expression was the highest among the four. In addition, the level of TRAIL-R2 expression was much higher than that of TRAIL-R1. Lastly, TRAIL-R3 decoy receptor expression was the lowest These results suggested that high levels of TRAIL-R4 decoy receptor expression correlated well with TRAIL resistance. However, as the gene expression detected inside the cell may not necessarily correlate with the receptor expression on cell surface, we decided to perform flow cytometry analysis using antibodies specific to four different TRAIL receptors. As shown in Figure 7 Panel B, MCF7 cells expressed all TRAIL receptors excluding TRAIL-R3 on cell surface While similar levels of TRAIL death receptors TRAIL-R1 and TRAIL-R2 were expressed, there were still considerable levels of TRAIL-R4 decoy receptor expression on the surface of MCF7 cells

TRAIL sensitive MDA-MB-231 cells displayed very low levels of TRAIL-R4 decoy receptor expression on cell surface

In order to solidify the importance of TRAIL-R4 expression and its connection to TRAIL resistance, another breast cancer cell line, MDA-MB-231, was also analyzed in terms of TRAIL receptor expression profile Real Time RT-PCR assays revealed that while TRAIL-R2 expression was the highest on transcript levels, TRAIL-R4 decoy receptor expression was the lowest TRAIL receptor expressed in MDA-MB-231 breast cancer cells (Figure 8, Panel A) Furthermore, flow cytometry analysis indicated that insignificant levels of TRAIL-R4 expression were detected on the surface of MDA-MB-231 breast cancer cells (Figure 8, Panel B) TRAIL-R3 decoy receptor expression, however, was not detectable using flow cytometry Intriguingly, in contrast to what was observed with MCF7, adenovirus delivery of TRAIL alone killed significant proportions of MDA-MB-231 breast cancer cells (Figure 9)

Lowering of TRAIL-R4 gene expression sensitized MCF7 breast cancer cells to TRAIL

In order to solidify the connection between TRAIL-R4 decoy receptor gene expression and TRAIL resistance, a DcR2 siRNA approach was executed in TRAIL resistant MCF7 breast cancer cells Flow cytometry analysis conducted 35 hours following the transfection revealed that the level of TRAIL-R4 protein expression on surface went down drastically (Figure 10, Panel A) At this stage, MCF7 cells were further infected with either Ad5hTRAIL or AdCMVLacZ vector at increasing doses Cell viability assays were conducted 48 hours following the infection Only Ad5hTRAIL infected cells exhibited considerable amount of cell death following transfection (Figure 10, Panel B) No such effect was observed when cells were infected with AdCMVLacZ virus (data not shown)

Discussion

Although, conventional treatment modalities could not satisfactorily improve the survival rates of patients with locally advanced and metastatic disease, adenovirus delivery of death ligands represents a feasible choice for the treatment of patients with breast cancer However, recent observations demonstrating that a considerable portion of human cancers including those of the breast [27,28] were TRAIL resistant undermined the potential application of TRAIL against cancer Accordingly, the understanding of the mechanism of TRAIL resistance is the key to resolve primary obstacles in TRAIL mediated gene therapy approach. Based on recent findings from our laboratory and others, we think that NF-kB signaling is one of the most crucial pathways involved in the constitution of TRAIL resistance [26]. Despite the fact that TRAIL-R1, TRAIL-R2 and TRAIL-R4 induced NF-kB activation has been shown to be primarily mediated by TRAF2-NIK-IkappaB kinase alpha/beta signaling cascade [35], there is some doubt on whether or not NF-kB activation can block TRAIL mediated apoptosis For example, in one particular study it was reported that NF-kB inhibition by way of IkappaBalpha mutant expression sensitized MCF7 cells to TNF but not TRAIL-induced apoptosis [35] Considering the fact that there are different ways to activate NF-kB pathway (IkB dependent and independent ways) [46] we decided to inhibit IKK activity rather than targeting IkappaBalpha itself to look for the possibility of sensitizing MCF7 breast cancer cells to TRAIL.

First of all, in order to find out the efficacy of adenovirus transduction in breast cancer cells, MCF7 cells were infected with increasing MOIs of AdEGFP virus The transduction profiles analyzed by flow cytometry showed that nearly 100 % of the cells were transduced with AdEGFP at an MOI of 10,000 DNA particles/cell (Figure 1) The efficacy of TRAIL in mediating apoptosis of MCF7 breast cancer cells was assessed using Ad5hTRAIL

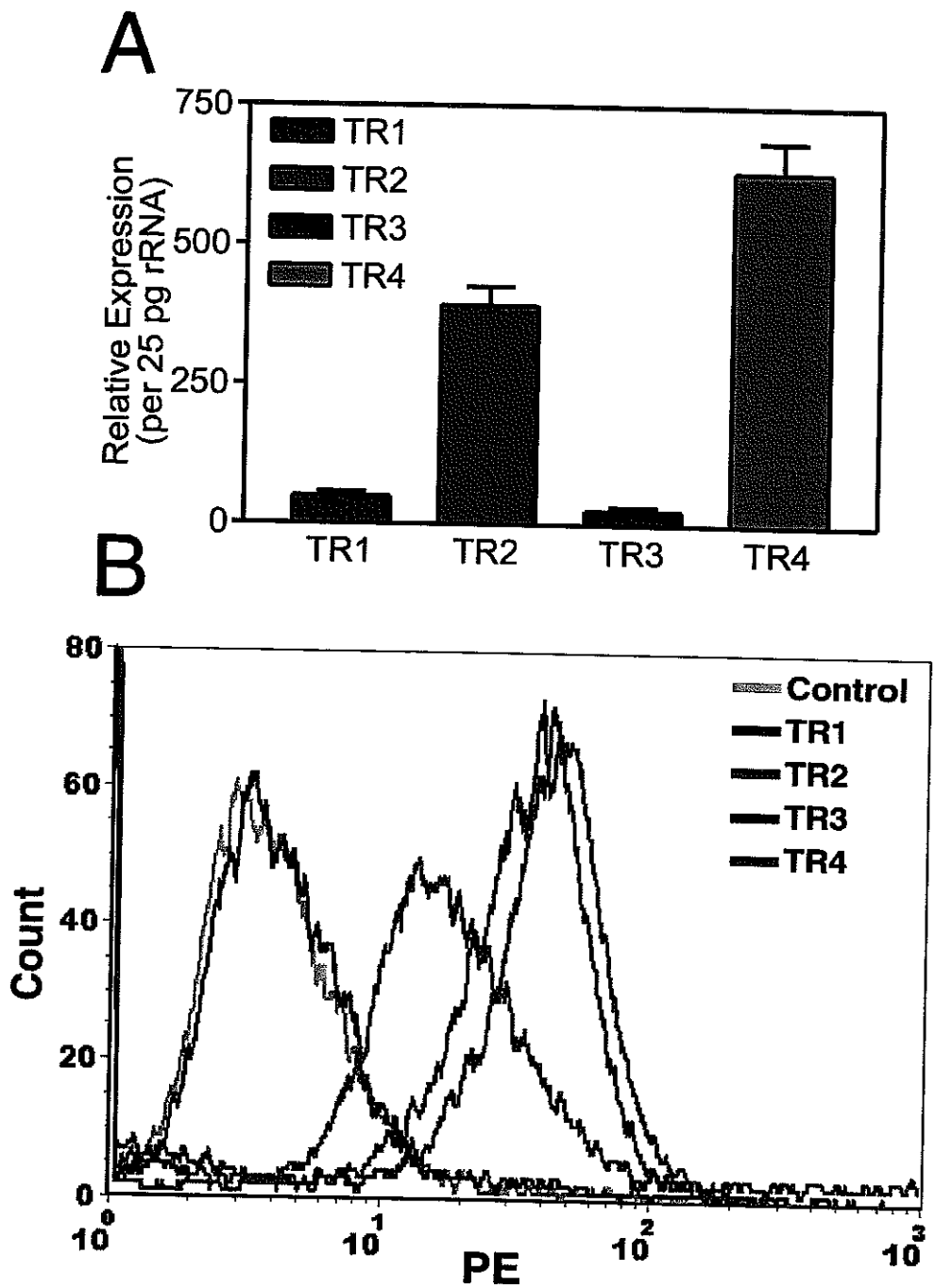

Figure 7

MCF7 breast carcinoma cell line displayed substantial levels of TRAIL-R4 decoy receptor expression. Quantitative Real Time RT-PCR of TRAIL receptors was performed as described in Methods (Panel A). TRAIL receptor levels per 25 pg of ribosomal cDNA are presented in the graph for clarity. Ribosomal RNA primers and probes were included in each TaqMan reaction as an internal control. Panel B depicts the surface TRAIL receptor expression pattern of MCF7 cells using flow cytometry. Experimental parameters are defined in colored lines. 10^4 cells were gated for each histogram. Only one representative assay for each experiment (independently repeated three times) is shown.

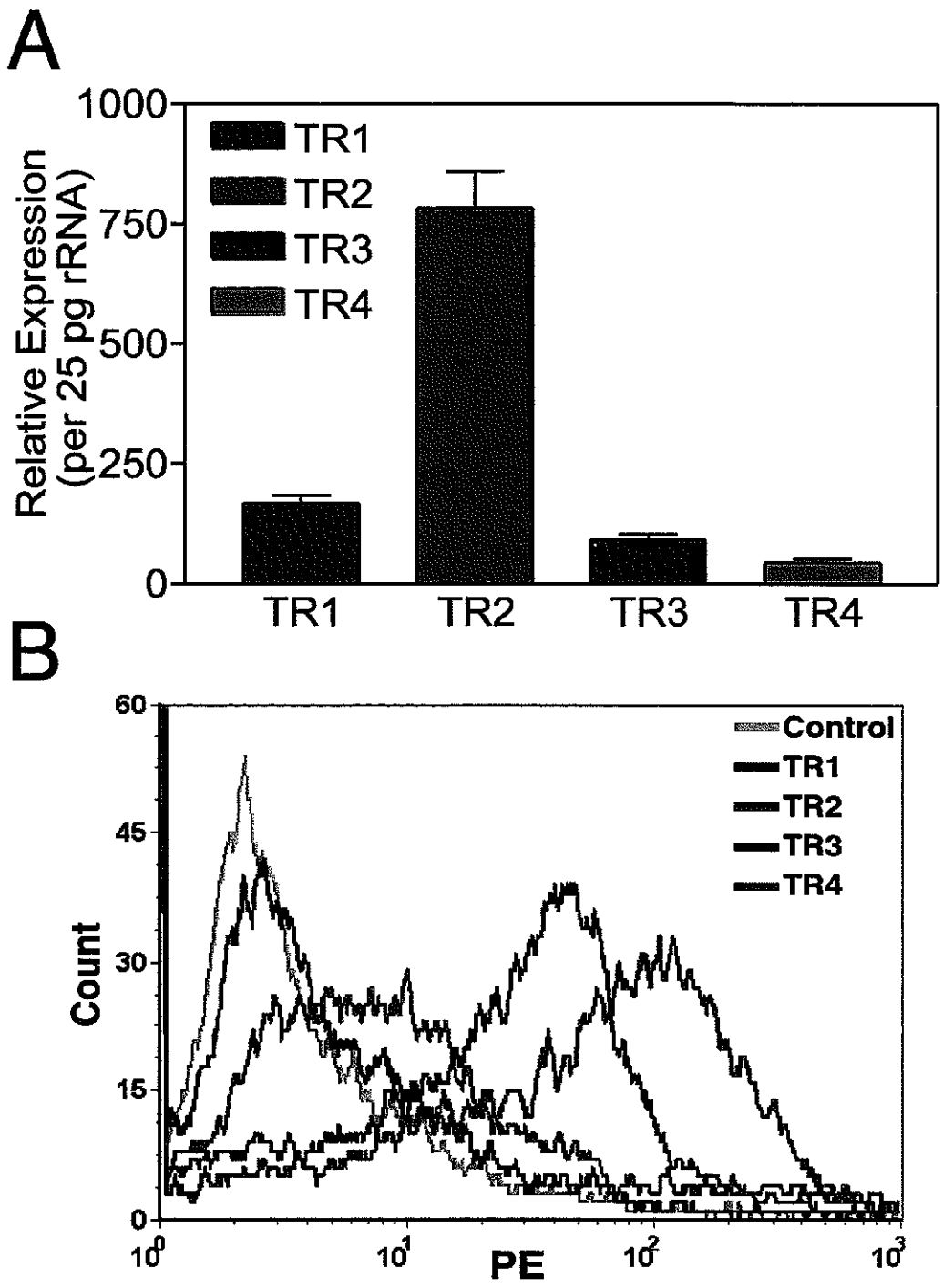

Figure 8
MDA-MB-231 breast cancer cells displayed trivial levels of TRAIL-R4 decoy receptor expression on surface. TRAIL receptor composition of MDA-MB-231 breast cancer cells revealed by Real Time RT-PCR assay is displayed in Panel A. Panel B illustrates flow cytometry analysis showing the surface expression pattern of TRAIL receptors. 10^4 cells were gated for each histogram. Only one representative assay out of three is shown.

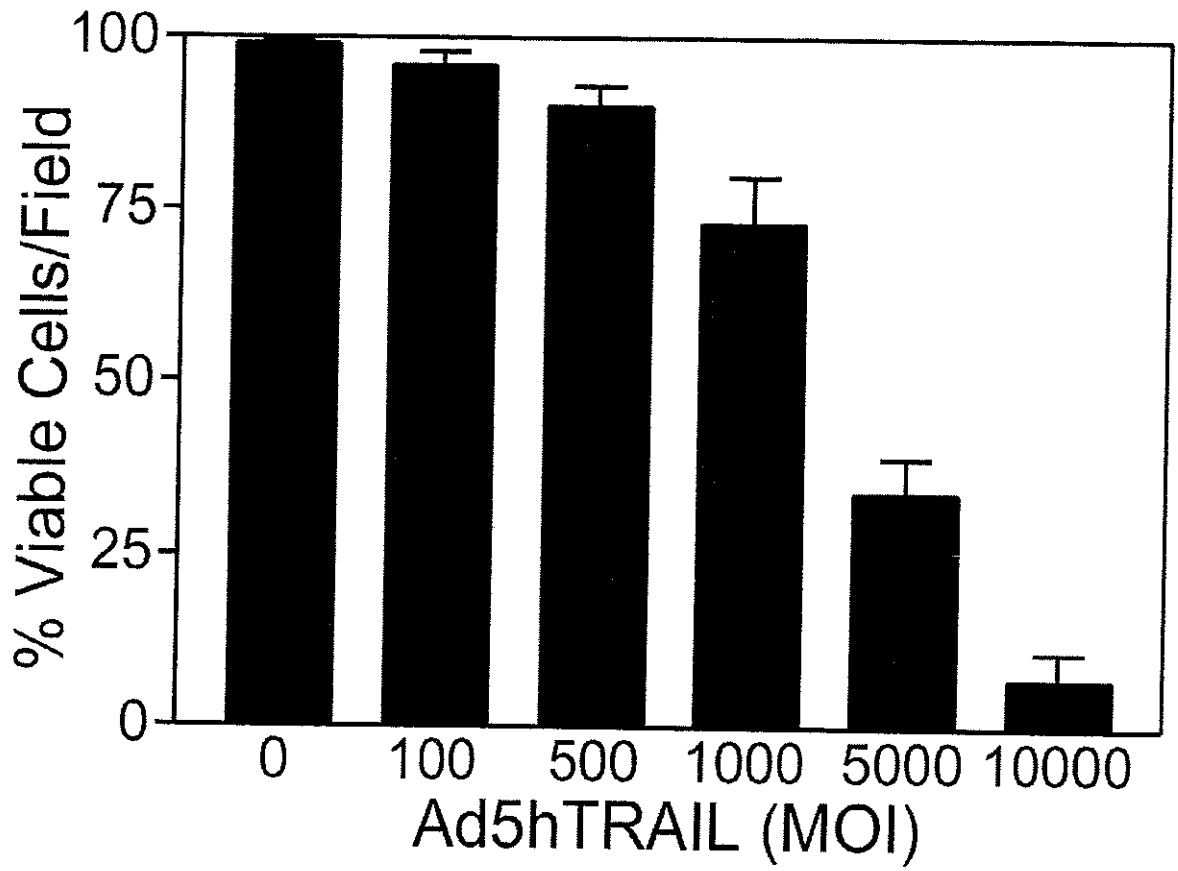

Figure 9
MDA-MB-231 breast cancer cell line is sensitive to Ad5hTRAIL infection. MDA-MB-231 breast cancer cells were infected with increasing MOIs of Ad5hTRAIL construct. Molecular Probe's Live/Dead Cellular Viability/Cytotoxicity Kit was used to detect % viable cells 48 hours following the infection. Numbers represent viral doses applied in MOI values as DNA particles/cell. Values represent the mean (± SEM) of six independent data points (n = 6)

construct. Interestingly, MCF7 cells displayed complete resistance to TRAIL as no reduction in the level of viable cells was observed even at an MOI of 10,000 DNA particles/cell (Figure 2). IKK inhibiting strategy alone proved inefficient in reducing the viability of MCF7 cells suggesting that an apoptotic stimulus was required in order to induce cell killing (Figure 2). Interestingly, in order to break down TRAIL resistance and to induce cell death, a coinfection of MCF7 cells with Ad5hTRAIL and AdIKKβKA was required (Figures 3 and 4). Luciferase assays confirmed that both the TRAIL induced and endogenous NF-kB activities were drastically reduced by the infection of MCF7 cells with AdIKKβKA virus (Figure 5). Moreover, IKKβKA sensitization of MCF7 breast carcinoma cells resulted in TRAIL induced apoptosis as revealed by Annexin V binding assays (Figure 6). These results suggested that NF-kB activation pathway has a hampering effect on TRAIL-induced cell death in MCF7 cells, and blocking this pathway is essential to sensitize breast cancer cells to TRAIL mediated apoptosis.

So far, no correlation between TRAIL resistance and TRAIL decoy receptor gene expression has been reported. For example, analysis of breast cancer cell lines by just examining the expression levels of TRAIL death receptors (TRAIL-R1 and TRAIL-R2) and TRAIL-R3 decoy receptor using RNase protection assay did not reveal any connection between the expression pattern of TRAIL receptors and TRAIL resistance [28]. But whether or not TRAIL-R4 decoy receptor gene expression in any way contributes to

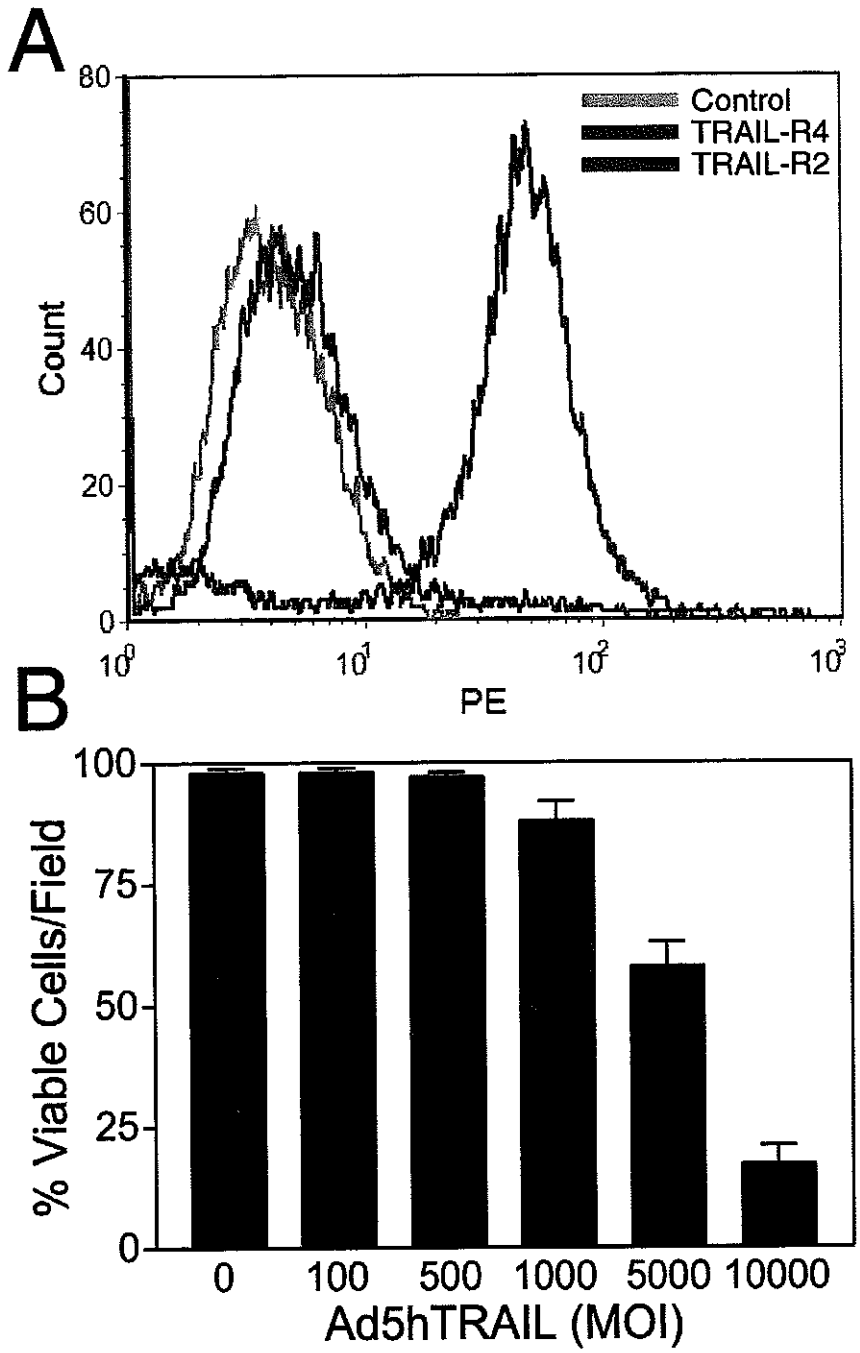

Figure 10
Knocking down TRAIL-R4 expression sensitized MCF7 breast cancer cells to TRAIL. A DcR2 siRNA approach was administered as described in Methods using TRAIL resistant MCF7 breast cancer cell line. Panel A depicts a flow cytometry analysis confirming strong attenuation of TRAIL-R4 expression on cell surface. TRAIL-R2 death receptor expression was also detected as a control. Sensitization of MCF7 breast cancer cells to TRAIL following a DcR2 siRNA approach is provided in Panel B. MCF7 breast cancer cells were infected with increasing doses of Ad5hTRAIL alone following a DcR2 siRNA transfection. Cell death was detected 48 hours following the infection (Panel B). Data represent the mean (± SEM) of 6 independent data points.

TRAIL resistance in breast cancer cells remains to be tested yet Quantitative Real Time RT-PCR assays were developed in order to assess the level of TRAIL receptor gene expression in breast carcinoma cells While all TRAIL receptors were detectable in MCF7 breast carcinoma cell line, the level of TRAIL-R4 decoy receptor gene expression was the highest among the four (Figure 7, Panel A) This intriguing observation is consistent with a previous report suggesting that transient TRAIL-R4 overexpression protected target cells from TRAIL induced cytotoxicity [45] TRAIL R4 is known to protect cells from apoptosis by acting both as a decoy receptor and an antiapoptotic signal provider While Real Time PCR assay is useful in assessing the level of gene expression on mRNA levels, obviously this assay does not necessarily reflect TRAIL receptor composition on cell surface For this reason, conventional flow cytometry analysis was carried out in order to determine the level of TRAIL receptor protein expression on cell surface Despite the presence of TRAIL death receptors, substantial levels of TRAIL-R4 decoy receptor expression were detectable on the surface of MCF7 breast carcinoma cells (Figure 7, Panel B). On top of that, TRAIL sensitive MDA-MB-231 cell line (Figure 9) displayed very low levels of TRAIL-R4 decoy receptor expression on cell surface (Figure 8, Panel B). Neither of the cell lines expressed detectable levels of TRAIL-R3 decoy receptor on surface Intriguingly, administration of a DcR2 siRNA approach lowered surface TRAIL-R4 expression and sensitized MCF7 breast cancer cells to TRAIL (Figure 10)

Conclusion

Our results demonstrated that the expression of TRAIL-R4 decoy receptor but not TRAIL-R3 appeared to correlate well with TRAIL resistance phenotype observed in MCF7 breast cancer cells. Further screening of another breast cancer cell line, MDA-MB-231, revealed that low levels of TRAIL-R4 expression on surface were correlated with TRAIL sensitivity These results strengthen our argument that TRAIL-R4 but not TRAIL-R3 is the decoy receptor which appeared to influence TRAIL sensitivity in breast cancer cells This is further confirmed by a DcR2 siRNA assay which suggested that down regulation of TRAIL-R4 expression sensitized MCF7 breast cancer cells to TRAIL. In addition, the inhibition of IKK pathway thereby NF-kB sensitized MCF7 cells to TRAIL induced apoptosis despite the expression of TRAIL-R4 decoy receptor on cell surface Consequently, this complementary gene therapy approach involving IKK inhibition might be necessary to breakdown TRAIL resistance encountered in patients with breast cancer

Abbreviations

TRAIL= Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand, EGFP= Enhanced Green Fluorescent Protein, MOI= Multiplicity of Infection, DcR2= Decoy receptor 2

Competing interests

The author(s) declare that they have no competing interests

Authors' contributions

ADS performed cell viability, Luciferase, Flow Cytometry, Real Time RT-PCR and siRNA assays, ED assisted ADS with adenovirus preparation, CA performed AdEGFP transduction assays, NE cultured breast cancer cells, SK optimized flow cytometry assays, SS participated in the coordination and execution of the study All authors read and approved the final manuscript

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Current progress in adenovirus mediated gene therapy for patients with prostate carcinoma

Review Article

Ahter D. Sanlioglu[1,3], Turker Koksal[2,3], Mehmet Baykara[2,3], Guven Luleci[1,3], Bahri Karacay[4] and Salih Sanlioglu[1,3,*]

[1]Departments of Medical Biology and Genetics, [2]Department of Urology and [3]The Human Gene Therapy Unit of Akdeniz University, Faculty of Medicine, Antalya, Turkey, 07070; [4]Department of Pediatrics, University of Iowa, College of Medicine, Iowa City, IA, 52240, USA

***Correspondence:** Salih Sanlioglu V.M.D., Ph.D., Director of The Human Gene Therapy Unit of Akdeniz University, Faculty of Medicine, B- Block, 1st floor, Campus, Antalya, 07070 Turkey; Phone: (90) 242-227-4343/ext: 44359, Fax: (90) 242-227-4482; e-mail: sanlioglu@akdeniz.edu.tr

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Summary

Prostate cancer is the most frequently diagnosed male cancer in the world. Like all cancers, prostate cancer is a disease of uncontrolled cell growth. In some cases tumors are slow growing and remain local, but in others they may spread rapidly to the lymph nodes, other organs and especially bone. Although surgery and radiation can cure early stages of organ confined prostate carcinoma (stages I and II), there is no curative therapy at this time for locally advanced or metastatic disease (stages III and IV). The likelihood of postsurgical local recurrence increases with capsular penetration as detected in 30 % of the patients at the time of radical prostatectomy. Moreover, 10-15 % of patients have metastatic cancer at the time of diagnosis. Considering the fact that 60 % local recurrence is observed in patients receiving radiation therapy with or without adjuvant hormonal ablation therapy, it is generally believed that androgen ablation therapy simply delays the progression of prostate carcinoma to a more advanced stage. In addition, the overall ten-year survival rate of patients with locally recurrent prostate cancer is only around 35 %; thus; the ultimate progression into androgen independent prostate carcinoma appears to be inevitable. Gene therapy arose as a novel treatment modality with the potential to decrease the morbidity associated with conventional therapies. Therefore, gene therapy is expected to lower the incidence of tumor recurrence and finally improve the outcome of patients with recurrent and androgen independent prostate carcinoma. Viral vectors are most commonly used for the purpose of gene therapy. Currently, there are a total of 40 clinical trials being conducted using viral vectors for the treatment of prostate carcinoma. 22 out of 40 clinical protocols (55 %) approved for the treatment of prostate cancer utilize adenovirus vectors. Most of these adenovirus mediated therapeutic approaches employ either selectively replicating adenoviruses or suicide gene therapy approaches. In this review, we mainly concentrated on the progress in adenovirus mediated gene therapy approaches for prostate cancer. Analysis of the death ligand mediated gene therapy approach was also discussed in detail, while our novel findings were incorporated as an example for up-to-date approaches used for adenovirus mediated gene therapy against prostate carcinoma.

I. Introduction

Prostate cancer is the second leading cause of death in men from cancer following lung carcinoma with an annual mortality rate of 38,000 (Yeung and Chung, 2002). There are 200,000 newly diagnosed cases of prostate carcinoma every year in the United States alone (Boring et al, 1994; Greenlee et al, 2001). As a result, prostate carcinoma is claimed to be the most frequently diagnosed male cancer in the United States (Powell et al, 2002). Despite the fact that there has been a considerable effort for screening and early detection of prostate cancer in recent years, the lifetime risk of being diagnosed with prostate cancer is still reported to be 1 in 5 (Grumet and Bruner, 2000). Several hundred clinical studies using experimental or approved chemotherapeutics failed to improve survival rates of patients with prostate cancer (Devi, 2002). Because prostate cancer is a heterogeneous

disease, treating patients with prostate cancer still remains a formidable task. In addition, the molecular mechanism responsible for the onset of the disease is poorly understood. However, earlier detection of prostate cancer has been associated with an improved outcome (Perrotti et al, 1998). Thus, the detection of prostate cancer at an earlier stage remains to be the most realistic chance for therapy.

For this purpose, different molecular screening methods (Ross et al, 2002a, 2002b) have been employed, but the most effective method is yet to be established. The most commonly used screening assays are based on the detection of up-regulated prostate specific markers such as prostate specific antigen (PSA). Currently, prostate specific antigen, (Farkas et al, 1998) when it is used in conjunction with other markers such as Gleason Scoring (Koksal et al, 2000) and TNM grading (Schroder et al, 1992), is considered to be a valuable tool to evaluate the histological grade of prostate carcinomas (Xess et al, 2001). As a result, patients were provided with various treatment options based on the results obtained with these parameters. These treatment options included but were not limited to operation, (Klotz, 2000b) radiotherapy, (Do et al, 2002) chemotherapy (Wang and Waxman, 2001) and hormone therapy (Klotz, 2000a; Smith et al, 2002). Regrettably, these conventional treatment modalities could not decrease the casualties from prostate cancer (Hsieh and Chung, 2001). Hence, there is a great need for development of novel treatment modalities to fight against prostate cancer. These remorseful facts ignited the initiation of gene therapy trials for prostate carcinoma (Sanda, 1997). So far, various viral vectors including lentivirus (Yu et al, 2001a), herpes simplex virus (Jorgensen et al, 2001), adeno-associated virus (Vieweg et al, 1995) and adenovirus (Loimas et al, 2001) were tested as carriers for therapeutic genes against prostate cancer. Other types of viruses such as Semliki Forest virus and Sindbis virus were also tested for gene delivery to prostate cancer cells (Loimas et al, 2001), but these viruses were unable to transduce prostate cells efficiently. Due to its antigenic properties and tissue transduction characteristics, adenovirus arose as a favored transporter vector. The exploitation of the tissue specific promoter in gene therapy especially eased adenovirus use in clinical trials (Lu and Steiner, 2000). In this review, we mainly highlighted the progress in adenovirus mediated prostate cancer gene therapy within the last three years with a particular emphasis in death ligand mediated gene therapy approach.

II. Immunomodulation

Tumors exhibit some degree of immunogenicity and the human immune system responds to these tumor specific antigens by mounting humoral and cellular responses, which are essential for the eradication of tumors. Adenovirus is commonly used for the delivery of genes encoding tumor-associated antigens in order to augment tumor-specific immune responses. However, antiviral immunity against adenovirus is a big concern, challenging its application in gene therapy. Various methods were employed in order to get around the antiviral immunity barrier to increase the efficacy of adenovirus mediated gene delivery. One of these methods involves the testing of a collagen-based matrix (Gelfoam) (Siemens et al, 2001). Coinjection of Gelfoam with adenovirus vectors carrying prostate-specific antigen (Ad5-PSA) into mice naive to PSA but immune to adenovirus, relinquished the inhibitory effects of adenoviral immunity on CTL activation. Viral vectors are also being tested to deliver tumor specific peptides into dendritic cells (DCs) to evoke an immune response. The degree of immune response generated relies on the functionality of DCs following viral transduction. To prove this, adenovirus and retrovirus vectors were compared on the basis of their influence on the functionality of DCs (Lundqvist et al, 2002a). Adenovirus-transduced monocyte-derived DCs (MO-DCs) stimulated allogenic lymphocytes and produced high levels of TNF and IL12. In addition, the expression of NF-κB and antiapoptotic molecules such as Bcl-X(L) and Bcl-2 (Lundqvist et al, 2002b) were also increased in adenovirus-transduced MO-DCs. Consequently, these cells became more resistant to spontaneous as well as Fas-mediated cell death. In contrast, retroviruses failed even to transduce MO-DCs. Although CD34(+) cell-derived DCs were transducable with retroviruses to a lesser extent, they were less potent in their ability to stimulate allogenic lymphocytes in comparison to nontransduced DCs. These results suggest that adenovirus transduction of DCs increased the survival and the potency of DC mediated activation of the immune system. This might be important for prolonging the antigen presentation to generate a greater degree of immune response.

Cytokine stimulated tumor infiltrating macrophages also play a major role in the generation of the cellular immune response against the tumor. The role of tumor-infiltrating macrophages in IFN-β-induced host defense against prostate cancer was revealed using xenograft mice models injected with adenovirus carrying IFN-β gene (Zhang et al, 2002a). Injection of an adenoviral vector encoding murine IFN-β (AdIFN-β) directly into the tumor suppressed the growth of PC-3MM2 tumors as well as prevented metastasis and prolonged the survival of tumor-bearing mice. Based on immunohistochemical staining, AdIFN-β infection resulted in the reduction of microvessel density of the tumor and increased apoptotic cell death (Cao et al, 2001). On the contrary, macrophage-selective anti-Mac-1 and anti-Mac-2 antibodies significantly reduced the antitumor effect of AdIFN-β induced therapy. Therefore, it was concluded that tumor-infiltrating macrophages must be involved in IFN-β induced suppression of tumor growth and metastasis.

III. Suicide Gene Therapy

Suicide strategy is a combined treatment modality involving chemotherapy and the gene transfer technology. The underlying principle is to limit the cytotoxicity of a drug to the local area of the tumor. To achieve this, the cDNA of a prodrug-converting enzyme is delivered into the tumor using viral vectors followed by regional or systemic application of the corresponding prodrug. As

soon as the prodrug reaches the tumor, it is taken up and converted to a cytotoxic drug by tumor cells expressing the prodrug-converting enzyme. For example, 5-Fluorouracil (5-FU) is widely used as a chemotherapeutic agent for the treatment of various malignancies. Although clinical trials have been conducted, so far 5-FU manifested a poor therapeutic index, which drastically limited its clinical use for cancer therapy. It is still not known whether the lack of success was due to problems associated with drug delivery or inherent insensitivity of cancer cells to this metabolite. However, adenovirus (Ad) vector-mediated cytosine deaminase (CD)/5-fluorocytosine (5-FC) gene therapy had the potential to overcome pharmacokinetic issues associated with systemic 5-FU administration. Escherichia coli cytosine deaminase converts the prodrug 5-FC to the cytotoxic product 5-FU. Adenovirus encoding cytosine deaminase (AdCD) gene was injected into the prostate cancer cells transplanted orthotopically on mice followed by the systemic use of 5-FC in order to investigate the antitumor and anti-metastatic effects of this approach (Zhang et al, 2002c). An effective inhibition on tumor growth and metastasis was observed through in situ injection of AdCD followed by systemic use of 5-FC in the xenograft mouse model of prostate cancer. The use of E. coli uracil phosphoribosyltransferase (UPRT), a pyrimidine salvage enzyme, which modifies 5-FU into 5-fluorouridine monophosphate, improved the activity of AdCD through enhancing the anti-tumoral effect of 5-FU. In order to assess the efficacy of the combined suicide gene therapy approach, two separate adenovirus constructs expressing either the E. coli CD or E. coli UPRT genes were infected into androgen refractory prostate cancer cell line DU145 bearing mice. This combined gene therapy approach drastically regressed the growth of tumors in these animals better than what was achieved with AdCD alone (Miyagi et al, 2003).

The most commonly used prodrug-converting enzyme for clinical approaches is the herpes simplex virus thymidine kinase gene (HSV-tk). The enzyme thymidine kinase phosphorylates the prodrug ganciclovir (GCV) to ganciclovir monophosphate, which is then further phosphorylated by cellular enzymes to ganciclovir triphosphate, a toxic metabolite and inhibitor of DNA polymerase. The efficacy of this approach was evaluated in an extended phase I/II study involving 36 prostate cancer patients with local recurrence after radiotherapy. These patients received single or repeated cycles of replication-deficient adenoviral mediated HSV-tk plus GCV in situ gene therapy (Miles et al, 2001). The study concluded that the repeated cycles of in situ HSV-tk plus GCV gene therapy can safely be administered to patients with prostate cancer who failed radiotherapy and have a localized recurrence. The therapeutic parameters such as PSA doubling time (PSADT), the mean PSA reduction (PSAR), and return to initial PSA (IR-PSA) values were all increased as a response to the treatment, indicating a therapeutic effect. A combined gene therapy approach using a recombinant adenovirus containing a fusion gene of CD and HSV-tk controlled by a cytomegalovirus (CMV) enhancer-promoter was designed to explore new frontiers in prostate cancer gene therapy (Lee et al, 2002b). Both of the prostate carcinoma cell lines tested (DU-145 or PC-3 cells) were effectively transduced and killed by this replication-incompetent adenovirus encoding CD-TK fusion protein in the presence of prodrugs. The effect of radiation and heat treatment was also tested using this vector system. Interestingly, heat treatment not only increased the expression of CD-TK but sensitized prostate cancer cells to radiation as well. These results suggested that combining heat treatment with radiation therapy improved the efficacy of the adenovirus mediated suicide gene therapy approach for prostate carcinoma. The CD-TK fusion fragment was also cloned into a lytic, replication-competent adenovirus (Ad5-CD/TKrep) and administered into patients with prostate carcinoma in a Phase I trial. This was the first gene therapy study in which a replication-competent virus was used to deliver a therapeutic gene to humans (Freytag et al, 2002a). This study demonstrated that intraprostatic administration of the replication-competent Ad5-CD/TKrep virus followed by 2 weeks of 5-fluorocytosine and ganciclovir prodrug therapy led to the destruction of tumor cells in patients without safety concerns. In addition, the efficacy and the toxicity of replication-competent adenovirus-mediated double suicide gene therapy (AdCD-TK) combined with an external beam radiation therapy (EBRT) approach was tested as a trimodal treatment modality in a preclinical study (Freytag et al, 2002b). Animals bearing prostate tumors were first injected with the lytic, replication-competent Ad5-CD/TKrep virus, then received 1 week of 5-fluorocytosine + ganciclovir (GCV) prodrug therapy supplemented with EBRT. The results from this study suggested that replication-competent adenovirus-mediated double suicide gene therapy combined with EBRT is very effective in eliminating tumors and reducing metastasis in an orthotropic mouse model of prostate carcinoma.

The efficacy of another gene-directed enzyme pro-drug therapy based on the Escherichia coli enzyme purine nucleoside phosphorylase (PNP) was tested in androgen-independent prostate cancer cells. PNP modifies the prodrug fludarabine to 2-fluoroadenine (Voeks et al, 2002). In this study, a recombinant ovine adenovirus vector (OAdV220) with a different receptor choice than that of human adenovirus type 5 carrying the PNP gene under the control of RSV promoter was used for functional studies. OAdV220 manifested a higher transgene expression compared to human Ad5 vector in infected murine RM1 prostate cancer cells during in vitro studies. Furthermore, the OAdV220 construct dramatically inhibited subcutaneous tumor growth when fludarabine phosphate was administered systemically in immunocompetent mice. Similar results were obtained using human PC3 xenografts in mice. PNP is also known to convert the prodrug 6MPDR to a toxic purine (6MP) causing cell death. In order to assess the efficacy of this approach for prostate cancer, replication-deficient human type-5 adenovirus (Ad5) carrying the PNP gene (Ad5-SVPb-PNP) was directly injected into PC3 tumors (Martiniello-Wilks et al, 2002). The specificity and the level of transgene expression from this recombinant adenoviral vector were controlled by the promoter from

the androgen-dependent, prostate-specific rat probasin (Pb) gene hooked up to the SV40 enhancer (SVPb). Unexpectedly, the SVPb element confirmed substantial prostate specificity even in the absence of androgens Intratumoral delivery of Ad5-SVPb-PNP followed by 6MPDR administration significantly suppressed the growth of human prostate tumors in nude mice These results suggested that Ad5-SVPb-PNP has therapeutic potential even in the absence of androgens for the treatment of prostate carcinoma.

Another non-toxic prodrug, CB1954, which is converted to a toxic metabolite by the Escherichia coli nitroreductase gene (NTR), was tested as a suicide gene therapy approach for prostate cancer. Adenovirus vector expressing NTR (CTL102) was injected into subcutaneous prostate cancer xenografts followed by systemic CB1954 administration (Djeha et al, 2001). A clear anti-tumor effect of the approach was observed. In addition to all the methods mentioned above, a novel approach inspired from radioiodine therapy for thyroid cancer was developed using sodium iodide symporter (NIS). NIS is normally exclusively expressed in thyroid glands. Adenovirus carrying the NIS gene (AdCMVNIS) was constructed and tested for the treatment of prostate cancer following ^{131}I administration (Spitzweg et al, 2001). Injection of AdCMVNIS construct to prostate cancer xenografts manifested highly active radioiodine uptake resulting in a drastic reduction in the tumor size following ^{131}I administration in nude mice. This new approach represented an effective and potentially curative modality leading to the accumulation of therapeutically effective radioiodine in prostate.

Diphtheria toxin (DT) is known to be a potent inhibitor of protein synthesis The fact that a single molecule of DT can result in cell death complicated the utilization of DT as a suicide gene for cancer therapy. Thus, the feasibility of using DT gene therapy would greatly be influenced by tissue specific gene expression. Adenovirus vector carrying the catalytic domain (A chain) of DT under the control of the prostate-specific antigen (PSA) promoter (Ad5PSE-DT-A) induced apoptosis in PSA-positive prostate cancer cells in the presence of exogenous androgen (R1881) (Li et al, 2002a). In addition, Ad5PSE-DT-A injection regressed the growth of a PSA-positive LNCaP xenograft in nu/nu mice. Non-PSA-secreting DU-145 cells did not manifest the same effect due to the lack of activation of PSA promoter in these cells. Therefore, the Ad5PSE-DT-A viral gene therapy approach might be a viable alternative in the treatment of PSA-secreting androgen-dependent prostate carcinoma.

IV. Joint approaches involving immunomodulation-hormonal or radiation therapy in combination with suicide gene approach

AdHSV-tk suicide gene therapy was coupled to adenovirus-mediated IL-12 delivery as a combined gene therapy approach in order to enhance NK activity induced by HSV-tk gene expression and ganciclovir (GCV) treatment (Hall et al, 2002). This dual treatment generated radical local and systemic growth suppression in a metastatic model of mouse prostate cancer (RM-1) The unification of AdHSV-tk/GCV + Ad mIL-12 gene therapy approaches resulted in the induction of apoptosis due to increased expression of Fas and FasL and improved anti-metastatic activity secondary to a strong NK effect Intra-tumoral injection of AdHSV-tk vector followed by systemic ganciclovir or local radiation therapy or the combination of gene and radiation therapy was administered to subcutaneously transplanted mouse prostate tumors (Chhikara et al, 2001) The combined treatment reduced tumor growth by 61% compared to 38% obtained by single therapy modalities. Combined therapy also increased the mean survival time. In order to analyze systemic anti-tumor activity, lung metastases were generated by tail vein injection of RM-1 prostate cancer cells. While radiotherapy alone had no effect on the metastatic growth, the number of lung nodules was reduced by 37% following treatment with AdHSV-tk. The combinational therapy led to an additional 50% reduction in lung colonization. This was the first study demonstrating a significant systemic effect of AdHSV-tk administration combined with radiation. A Phase I/II study of radiotherapy and in situ gene therapy (adenovirus/herpes simplex virus thymidine kinase gene/valacyclovir) in combination with or without hormonal therapy in the treatment of prostate cancer was conducted recently (Teh et al, 2001). Based on the preliminary results, no serious side effect of the combined therapy was observed. This was reported as the first trial of its kind in the field of prostate cancer, and is expected to enlarge the curative index of radiotherapy by merging in situ gene therapy.

V. Molecular signaling pathways modulating the efficacy of adenovirus mediated therapeutic gene delivery

Expression of certain hormone and growth factor receptors as well as cytokines and related downstream molecules can affect the efficacy of adenovirus-mediated gene therapy for prostate cancer. For example, gonadotrophin-releasing hormone (GnRH) restrains cell growth of reproductive tissue via gonadotrophin-releasing hormone receptors (GnRH-Rs) expressed in most cancers of reproductive tissues like that of prostate. Unfortunately, endogenous GnRH-R expression was not detected in PC3 cells, indicating that the cells are insensitive to GnRH. Exogenous expression of high affinity GnRH-R using adenovirus vectors (AdGnRH-R) facilitated antiproliferative effects of GnRH agonists in prostate cancer cells (Franklin et al, 2003) In addition, most of the prostate cancer cell lines overexpress fibroblast growth factors (FGFs). FGF signaling controls cell proliferation and inhibits cell death. A recombinant adenovirus expressing a dominant-negative FGF receptor (AdDNFGFR-1) was created in order to determine the biological significance of altered FGF signaling in human

prostate cancer (Ozen et al, 2001). AdDNFGFR-1 infection of LNCaP and DU145 prostate cancer cells induced extensive cell death within 48 hours. Some of the prostate cancer cell lines are androgen dependent (LNCaP) whereas some are androgen independent (DU145 or PC3). Androgen ablation therapy, surgery, and radiation therapy are relatively effective in treating androgen dependent prostate carcinoma. However these treatments were ineffective for androgen-insensitive prostate carcinoma. Upregulation of IL6 cytokine induced by the constitutive NF-κB and Jun D activation is one of the distinctive parameters of androgen independent cell lines (Giri et al, 2001). IL6 is known to function as a proliferation and differentiation factor for prostate carcinoma. The infection with adenovirus vectors encoding either the dominant negative form of IκBα gene or Jun D reduced IL6 gene expression, leading to growth suppression of prostate cancer cells (Zerbini et al, 2003). Some but not all prostate cancer cells respond to vitamin D treatment. 1α, 25-Dihydroxyvitamin D(3) (1α, 25-(OH)(2)D(3)) is known to have significant antiproliferative effects on certain prostatic carcinoma (PC) cell lines. 1α, 25-(OH)(2)D(3) inhibited cell growth and upregulated p21 expression in PC cell lines such as ALVA-31 and LNCaP (Moffatt et al, 2001). Stable transfection with a p21 antisense construct abolished the growth inhibition of ALVA-31 cells without altering vitamin D receptor expression. On the contrary, adenovirus-mediated expression of a sense p21 cDNA significantly reduced the proliferation of 1α, 25-(OH)(2)D(3) unresponsive ISU-Pr1 and JCA-1 prostate cancer cell lines. Therefore, Adp21 gene therapy may be useful even for prostate cancer patients not responding to vitamin D treatment.

Molecular signaling pathways are also altered in cancer cells. For instance, highly metastatic tumor cell lines display increased activity for focal adhesion kinase (FAK). The role of FAK in regulating migration of prostate carcinoma cell lines with increasing metastatic potential was studied in detail (Slack et al, 2001). Highly tumorigenic PC3 and DU145 cells displayed intrinsic migratory capacity correlating with an increased FAK expression and activity. On the contrary, poorly tumorigenic LNCaP cells required a stimulus to migrate. Inhibiting the FAK/Src signal transduction pathway by overexpressing FRNK (Focal adhesion kinase-Related Non-Kinase), an inhibitor of FAK activation, significantly inhibited migration of prostate carcinoma cells. Modulation of phosphatidylinositol 3'-kinase (PI3'-kinase), leading to Akt activation, frequently occurs in prostate cancer and disrupts apoptotic signaling induced by various cytokines such as tumor necrosis factor. TNF and TNF-related apoptosis-inducing ligand (TRAIL). Two prostate cancer cell lines with constitutively activated PI3'-kinase cascades (LNCaP and PC-3) were examined in order to study the role of PI3' phosphorylation in cellular response to TNF or TRAIL alone. Both TNF and TRAIL failed to activate apoptosis in either LNCaP or PC-3 cells. Interestingly, downregulation of PI3'-kinase/Akt signaling significantly enhanced the apoptotic activity of both TNF and TRAIL in LNCaP cells but not in PC-3 cells. Infection with adenovirus delivered PTEN/MMAC1 (phosphatase and tensin homologue/mutated in multiple advanced cancers) reduced Akt activation, activated apoptosis and sensitized cells to TNF but not to TRAIL in LNCaP cell line (Beresford et al, 2001). Therefore, it was concluded that although PI3'-kinase signaling inhibits both TNF and TRAIL mediated apoptosis, this may only represent one of the several apoptotic resistance mechanisms in signaling pathways.

Selenium compounds are known to be potential chemotherapeutic agents for prostate cancer. NF-κB has been categorized as the key antiapoptotic signaling molecule often activated in transformed cells. Testing of selenium compounds on DU145 and JCA1 prostate carcinoma cells revealed that these compounds induced apoptosis through the inhibition of NF-κB pathways in these cell lines (Gasparian et al, 2002b). Increased IKK activity was blamed for constitutive NF-κB activation responsible for survival of androgen independent prostate carcinoma cell lines (Gasparian et al, 2002a).

60-80 % of prostate cancers acquire the PTEN mutation during tumorigenesis. This results in the constitutive activation of the PI3'-kinase pathway and prostatic cell proliferation. The loss of PTEN activity is also correlated with the loss of activity of the FOXO family of forkhead transcription factors such as FKHRL1 and FKHR. Interestingly, these transcription factors are shown to control the expression of apoptosis inducing ligand TRAIL. Not surprisingly, the expression of TRAIL was also reduced in PTEN-lacking prostate cancer cells, leading to decreased apoptosis. Restoration of TRAIL expression using adenovirus-mediated overexpression of these transcription factors in LAPC4 prostate cancer cell line induced apoptosis (Modur et al, 2002).

VI. Apoptosis Modulators

A. The exploitation of death ligands to induce apoptosis in cancer cells

Apoptosis, known as programmed cell death (Reed, 2000) is defined as cell's preferred form of death under hectic conditions (Sears and Nevins, 2002). In reality, it is also a key mechanism for homeostasis throughout embryonic and adult life. Genetic aberrations disrupting programmed cell death underpin tumorigenesis and drug resistance. Therefore, the specific activation of apoptosis within tumor cells could be a highly effective therapeutic intervention for prostate cancer. Currently, chemotherapy (Stein et al, 2002) and radiotherapy (Wang et al, 2002) are among the most commonly used treatment modalities against prostate cancer. The tumor suppressor gene, p53, is required in order for both of these treatment methods to work as anti-tumor agents (Levine, 1997). However, more than half of the human tumors acquire p53 mutations during tumorigenesis (Horowitz, 1999; Zeimet et al, 2000). As a result, tumors lacking p53 display resistance to both chemotherapy and radiotherapy (Obata et al, 2000). Intriguingly, death ligands induce apoptosis independent of p53 status of the cells (Ehlert and Kubbutat, 2001; Norris et al, 2001). Thus, these methods constitute somewhat of a complementary treatment modality to currently employed conventional treatments.

At present, death ligands are being evaluated as potential cancer therapeutic agents (Herr and Debatin, 2001). Previously, several studies using external Fas agonists, anti-Fas antibodies and membrane-bound FasL failed to induce Fas L mediated apoptosis in prostate cancer cells. Although the down regulation of c-FLIP expression through the use of anti-sense oligonucleotides sensitized DU145 cells to an anti-Fas monoclonal antibody (Hyer et al, 2002), efficient cell killing was not observed by this approach. However, intracellular expression of FasL using adenoviruses efficiently killed 70-90% of various human prostate cancer cell lines tested (Hyer et al, 2000). Furthermore, part of this cell killing was attributed to the bystander effect mediated by FasL carried within the apoptotic bodies and cellular debris (Hyer et al, 2003). Despite the fact that human prostate cancer cells express apoptotic FasL, some of the cell lines, such as LNCaP, are resistant to Fas L mediated cell death. Even so, prior exposure to IFNγ sensitized orthotropic prostate primary tumors to recombinant adenovirus mediated FasL delivery (Selleck et al, 2003). Despite the fact that tumor necrosis factor (TNF) (Terlikowski, 2001) and FasL (Nagata, 1997) have been studied extensively and were shown to effectively induce apoptosis in cancer cells, their systemic use in cancer gene therapy is not recommended due to the systemic toxicity.

With the discovery of a novel death ligand, TRAIL/Apo2L, (Wiley et al, 1995; Pitti et al, 1996) a new era emerged for the deployment of death ligands for cancer gene therapy (Nagane et al, 2001). The fact that TRAIL does not cause any harm to normal cells but can selectively induce apoptosis in cancer cells brought up the possibility of TRAIL testing for systemic use (Griffith and Lynch, 1998). Five different receptors were identified to interact with TRAIL; TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4 and osteoprotegrin (Abe et al, 2000; Sheikh and Fornace, 2000). TRAIL-R1 and TRAIL-R2 function as authentic death receptors inducing apoptosis while TRAIL-R3 and TRAIL-R4 are unable to induce such signaling but can serve as decoy receptors (Meng et al, 2000). However even today, no single mechanism has been found to account for TRAIL resistance observed in normal cells. The soluble form of TRAIL has successfully been tested and no toxicity due to systemic use was observed in animal models. However, large quantities of TRAIL were needed in order to suppress the tumor growth. A replication-deficient adenovirus encoding human TRAIL (TNFSF10; Ad5-TRAIL) was generated as an alternative to recombinant, soluble TRAIL protein (Griffith and Broghammer, 2001). Ad5-TRAIL infection into TRAIL-sensitive prostate tumor cells induced apoptosis through the activation of Caspase 8 pathways. Normal prostate epithelial cells were not harmed by Ad5-TRAIL infection. Moreover, in vivo Ad5-TRAIL administration suppressed the outgrowth of human prostate tumor xenografts in SCID mice. Eight prostate cancer cell lines (CWR22Rv1, Du145, DuPro, JCA-1, LNCaP, PC-3, PPC-1, and TsuPr1) and primary cultures of normal prostate epithelial cells (PrEC) were tested for sensitivity to soluble TRAIL induced cell death in another study (Voelkel-Johnson et al, 2002). 100 ng/mL of soluble TRAIL administration did not induce apoptosis in Du145, DuPro, LNCaP, TsuPr1, and PrEC. Interestingly, treatment with the chemotherapeutic agent doxorubicin sensitized almost all prostate cancer cells to TRAIL-induced cell death. On the other hand, an adenoviral vector expressing full-length TRAIL (AdTRAIL-IRES-GFP) killed prostate cancer cell lines and, unexpectedly, PrEC as well, independent of doxorubicin cotreatment. This study suggested that the AdTRAIL-IRES-GFP gene therapy approach, complemented with tissue-specific promoters, would be useful for the treatment of prostate carcinoma. However, the mechanism of TRAIL resistance in normal cells is not understood and some prostate cancer cells appeared to be TRAIL-resistant (Nesterov et al, 2001). In one study, ALVA-31, PC-3, and DU 145 cell lines were highly sensitive to apoptosis induced by TRAIL, while TSU-Pr1 and JCA-1 cell lines were moderately sensitive, and the LNCaP cell line was resistant (Nesterov et al, 2001). Due to the lack of active lipid phosphatase PTEN, LNCaP cells demonstrated a constitutive Akt activity. Akt is a negative regulator of the phosphatidylinositol (PI)3-kinase/Akt pathway. PI3-kinase inhibitors sensitized LNCaP prostate cancer cells to TRAIL. In addition, adenovirus expressing a constitutively active Akt reversed the ability of wortmannin to potentiate TRAIL-induced BID cleavage. This suggested that constitutive Akt activity inhibits TRAIL-mediated apoptosis (Nesterov et al, 2001).

B. NF-κB inhibiting approaches used to breakdown TRAIL resistance in prostate cancer cells

The mechanism of TRAIL induced apoptosis and resistance is outlined in **Figure 1**. So far, at least two different hypotheses that may partly explain TRAIL resistance are asserted. The first hypothesis advocates that normal cells carry decoy receptors (TRAIL-R3, TRAIL-R4), which compete with apoptosis inducing TRAIL receptors (TRAIL-R1, TRAIL-R2) for binding to TRAIL (Pan et al, 1997; Sheridan et al, 1997). In this hypothesis, it is believed that decoy receptors either function to dilute out TRAIL ligands (like TRAIL-R3) or supply anti-apoptotic signals (like TRAIL-R4) to cells. As reported previously, TRAIL-R4 binding activates the anti-apoptotic NF-κB signaling pathway, leading to the blockade of TRAIL induced apoptosis (Degli-Esposti et al, 1997). In addition, the expression of decoy receptors is down-regulated in cancer cells through promoter hypermethylation leading to differential sensitivity to TRAIL (van Noesel et al, 2002). However, the link between TRAIL resistance and the expression of decoy receptors has not been clearly established in human cells (Griffith and Lynch, 1998). Interestingly, activation of death receptors such as TRAIL-R1 and TRAIL-R2 also stimulated the NF-κB pathway (Chaudhary et al, 1997; Schneider et al, 1997). Under these circumstances, the reason(s) for cells undergoing apoptosis despite the induction of anti-apoptotic pathways through the same death receptors is not fully understood.

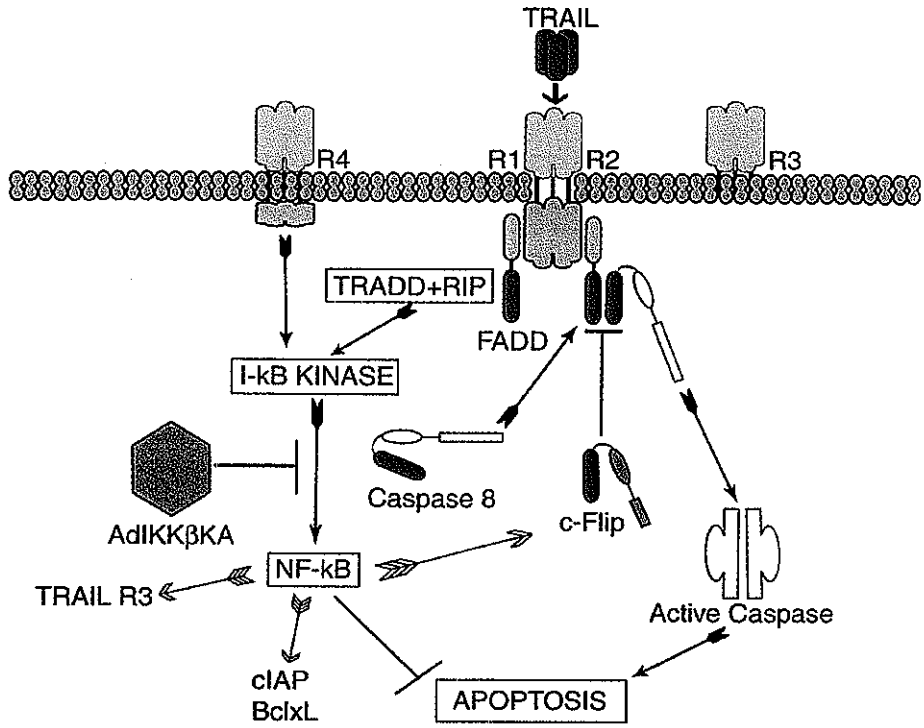

Figure 1: A gene therapy strategy to block anti-apoptotic NF-κB signaling pathway to induce TRAIL sensitivity in prostate cancer cells Activation of TRAIL receptor 1 (R1) or 2 (R2) by trimeric TRAIL ligands leads to the recruitment of Fas associated death domain protein (FADD) to the membrane. Then, FADD recruits procaspase 8 to form death inducing signaling complex (DISC). DISC induced signaling activates caspase pathway inducing cells into apoptosis. TRAIL receptor 3 (R1) and 4 (R4) serve as decoy receptors. R4 activates NF-κB signaling pathways as well. In addition, NF-κB pathway is also activated by R1 and R2 via TNFR-associated death domain protein (TRADD) and receptor interacting protein (RIP). Consequently, NF-κB activation augments expressions of various anti-apoptotic genes such as cIAP, BclxL and cFlip in addition to R3. c-Flip, a procaspase 8 homologue, competes with procaspase 8 for binding to FADD. Thereby it inhibits apoptotic signaling. The expression of adenovirus delivered IKKβKA mutant prevented the activation of anti-apoptotic NF-κB signaling. This method sensitized prostate cancer cells to TRAIL.

The second hypothesis claims the presence of apoptosis inhibitory substances in these cells. Such a molecule, cFLIP (FLICE Inhibitory Protein), a caspase 8 homologue, has been shown to obstruct death ligand induced apoptosis (Irmler et al, 1997; Griffith et al, 1998). Intriguingly, NF-κB activating agents up-regulated cFLIP synthesis (Kreuz et al, 2001). Furthermore, the NF-κB pathway has been proven to increase TRAIL-R3 synthesis, a decoy receptor for TRAIL, (Bernard et al, 2001) and the expression of apoptosis inhibitor Bcl-xL (Hatano and Brenner, 2001; Ravi et al, 2001) resulting in the obstruction of TRAIL mediated apoptosis. Apoptosis inhibitors such as cIAP are also activated by NF-κB pathways (Mitsiades et al, 2002). Based on these results, we can clearly state that the active NF-κB signaling pathway may provide cells with TRAIL resistance by at least four different ways (**Figure 1**). Additionally, it has been reported that a novel tumor suppressor gene, PTEN/MMAC1 (Steck et al, 1997; Simpson and Parsons, 2001) negatively regulated TNF induced NF-κB activity (Ozes et al, 1999; Mayo et al, 2002) through the IKK complex (Gustin et al, 2001). The observation in which IKK activity is required for PI3K-Akt induced NF-κB activation (Burow et al, 2000; Demarchi et al, 2001) confirmed this report (Madrid et al, 2001; Sizemore et al, 2002). Due to a negative correlation between the expression of PTEN and the progression of prostate cancer, advanced prostate cancer cells might have intrinsically higher NF-κB activity due to the progressive loss of PTEN. Absence of PTEN function may result in increased Akt activity induced by PI3K. Since NF-κB is a downstream target for Akt, (Kane et al, 1999; Romashkova and Makarov, 1999; Andjelic et al, 2000; Jones et al, 2000) TRAIL resistance would ultimately be ensured in cells by way of the NF-κB pathway. In agreement with this hypothesis, PTEN sensitized prostate cancer cells to TRAIL induced apoptosis (Yuan and Whang, 2002). Thus, these possible scenarios make NF-κB inhibiting vectors such as Ad IKKβKA (Sanlioglu et al, 2001a) or Ad.IκBαSR (Batra et al, 1999; Sanlioglu and Engelhardt, 1999) ideal candidates for overcoming the TRAIL resistance in PTEN mutant prostate cancer cells. In a similar manner, TNF induced apoptosis can also be prevented by NF-κB activation as reported (Beg and Baltimore, 1996; Van Antwerp et al, 1996). Previously, NF-κB inhibiting approaches such as adenovirus mediated transfer of IKKβ (Ad.IKKβKA) (Sanlioglu et al, 2001a, 2001b) or IκBα (Ad.IκBαSR) (Batra et al, 1999; Sanlioglu and Engelhardt, 1999) dominant negative mutants were successfully deployed in order to sensitize lung cancer cells to TNF. Since some tumor cells have intrinsically high NF-κB activity, which might be responsible for TRAIL resistance, NF-κB blocking agents can potentially be useful to overcome TRAIL resistance. For example, a constitutive NF-κB activation was observed in renal carcinoma (Oya et al, 2001). Not surprisingly, melanoma cells having a constitutive NF-κB

activity exhibit TRAIL resistance (Franco et al, 2001). Resistant melanoma cells were sensitized to TRAIL either with proteasome inhibitors or transfections with plasmids encoding degradation resistant IκBα protein (Franco et al, 2001). In accordance with these studies, we have tested if adenovirus mediated NF-κB inhibiting approach would sensitize prostate cancer cells to TRAIL. Consequently, adenovirus mediated delivery of IKKβKA mutant (Ad.IKKβKA) sensitized PTEN mutant prostate cancer cells (PC3) to TRAIL as shown in **Figure 2**. At first, PC3 cells appeared to be relatively resistant to pro-apoptotic effects of TRAIL when cells were infected with adenovirus vector encoding hTRAIL (Ad.hTRAIL) even at an MOI of 1000 DNA particles/cell (**Figure 2 Panel A**). Infection with Ad.IKKβKA vector alone did not yield any cell death either (**Figure 2, Panel B**). However, when the dose of Ad.hTRAIL vector was kept constant at an MOI of 1000 DNA particles/cell, increasing the amount of Ad.IKKβKA construct sensitized PC3 cells to TRAIL mediated apoptosis (**Figure 2, Panel C**).

C. Intracellular proapoptotic regulators

Although caspases are the effector mediators of apoptosis, the expression of proapoptotic molecules such as procaspase 3 or 7 using adenovirus constructs did not induce apoptosis in prostate cancer cells due to the inability of these caspases to undergo autocatalytic activation (Li et al, 2001). A novel suicide gene therapy approach was developed using chemically inducible effector caspases to trigger apoptosis in prostate cancer cells. Cell death was mediated by replication-deficient adenoviral vector expressing conditional caspase-1 (Ad-G/iCasp1) or caspase-3 (Ad-G/iCasp3) and the caspase activation was achieved by nontoxic, lipid-permeable, chemical inducers of dimerization (CID) (Shariat et al, 2001). Aggregation and activation of these recombinant caspases occurred, leading to rapid apoptosis only after vector transduction followed by CID administration in both human (LNCaP and PC-3) and murine (TRAMP-C2 and TRAMP-C2G) prostate cancer cell lines. Subcutaneous TRAMP-C2 tumors displayed focal but extensive apoptosis following direct injection of Ad-G/iCasp1 in vivo. In order to express caspase 9 exclusively in prostate, a recombinant adenovirus carrying iCaspase-9 was constructed with two copies of the androgen response region (ARR) placed upstream of the probasin promoter elements (ADV.ARR(2)PB-iCasp9) (Xie et al, 2001b). AP20187 is a chemical dimeric ligand, which causes dimerization and thereby activation of iCaspase-9 leading to rapid apoptosis in both dividing and nondividing cells. Testing of ADV.ARR(2)PB-iCasp9 construct in LNCaP tumor xenografts demonstrated that this construct induces apoptosis in prostate cancer cells only in the presence of AP20187.

The proapoptotic members of Bcl- 2 protein family including Bax, Bak, Bad, and Bik also mediate apoptosis. Apoptosis-inducing proteins were cloned into adenovirus constructs and shown to induce apoptosis in prostate cancer cell lines previously

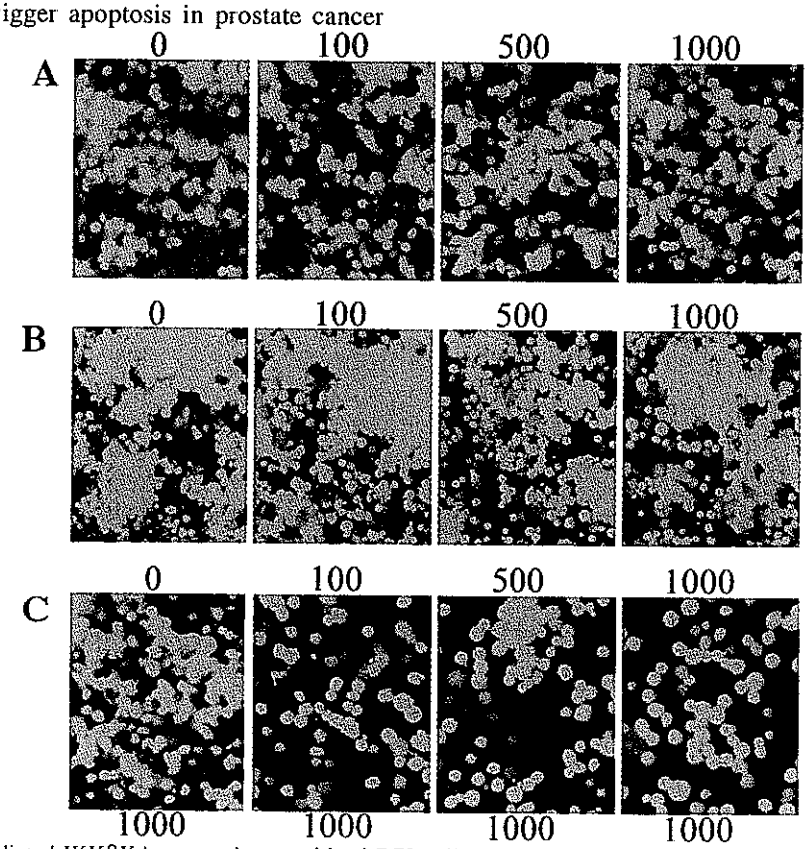

Figure 2. Adenovirus mediated IKKβKA expression sensitized PC3 cells to TRAIL mediated apoptosis. PC3 cells were infected with increasing MOIs of either Ad5hTRAIL (Panel A) or Ad.IKKβKA (Panel B). In panel C, the dose of Ad.IKKβKA vector was increased gradually (stated just above each panel) while the amount of Ad5hTRAIL was kept constant (as indicated under the panel). Cell death was detected using molecular probe's Live and Death Cellular viability and toxicity kit 48 hours following infection. Numbers indicate viral doses as MOI values of DNA particles/cell.

However, overexpression of proapoptotic genes without the use of tissue specific promoters could result in unwanted apoptosis even in normal cells. In order to provide tissue specificity, an adenoviral construct was generated containing Bax cDNA under control of the probasin promoter that included two androgen response elements (Av-ARR2PB-Bax). Av-ARR2PB-Bax construct drove Bax overexpression in an androgen-dependent way in androgen receptor (AR)-positive cell lines of prostatic origin but not in others. The androgen dihydrotestosterone activated apoptosis in LNCaP cells infected with Av-ARR2PB-Bax but not in those infected with control vectors. These results demonstrated that Av-ARR2PB-Bax induced apoptosis was androgen dependent and limited to AR positive cells of prostatic epithelium. On the other hand, using a binary co-transfection strategy involving Ad/GT Bax and Ad/PGK-GV16; overexpression of proapoptotic Bax protein induced apoptosis both in androgen-insensitive (DU145 and PC3), and androgen-sensitive (LNCaP) cell lines (Honda et al, 2002). The same binary approach was tested to assess the consequences of Bcl-2 overexpression in the progression of prostate carcinoma leading to apoptosis-resistant and androgen-independent phenotype in DU145, PC3 and LNCaP cell lines which represent models of advanced prostate carcinoma. Bax expression generated by the adenoviral co-transfection system induced apoptosis even in these Bcl-2 overexpressing cell lines. These results suggest that the Ad/GI Bax and Ad/PGK-GV16 combined expression system might represent a powerful gene therapy strategy for the treatment of androgen-independent and apoptosis-resistant prostate carcinoma. Moreover, monogene and polygene approaches were compared in an experimental prostate cancer model using apoptotic genes bad and bax driven by a prostate specific promoter (ARR(2)PB) in an adenovirus construct (Zhang et al, 2002b). The ARR(2)PB is a dihydrotestosterone (DHT)-inducible third-generation probasin-derived promoter. In this study, animals bearing tumors of prostatic origin responded better to combined bad and bax therapy than either of the vectors alone. Therefore, it was concluded that polygene therapy involving more than one apoptotic molecule is more effective in xenograft models of androgen-dependent or independent prostate cancer than monogene therapy alone. It is also known that overexpression of anti-apoptotic genes such as Bcl-2 in prostate carcinoma provides resistance to radiation therapy and androgen ablation. A second-generation adenoviral vector (ARR2PB.Bax GFP) was constructed with the modified prostate-specific probasin promoter (ARR2PB) directing the expression of a HA-tagged Bax gene in order to restore the balance of Bcl-2 family members to induce apoptosis in prostate cancer cells (Lowe et al, 2001). ARR2PB Bax.GFP vector induced significant levels of apoptosis in LNCaP cells 48 hours following infection even in the presence of high levels of Bcl-2 protein. No toxicity in liver, lung, kidney, and spleen was detected by systemic administration of ARR2PB.Bax.GFP in nude mice. Therefore, a second-generation adenovirus-mediated, prostate-specific Bax gene therapy appeared to be a very safe and efficient approach for the treatment of prostate cancer. Another member of the proapoptotic Bcl-2 family, namely "Bik", was cloned into adenovirus vectors to explore its therapeutic potential. AdBik infection also induced apoptosis and suppressed the growth of PC-3 xenografts established in nude mice (Tong et al, 2001).

Several other genes were also tested for their ability to induce apoptosis in prostate tumor cell lines as well as in xenograft models. The antiapoptotic protein CLN3 negatively regulates endogenous ceramide production, an inducer of apoptotic cell death. CLN3 protein is overexpressed in most of the cancer cell lines tested including those of prostate (Du145, PC-3, and LNCaP). An adenovirus-expressing antisense CLN3 (Ad-AS-CLN3) blocked CLN3 protein expression in prostate cancer cell lines as demonstrated by Western Blotting (Rylova et al, 2002). Ad-AS-CLN3 infection resulted in the inhibition of cell growth and reduction in cell viability of cancer cells through elevation of endogenous ceramide production. This study revealed CLN3 as a novel target to induce apoptosis in prostate cancer cells. A recombinant adenovirus containing pHyde cDNA gene (AdpHyde), a novel gene cloned from Dunning rat prostate cancer cells, was constructed in order to study its function (Zhang et al, 2001). Surprisingly, the AdpHyde construct inhibited the growth of human prostate cancer cells and induced apoptosis involving the caspase-3 pathway in human prostate cancer tumor xenografts in nude mice. Ionic movement also influences apoptosis. For instance, K^+ efflux is an early event in apoptosis, which is regulated by K^+ channel-associated protein (KChAP). A recombinant adenovirus encoding KChAP (Ad/KChAP) was constructed in order to determine if KChAP expression could induce apoptosis in prostate cancer cells (Wible et al, 2002). The LNCaP cell line displayed a reduction in cell size upon infection with Ad/KChAP. The Ad/KChAP construct also induced apoptosis in DU145 cells in a p53 independent manner. In addition, infection with Ad/KChAP prevented growth of DU145 and LNCaP tumor xenografts in nude mice.

VII. Tumor suppressor genes

Aberrations in the expression of tumor suppressor genes have been one of the key factors affecting the outcome of cancer therapy. Several studies examined the possible use of tumor suppressor genes as therapeutic agents for prostate cancer. Doxorubicin (Dx) is a commonly used chemotherapeutic agent in recurrent prostate cancer and is a strong inducer of p53 expression leading to p21(CIP1/WAF1) transactivation. As suggested by previous reports, p21 plays a role in the modulation of chemotherapy-induced apoptosis, prostate cancer progression and androgen regulation. Two androgen-regulated human prostate cancer cell lines (MDA PCa 2b and LNCaP) were exposed to Dx and growth factor withdrawal in order to investigate if p21 plays a role in the survival of prostate cancer cells under stress (Martinez et al, 2002). Infection with adenovirus vectors encoding the antisense strand of p21 reduced p21 levels, sensitized prostate cancer cells to Dx and facilitated apoptosis in response to growth factor withdrawal. These results suggest that modulation of p21 pro-survival gene

expression via adenovirus constructs sensitizes prostate cancer cells to chemotherapeutics and androgen withdrawal. Another tumor suppressor protein, p27, also known as cyclin-dependent kinase inhibitor (CDKI), is normally expressed in human prostate. However, the majority of human prostate cancers have reduced levels of p27. The down regulation of this putative tumor suppressor gene through proteolysis is mediated by SCFSKP2 ubiquitin ligase complex. Adenovirus-mediated overexpression of SKP2 induced ectopic down-regulation of p27 in LNCaP prostate carcinoma cells (Lu et al, 2002). This observation confirmed that SKP2 activity was the major determinant of p27 levels in human prostate cancer cells. Based on in vitro studies, it is believed that the overexpression of SKP2 might be one of the mechanisms allowing prostate cancer cells to escape growth control mediated by p27. Therefore, knocking out SKP2 function would be a logical novel approach to fight prostate cancer. In another study, an adenovirus construct carrying p27 coding sequences Adp27(Kip1) was generated to assess whether the overexpression of p27 has any affect on the prostatic tumor growth in vivo (Katner et al, 2002). Injection of Adp27(Kip1) vector reduced the growth of LNCaP tumor xenografts in mice. This study supported the idea that Adp27(Kip1) can serve as a potential therapeutic vector for the treatment of prostate carcinoma.

p14(ARF), encoded by the human INK4a gene locus, is another tumor suppressor protein which is frequently inactivated in human cancer. p14(ARF) has recently been implicated in p53-independent cell cycle regulation and apoptosis. A replication-deficient adenoviral construct carrying p14(ARF) coding sequence (Ad-p14(ARF)) was generated in order to explore the pro-apoptotic function of p14(ARF) in relationship to p53 function (Hemmati et al, 2002). Ad-p14(ARF) construct induced apoptosis in p53/Bax-mutated DU145 prostate cancer cells and HCT116 cells lacking functional Bax expression. This study demonstrated that overexpression of p14 through adenovirus vectors is sufficient to induce apoptosis in p53- and bax-deficient prostate cancer cells. Prostate carcinoma with p53 mutant phenotype represents a clear obstacle for irradiation therapy. Ionizing radiation (IR) and adenoviral p53 gene therapy (Ad5CMV-p53) were utilized individually as well as in combination in order to assess the effectiveness of combined therapy for prostate cancer (Sasaki et al, 2001). In this study, IR alone did not induce significant levels of apoptotic cell death in DU145 and PC-3 cells. However, after combined therapy, the proportion of apoptotic cells was greatly amplified in both of the cell lines tested. Therefore, it was concluded that the observed synergistic effect might be useful for the treatment of radio-resistant prostate carcinoma.

The loss of MMAC/PTEN tumor suppressor gene expression is frequently detected in human tumors. Survival signaling through the phosphatidylinositol-3 kinase/Akt pathway is constitutively activated in cells lacking functional PTEN expression. Therefore, the functional effect of MMAC/PTEN expression was examined in LNCaP cells, which are devoid of a functional PTEN product (Davies et al, 1999). Infection with an adenovirus construct driving the expression of MMAC/PTEN resulted in a specific inhibition of Akt/PKB activation. This is consistent with the phosphatidylinositol phosphatase activity of MMAC/PTEN. Compared to adenovirus delivered p53 expression, MMAC/PTEN expression induced apoptosis in LNCaP cells to a lesser extent. Interestingly, the growth suppression properties of MMAC/PTEN were significantly greater than those accomplished with p53. Moreover, Bcl-2 overexpression in LNCaP cells blocked both the adenovirus mediated MMAC/PTEN- and p53-induced apoptosis, but it did not affect the growth-suppressive properties of MMAC/PTEN. This is consistent with the fact that MMAC/PTEN may play multiple roles in the cell. Prostate cells were infected with adenovirus vector carrying PTEN coding sequence in order to determine if supplying PTEN function would sensitize these cells to various apoptotic stimuli (Yuan and Whang, 2002). As predicted, adenovirus-mediated PTEN delivery sensitized LNCaP prostate cancer cells to apoptosis through the inhibition of constitutive Akt activation. Since PTEN G129E mutant lacking lipid phosphatase activity was unable to sensitize cells to apoptosis, it was concluded that the lipid phosphatase activity of PTEN was required for apoptosis. The therapeutic effect of adenoviral delivery of MMAC/PTEN was tested on both the in vitro and in vivo growth of PC3 human prostate cancer cells (Davies et al, 2002). The in vitro growth of PC3 cells was repressed by adenovirus expression of MMAC/PTEN via blocking of cell cycle progression. Although this approach did not inhibit the tumor progression of orthotopically implanted PC3 cells, a significant reduction was observed in the tumor size in vivo, in addition to complete inhibition of metastases. Therefore, it was suggested that MMAC/PTEN might play a role mostly in the regulation of the metastatic potential of prostate cancer.

A considerable fraction of prostate tumors display an alteration of Mxi1 expression, an antagonist to c-Myc. This was confirmed by transgenic approaches in which prostatic hyperplasia was observed in mice deficient for Mxi1. Mxi1-expressing adenovirus (AdMxi1) was generated to study the ability of Mxi1 to act as a growth suppressor in prostate tumor cells (Taj et al, 2001). Overexpression of Mxi1 using adenovirus vectors in the DU145 prostate carcinoma cell line resulted in growth arrest and decreased colony formation on soft agar. All these studies emphasize that the modulation of tumor suppressor gene function might be necessary for an optimum therapeutic response to fight against prostate cancer.

VIII. Cell adhesion molecules and anti-angiogenic approaches

Cell adhesion molecules play major roles especially in metastasis of cancer cells. Therefore, aberrant expression patterns of cell adhesion molecules are frequently associated with poor prognosis. For instance, the expression of a well-known cell adhesion molecule, C-CAM1, is downregulated during the early stages of prostate carcinoma in an animal model (TRAMP) (Pu et al, 1999). C-CAM1 was cloned into an adenovirus

construct and its efficacy was tested both in vitro and in vivo using PC3 xenograft murine model (Lin et al, 1999). AdC-CAM1 construct manifested a strong antitumoral activity on PC3 tumor cells grown in nude mice. Therefore, selective use of cell adhesion molecules might be beneficial for the treatment of prostate carcinoma. Moreover, combining C-CAM1-based therapy with TNP-470, a potent angiogenesis inhibitor, induced greater growth suppression on DU145 tumor xenografts than by either Ad-C-CAM1 or TNP-470 application alone (Pu et al, 2002)

Vascularization of a solid tumor is required for cancer growth. Recently, preventing vascularization through inhibition of angiogenesis was a popular target for cancer gene therapy. For example, a 16-kDa prolactin protein (PRL) has previously been shown to possess an antiangiogenic activity (Galfione et al, 2003). Not surprisingly, adenovirus delivery of PRL protein manifested a significant antitumoral activity in vivo (Kim et al, 2003). In addition, vascular endothelial growth factor (VEGF) receptor signaling is another relevant pathway, which modulates the vascularization of newly growing tumors. Interfering with such a signaling pathway might be valuable in controlling the tumor growth. In fact, when fused to an Fc domain and cloned into the recombinant adenovirus construct, the ligand-binding ectodomain of VEGF receptor 2 (Flk1) manifested a considerable reduction in tumor growth induced by a drastic decline in the microvessel density in SCID mice carrying human LNCaP xenografts (Becker et al, 2002).

Growth factors are needed for survival of cancer cells and molecular chaperones are required for functional production of these molecules. A new member of the heat shock protein family functioning as a molecular chaperone in the endoplasmic reticulum was recently discovered and named as 150-kDa oxygen-regulated protein (ORP150). Since prostate cancer cells exhibited an upregulation of ORP150 protein and VEGF, adenovirus delivery of an antisense ORP150 cDNA approach was used to reduce angiogenicity and tumorigenicity through inhibition of VEGF secretion. This approach indeed suppressed the growth of DU145 prostate carcinoma cell line in a xenograft model (Miyagi et al, 2002).

IX. Replication competent adenovirus vectors

Replication competent adenoviral vectors provide powerful means to kill cancer cells through cell lysis. Since they only replicate in tumor cells, the therapeutic range is limited to cancer cells. Two replication-competent adenoviruses, CV706 and CV787, were generated in order to selectively destroy PSA producing prostate cancer cells. It has been demonstrated earlier that prostate-specific antigen (PSA)-selective replication-competent adenovirus variant CV706 specifically eliminated tumors in human prostate cancer xenografts in preclinical models (Rodriguez et al, 1997) Since adenovirus E1A is known to be a potent inducer of chemosensitivity and radiosensitivity through p53-dependent and independent mechanisms, the potential radiosensitizing effects of CV706 on prostate cancer cells were evaluated (Chen et al, 2001). The CV706 construct demonstrated a synergistic antitumoral effect both on irradiated human prostate cancer cells and tumor xenografts. Moreover, in order to investigate the safety and the functionality of intraprostatic delivery of CV706 for the treatment of patients with locally recurrent prostate cancer following radiation therapy, a Phase I dose-escalation study was conducted (DeWeese et al, 2001). Results from this study suggested that even at high doses, intraprostatic delivery of the CV706 was relatively safe for patients and CV706 construct demonstrated high therapeutic activity as reflected by the reduction in serum PSA. This was the first clinical trial of a prostate-specific, replication-restricted adenovirus for the treatment of prostate cancer. Another prostate-specific replication-competent adenovirus carrying not one, but two, cell type specific promoters (CV787) was constructed. This construct contained E1B gene driven by the human prostate-specific enhancer/promoter and the adenovirus type 5 (Ad5) the E1A gene under the control of prostate-specific rat probasin promoter. The Ad5 E3 region was also conserved in the vector to improve the efficacy. A single tail vein injection of CV787 eliminated LNCaP xenografts within 4 weeks in nude mice (Yu et al, 1999) When the prostate cancer-specific adenovirus CV787 was combined with chemotherapeutic agents like taxanes (paclitaxel and docetaxel), a synergistic antitumoral effect was observed in mice carrying human prostate cancer xenografts (Yu et al, 2001b)

Heat-inducible gene expression is another approach used in the context of suicide gene therapy. A recombinant adenovirus containing the CD-TK fusion gene controlled by the human inducible heat shock protein 70 promoter (Ad.HS-CDTK) was generated for this purpose. Heat application at 41°C for 1 hour induced therapeutic gene expression from this vector. Despite the fact that the Ad.HS-CDTK construct induced CD-TK expression in human prostate cancer cells, a therapeutic benefit was not observed due to lower transduction efficiency of tumors in vivo Instead, a replication-competent, E1B-attenuated adenoviral vector containing the hsp70 promoter-driven CD-TK gene (Ad.E1A$^+$HS-CDTK) was generated to increase CD-TK gene expression to achieve a therapeutic effect (Lee et al, 2001). Contrary to replication incompetent Ad.HS-CDTK, replication competent Ad.E1A$^+$HS-CDTK construct yielded severe cytotoxicity and greater levels of therapeutic index in the presence of prodrugs This approach revealed the beneficial effects of using replication competent virus complemented with a heat inducible suicide gene therapy approach for prostate carcinoma.

X. Adenovirus vectors with cell type specific and inducible promoters

Even though adenovirus-mediated HSVTK suicide gene therapy approach manifested a satisfactory toxicity profile in Phase I clinical trials, the toxicity studies using adenovirus vectors were very restricted in numbers.

However, it was known that the promoter of choice might influence the level of toxicity In order to study the promoter effect on adenovirus mediated toxicity the mouse caveolin 1 promoter was cloned into the adenovirus HSV-tk vector (Adcav-1tk) because this promoter was highly active in metastatic and androgen-resistant prostate cancer cells (Pramudji et al, 2001) The efficacy of this vector for suicide gene therapy was compared to those of AdHSV-tk vectors carrying either cytomegalovirus (AdCMV-tk) or rous sarcoma virus (AdRSV-tk) promoters in mice transplanted with mouse prostate cancer cells. Following GCV administration, all the HSV-tk expressing vectors regressed the tumor growth in situ Interestingly, the efficacy of Adcav-1tk vector was much greater in terms of inducing necrosis and microvessel density. In order to evaluate the toxicity profile of adenovirus vectors carrying CMV, RSV or mouse caveolin promoter-driven HSV-tk transgenes, these vectors were also injected systemically into mice (Ebara et al, 2002). Adenovirus vectors with CMV and RSV promoters, but not caveolin promoter, exhibited significant levels of liver damage. These results suggested that the promoter selection greatly influences the toxicity profile of adenovirus-mediated suicide gene therapy approach. In order to increase the number of promoters available for prostate specific gene expression, transgenic mice were generated expressing a reporter gene (SV40 Tag) directed by prostate secretory protein of 94 amino acids (PSP94) (Gabril et al, 2002). PSP94 gene promoter/enhancer region directed SV40 Tag expression exclusively in prostate leading to prostatic intraepithelial neoplasia and eventually to high-grade prostate carcinoma These studies suggested that this PSP94 gene promoter/enhancer strategy could be employed for the treatment of prostate carcinoma

One conventional way to limit the toxicity of virus mediated suicide gene therapy is to use cell type specific promoters as suggested above Although adenovirus vectors with the native PSA enhancer and promoter (PSAP) provided prostate-specific expression, lower transcriptional activity observed in prostate challenged its use in prostate-targeted gene therapy To improve the activity and specificity of the prostate-specific PSA enhancer for gene therapy, various studies were carried out by exploring the properties of the natural PSA control regions. Chimeric PSA enhancer constructs were generated with tandem copies of the proximal ARE elements and then inserted into adenovirus constructs (Ad-PSE-BC-luc) (Wu et al, 2001) This construct was highly inducible with androgens as shown by systemic administration into SCID mice carrying LAPC-9 human prostate cancer xenografts while retaining prostate specific gene expression. Furthermore, the CreLoxP system was also utilized to enhance the activity of PSAP. CD suicide gene therapy approach using adenoviral vectors with CRELoxP augmented PSAP activity effectively inhibited subcutaneous LNCaP tumor growth in nude mice (Yoshimura et al, 2002). In addition, hormone refractory prostate cancer cells retain the expression of prostate-specific membrane antigen (PSMA) and prostate-specific antigen (PSA) An adenovirus construct with an artificial chimeric enhancer (PSES) composed of two modified regulatory elements of PSA and PSMA genes (Ad-PSES-luc) was generated and tested for its promoter activity for the treatment of prostate cancer (Lee et al, 2002a). Systemic injection of Ad-PSES-luc construct into mice produced very low levels of reporter gene expression in major organs However, when injected directly into prostate, only the prostate but not other tissues produced high levels of reporter gene expression These results encouraged the use of PSES for the treatment of androgen-independent prostate carcinoma Even though prostate-specific antigen (PSA/hK3) provided prostate specific gene expression, its expression displayed an inverse correlation with prostate cancer grade and stage, giving reason to doubt its effectiveness for advanced stage of prostate carcinoma A new approach was developed in order to generate gene therapy vectors targeting higher grades especially of prostate carcinoma. The human glandular kallikrein 2 (hK2) is upregulated in an advanced form of prostate cancer with a higher grade. Therefore the hK2 promoter was cloned into adenovirus construct in combination with EGFP reporter gene (ADV.hK2-E3/P-EGFP) in order to obtain preferential expression of EGFP in prostate cancer (Xie et al, 2001a) Indeed ADV.hK2-E3/P-EGFP injection led to a robust but tumor-restricted EGFP expression in subcutaneously generated LNCaP tumors. These results showed that adenovirus constructs with the hk2 multienhancer/promoter driven therapeutic genes might be a powerful tool for gene therapy of advanced prostate cancer

Previous studies have shown that the bone matrix protein osteocalcin is predominantly expressed in prostate cancer epithelial cells, fibromuscular stromal cells and osteoblasts. A conditional replication competent adenovirus vector carrying the osteocalcin promoter driven early E1A gene (AdOCE1A) was generated to co-target both prostate cancer cells and their surrounding stromal cells (Matsubara et al, 2001) Both PSA-producing (LNCaP) and non-producing (DU145 and PC3) human prostate cancer cell lines as well as human stromal cells and osteoblasts were effectively killed by this recombinant virus in vitro. In addition a single systemic intravenous injection of the AdOCE1A construct significantly destroyed prostate tumor cells transplanted in SCID mice. This co-targeting strategy appeared to have a broader effect compared to other recombinant constructs tested on the preclinical models of human prostate cancer These promising results initiated first gene therapy trial (phase I) in which adenoviruses carrying the osteocalcin promoter driven HSV-tk gene (AdOCHSVTK) were directly injected into prostate cancer lymph node and bone metastasis (Kubo et al, 2003) The results of this trial suggested that adenoviruses did not display any adverse effects and the treatment was well tolerated in all patients. In addition, 63 % of the patients had local cell death in treated lesions. Further studies are suggested in order to assess the efficacy of this approach for androgen-independent prostate carcinoma. A new treatment modality to enhance adenoviral replication by vitamin D_3 in androgen-independent human prostate cancer cells and tumors was tested using a novel replication-competent adenoviral vector, Ad-hOC-E1, carrying the human

osteocalcin (hOC) promoter to drive both the early viral E1A and E1B genes (Hsieh et al, 2002). While the replication properties of Ad-hOC-E1 vector were restricted to OC-expressing cells, vitamin D_3 exposure further enhanced viral replication by 10 fold. The growth of both androgen-dependent and androgen-independent prostate cancer cells was suppressed by Ad-hOC-E1 infection, irrespective of the cells' androgen responsiveness and PSA status. This is in contrast to Ad-sPSA-E1 vector, which only replicated in PSA-expressing cells with androgen receptor (AR). Ad-hOC-E1 injection inhibited the growth of DU145 (an AR and PSA-negative cell line) tumor xenografts in mice. Consequently, vitamin D_3-enhanced Ad-hOC-E1 viral replication represented an alternative for the treatment of localized or osseous metastatic prostate cancer. Prostate specific antigen promoter (PSAP) and rat probasin (rPB) promoter are currently employed to drive the therapeutic transgene expression in prostate cancer cells. However, since these promoters require the binding of androgen to androgen receptor for activation, they were only functional in androgen-dependent prostate carcinoma cells. Because androgen refractory prostate carcinoma cells lose the expression of androgen receptor along the way, constructs with PSAP or rPB promoters are not useful for treating patients with androgen-independent prostate carcinoma. In order to circurment this problem, prostate specific promoters were modified so that they were activated in response to the retinoids-retinoid receptor complex in place of the androgen-AR complex. As a result, retinoid treated androgen-independent prostate cancer cells were sensitized to HSVTK-ganciclovir gene therapy using promoters responding to retinoids (Furuhata et al, 2003).

Apart from promoters providing tissue specific gene expression, expression inducible promoters were cloned into adenovirus constructs to control the onset and the duration of gene expression. Tetracycline-inducible adenovirus vectors expressing the cytokine interleukin-12 were successfully tested in an immunotherapy model for prostate cancer (Nakagawa et al, 2001). Thus, recombinant adenovirus vectors with tetracycline-inducible gene expression opened up new avenues while improving the safety of viral vector administration for cancer gene therapy. Limitation of cytotoxic gene expression only to tumor cells is very much desired in adenovirus-mediated gene therapy approach for cancer. Unfortunately, the expression levels of many tumor and tissue-specific promoters are much lower than the constitutively active promoters. A complex adenoviral vector was generated by fusing the tetracycline transactivator gene to a prostate-specific ARR2PB promoter while placing a mouse FASL-GFP fusion gene under the control of the tetracycline responsive promoter. This allowed the joining of cell-type specificity with high-level regulation of transgene expression (Rubinchik et al, 2001). The doxycycline regulated, ARR2PB driven FASL-GFP vector generated higher levels of prostate-specific FASL-GFP expression than FASL-GFP expression directed with ARR2PB alone, leading to apoptosis in LNCaP cells. Systemic delivery of both the prostate-specific and the prostate-specific/tet-regulated vectors was well tolerated in animals at doses that were lethal for adenovirus vectors with CMV-driven FASL-GFP expression. This approach improved the safety and efficacy of adenovirus-mediated cytotoxic gene delivery for the treatment of prostate carcinoma.

The prostate-specific adenovirus gene expression technology can also be used for the identification of metastatic lesions of prostate cancer through the use of non-invasive imaging. A prostate-specific adenovirus vector expressing a luciferase reporter gene (AdPSE-BC-luc) and a charge-coupled device-imaging system were employed for this purpose (Adams et al, 2002). A robust expression from AdPSE-BC-luc construct was found in the prostate, especially in the androgen-independent tumors. Furthermore, metastatic lesions in the lung and spine with prostatic origin were identified successfully through repetitive imaging over a three-week period after AdPSE-BC-luc injection into tumor-bearing mice. These results demonstrate that adenovirus gene delivery specific to the prostate can be coupled to a non-invasive imaging modality for therapeutic and diagnostic strategies for prostate cancer.

XII. Adenovirus vectors for vaccination and adjuvant gene therapy

CAR receptors and MHC class I heavy chains are important mediators of adenovirus entry into tumor cells. Contrary to the cell lines derived from other malignancies, down regulation of CAR or MHC class I expression is relatively rare in both human and murine prostate carcinoma cells. This brought the possibility of developing vaccine strategies for prostate cancer based on the modification of prostate cancer cells using recombinant adenovirus vectors (Pandha et al, 2003). The expression of prostate-specific antigen (PSA) is highly restricted to prostatic epithelial cells. In fact, 95 % of patients with prostate carcinoma express PSA, making this antigen a good candidate for targeted immunotherapy. A recombinant PSA adenovirus type 5 (Ad5-PSA) was generated in order to activate PSA-specific T-cell response with the potential of eliminating prostate cancer cells (Elzey et al, 2001). Ad5-PSA immunized mice displayed a PSA-specific cellular immunity involving $CD8^+$ T lymphocytes. This approach deterred subcutaneous tumor formation with RM11 prostate cancer cells expressing PSA (RM11psa). However, this did not affect the growth of existing RM11psa tumors. On the contrary, Ad5-PSA administration followed by intratumoral injection of recombinant canarypox viruses (ALVAC) encoding interleukin-12 (IL-12), IL-2, and tumor necrosis factor-α effectively eliminated established RM11psa tumors.

Surgery is one of the conventional treatment modalities used against solid tumors. Due to the fact that minor residual tumors following surgical operation may result in local recurrence, surgery is neither efficient nor plausible for the treatment of metastatic disease. Although AdHSV-tk gene therapy followed by ganciclovir administration has been evaluated extensively as a potential treatment modality for numerous tumors, it has not yet been proven to achieve a complete cure on its own.

Prostate-derived tumor models were used to evaluate the effects of AdHSV-tk gene therapy as an adjuvant to surgery (Sukin et al, 2001) Lung nodules of prostate cancer cells were generated by intravenous injection of tumor cells in order to evaluate systemic effects Following resection of subcutaneous tumors, AdHSV-tk was delivered to the resection site Toxicity, local tumor recurrence, survival, and lung nodule formation were evaluated in animals; increased survival and decreased recurrence accompanied by no systemic toxicity were observed Adjuvant AdHSV-tk gene therapy resulted in a significant reduction in lung nodules as well This study suggested that AdHSV-tk gene therapy might be beneficial as an adjuvant for patients undergoing surgical treatment of cancer.

XIII. Current progress to overcome rate-limiting steps in adenovirus-mediated gene therapy for prostate carcinoma

The success of adenovirus mediated gene therapy for prostate carcinoma is effected by several factors including the level of expression of the receptor which facilitates the entry of the viral vectors into the cells, penetration of transgenes to surrounding tissues, and finally the expression of the delivered gene. Enhancing these factors has been the focus of many laboratories working on adenovirus-mediated gene therapy for prostate carcinoma. Although a limited number of studies have been completed regarding these issues, effectiveness of prostate cancer gene therapy will certainly benefit from the progress in this field.

A. Receptor abundance

The presence of the coxsackie adenovirus cell surface receptor, CAR, is required for an effective adenovirus infection of target cells. CAR expression patterns of normal prostate and prostate carcinoma were compared using immunohistochemical approaches in order to assess the feasibility of adenovirus mediated gene therapy for prostate cancer (Rauen et al, 2002). While a robust membrane staining for CAR was detected in the metastatic prostate specimens with higher Gleason scores, just lumenal and lateral cell membrane staining were detected in the benign prostate epithelia Therefore, adenovirus mediated gene delivery should be more effective for aggressive prostate tumors than it is for benign cases

B. Penetration of hybrid therapeutic transgenes to the surrounding tissue

Despite the fact that adenovirus could transduce cells very efficiently in vitro, adenovirus mediated gene delivery is restricted by the inefficient transduction of surrounding cells for a given tumor In order to overcome this obstacle, an important intercellular transport protein named VP22, was first fused to the therapeutic transgene of interest (p53 gene) and then cloned into adenovirus vector (Roy et al, 2002). Infection of p53 negative human prostate cancer cells (LNCaP) by this approach generated very efficient gene delivery of p53, inducing apoptosis not only in the infected cells but also in the surrounding uninfected cells.

C. Enhancement of transgene expression through transcriptional regulation

Although the use of prostate specific promoters is necessary to limit the transgene toxicity, the low level of transgene expression directed by these promoters represents a barrier to gene therapy. The observation, which led to the idea that chemotherapeutics enhanced the transgene expression from viral promoters, represented a new approach to overcome this barrier Two recombinant adenovirus constructs were used to deliver p21WAF-1/CIP1 and p53 protein c-DNA under the control of cytomegalovirus promoter to the metastatic androgen independent prostate cancer cells treated with chemotherapeutic agents docetaxel or paclitaxel (Li et al, 2002b). Both chemotherapeutics appeared to enhance adenovirus mediated transgene expression in androgen independent prostate cancer cell lines. This increase in transgene expression was attributed to the enhancement of CMV promoter activity rather than the increased viral uptake. Therefore, the observed synergy of gene therapy with these chemotherapeutics may become useful when the transgene expression is a limiting factor for the treatment of the metastatic androgen independent prostate cancer. The possible use of other chemotherapeutic agents and their effect on prostate specific promoters should also be explored.

XIV. Summary of clinical trials

There are 636 clinical protocols involving 3496 patients employed in gene therapy worldwide as reported to the Journal of Gene Medicine website by the year 2002. 403 clinical studies (63.4 %) with regard to gene therapy for cancer were tested on 2392 (68.5 %) patients. Adenovirus was the vector of choice in 171 of these protocols (27 %), and 644 patients (18.4 %) received the adenovirus vector for gene therapy. 22 out of 171 clinical protocols were engaged in adenovirus mediated gene therapies targeting the prostate only as summarized in **Table 1** 13 of these were reported to be in Phase I, 3 trials in Phase II and the rest (5) were in Phase I/II There is no Phase III clinical study reported using adenovirus vectors targeting prostate yet. Some of the adenovirus mediated gene therapy approaches were complemented either with radiotherapy or radical prostatectomy The percentage of the choice of gene therapy modalities targeting prostate is provided in **Figure 3**. The use of selectively replicating adenovirus constructs leads other approaches followed by suicide gene therapy This is partly because not long ago astonishing results were obtained with selectively replicating adenovirus constructs in the preclinical animal models It is also interesting to note that two of these clinical trials utilize suicide gene therapy in combination with the selectively replicating adenovirus approach

(**Figure 3**). No clinical studies have been carried out using the death ligand-mediated gene therapy approach and adenovirus vectors up to date. However we should not be surprised if such trials are being initiated and we encounter some of these in the near future. Although preliminary results are very encouraging from these clinical investigations, clear conclusions can be drawn only upon completion of these studies.

Considering all these preclinical and clinical studies, we concluded that great progress in adenovirus mediated gene therapy for prostate carcinoma has been made within the last 3 years. While the molecular mechanisms responsible for prostate carcinoma are not fully understood, the effectiveness of gene therapy is still quite amazing. As more data become available on the understanding of prostate carcinoma, we anticipate that more effective treatment modalities will be developed using adenovirus to target prostate cancer.

Table 1. A summary of ongoing clinical trials of adenovirus mediated gene therapy targeting prostate as of 2002. The data was collected from the Journal of Gene Medicine web site (www.wiley co.uk/genmed/clinical) and published with the permission from ©John Wiley and Sons 2002.

Country	Investigator	Mode of Therapy	Phase
Canada	A. K. Stewart	Immunotherapy (IL-2)	I
Canada	J. Dancey	Immunotherapy (IL-2)	I
USA	Peter T. Scardino	Suicide gene therapy (HSV-tk) + radiotherapy	I
USA	Simon J. Hall	Neo-adjuvant suicide gene therapy (HSV-tk) + radical prostatectomy	I
USA	Arie Belldegrun	Tumor suppressor gene therapy (p53)	I
USA	Christopher J. Logothetis	Tumor suppressor gene therapy (p53)	I/II
USA	Dov Kadmon	Neo-adjuvant suicide gene therapy (HSV-tk) + radical prostatectomy	I
USA	Jonathan W. Simons	Selectively replicating adenovirus (CN706)	I
USA	Thomas A. Gardner	Suicide gene therapy (HSV-tk)	I
USA	Jae Ho Kim	Suicide gene therapy (CD/Tk) with selectively replicating adenovirus + radiotherapy	I
USA	E. Brian Butler	Suicide Gene Therapy (HSV-tk) + radiotherapy	I/II
USA	Jeffrey R. Gingrich	Neo-adjuvant CDK inhibitor (p16) + radical prostatectomy	I
USA	Martha K. Terris	Selectively replicating adenovirus (CV787) + Radiotherapy	I/II
USA	George Wilding	Selectively replicating adenovirus (CV787)	I/II
USA	Alan Pollack	Tumor suppressor gene therapy (p53) + radiotherapy	II
USA	Thomas A. Gardner	Selectively replicating adenovirus with osteocalcin promoter (Ad-OC-E1A)	I
USA	David M. Lubaroff	Immunotherapy (PSA)	I
USA	Brian J. Miles	Immunotherapy (IL-12) + radiotherapy	I
USA	Theodore L. DeWeese	Selectively replicating adenovirus (CV706)	II
USA	Eric J. Small	Selectively replicating adenovirus (CV787) + chemotherapy	II
USA	Svend O. Freytag	Neo-adjuvant suicide gene therapy (CD/Tk) with selectively replicating adenovirus + Radiotherapy	I
USA	John M. Corman	Selectively replicating adenovirus (CG7060) + radiotherapy	I/II

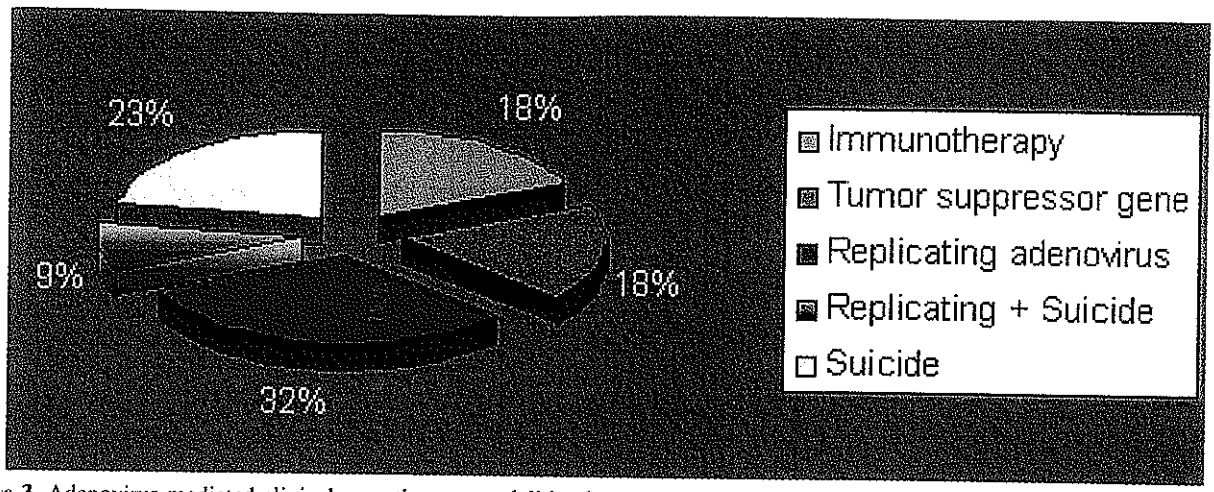

Figure 3. Adenovirus mediated clinical gene therapy modalities for prostate. The types of clinical gene therapy modalities for prostate are represented as percentages in a pie graph in order to better appreciate the contribution of each treatment modality.

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Dr Salih Sanlioglu