

T.C
AKDENİZ ÜNİVERSİTESİ
SAĞLIK BİLİMLERİ ENSTİTÜSÜ
Tıbbi Biyoloji ve Genetik Anabilim Dalı

**TİP 1 DİYABET TEDAVİSİNDE KULLANILMAK
ÜZERE ADENOVİRÜS ARACILI *TRAIL* GEN
TEDAVİ METODLARININ GELİŞTİRİLMESİ VE
ETKİNLİKLERİNİN DİYABETİK HAYVAN
MODELLERİNDE TEST EDİLMESİ**

Ercüment DİRİCE

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Doktora Tezi

Tez Danışmanı
Prof. Dr. Salih ŞANLIOĞLU

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3) Sanlioglu AD, Griffith TS, Omer A, **Dirice E**, Sari R, Altunbas HA, Balci MK, Sanlioglu S. Molecular mechanisms of death ligand-mediated immune modulation: a gene therapy model to prolong islet survival in type 1 diabetes. *J Cell Biochem* 2008;104(3):710-20.

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

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ÖZET

İnsülin üreten pankreatik beta hücrelerinin kalıcı yıkımı ile karakterize olan Tip 1 Diyabet, hastaya dışarıdan insülin enjeksiyonu ya da pankreas nakli yapılmadığı sürece ölümcüldür. Pankreas nakli ile karşılaştırıldığında pankreas adacık nakli, daha güvenilir ve ciddi bir operasyon gerektirmediğinden avantajlı bir yöntemdir. Adacık greftlerindeki fonksiyonel bozukluk ve nakil sonrasında oluşan greft reddi gibi sınırlayıcı faktörler pankreatik adacıkların genetik açıdan nakil öncesi *ex vivo* manipulasyon gerekliliğini doğurmuştur.

Bu nedenlerden dolayı pankreatik adacıklara adenovirus aracılı TRAIL geni aktarımını içeren yeni gen tedavi yaklaşımı test edilerek bu yöntemin STZ ile indüklenen Tip 1 Diyabetik sıçanları otoreaktif T-hücre saldırısından koruyup korumadığı araştırıldı. Bu kapsamda genetik olarak değiştirilmiş sıçan pankreatik adacıkları, STZ ile indüklenmiş diyabetik sıçanların böbrek kapsülü altına nakledildi.

Diyabet tablosu adacık nakli sonrasında 90 gün süresince takip edildi. Ad5hTRAIL ile enfekte edilmiş adacıkları taşıyan sıçanlar, AdCMVLacZ ile enfekte edilmiş ya da enfekte edilmemiş adacıkların nakledildiği diyabetik sıçanlar ile karşılaştırıldığında, Ad5hTRAIL grubunun daha uzun normoglisemi sağladığı görüldü. AdCMVLacZ ile enfekte edilmiş ya da enfekte edilmemiş adacıkların nakledildiği sıçanlarda şiddetli insülitis gözlenirken, Ad5hTRAIL ile transfeksiyona uğratılan adacıkları taşıyan hayvanlarda insülitis düzeyi daha az bulundu. Sonuç olarak, pankreatik adacıklarda aşırı *TRAIL* geni sentezi allograft sağkalım süresini ve fonksiyonunu artırarak STZ indüklü diyabetik sıçanlarda terapötik etki sağlamıştır.

Anahtar Kelimeler: TRAIL, Tip 1 Diyabet, gen tedavisi, pankreatik adacık nakli, adenoviral vektörler.

ABSTRACT

Type 1 Diabetes (T1D), characterized by permanent destruction of insulin-producing beta cells is lethal unless exogenous-insulin therapy or whole-organ transplantation is employed. While pancreatic islet transplantation is a safer and less invasive method compared to whole-organ transplant surgery, its treatment efficacy has been limited by islet graft malfunction and graft failure. Thus, *ex vivo* genetic engineering of beta cells is necessary to prolong islet graft survival.

For this reason, a novel gene therapy approach involving adenovirus-mediated TRAIL gene delivery into pancreatic islets was tested to determine whether this approach would defy auto-reactive T-cell assault in streptozotocin (STZ)-induced diabetic rats. To test this, genetically-modified rat pancreatic islets were transplanted under the kidney capsule of STZ induced diabetic rats.

The diabetic status (blood sugar and body weight) was followed up to 90 days after islet transplantation. Animals carrying Ad5hTRAIL-infected islets experienced prolonged normoglycemia compared to animals grafted with mock-infected or AdCMVLacZ infected islets in STZ-induced diabetic rats. While severe insulinitis was detected in animals transplanted with AdCMVLacZ-infected or mock-infected islets; the severity of insulinitis was reduced in animals engrafted with Ad5hTRAIL-infected islets. Thus, TRAIL over-expression in pancreatic islets extends allograft survival and function leading to a therapeutic benefit in STZ induced diabetic rats.

Key Words: TRAIL, Type 1 Diabetes, gene therapy, pancreatic islet transplantation, adenoviral vectors.

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SİMGELER ve KISALTMALAR DİZİNİ

AAV	: Adeno-associated Virus
Ad5hTRAIL	: TRAIL geni taşıyan recombinant adenovirüs
AdCTLA4-FasL	: FasL geni taşıyan rekombinant adenovirüs
APC	: Antigen Presenting Cell
CAR	: Coxsackie Adenovirüs Reseptor
CCF	: Cytospin Collection Fluid
CD	: Cluster of Differentiation
CMV-hFasL	: FasL geni taşıyan sitomegalovirüs
CsCl	: Caesium Chloride
DcR	: Decoy Receptor
DM	: Diabetes Mellitus
DMEM	: Dulbecco's Modified Eagle's Medium
DMSO	: Dimethyl Sulfoxide
DNA	: Deoxyribonucleic acid
DR	: Death Receptor
EGFP	: Enhanced Green Fluorescent Protein
FADD	: Fas-associated death domain
FasL	: Fas ligand
FDA	: Food and Drug Administration
FDA	: Flouresan Diasetate
FS	: Phosphatidylserin
GvHD	: Graft versus Host Disease
HBSS	: Hank's Buffered Salt Solution
H&E	: Hemotoxylen/Eosin
IDF	: International Diabetes Foundation
IE	: Islet Equivalent
IFN-α	: Interferon alfa

IFN-γ	: Interferon gamma
MOI	: Multiplicity Of Infection
NOD	: Non-Obese Diabetic
OPG	: Osteoprotegrin
PBS	: Phosphate Buffered Saline
PE50	: Poly-ethylene 50
PI	: Propidium iodide
RFI	: Relative Fluorescent Intensity
RPMI-1640	: Rosewell Park Memorial Institute – 1640 besiyeri
SCID	: Severe Combined Immunodeficiency
sDR5	: Soluble Death Receptor 5
SEM	: Standard Error of Mean
STZ	: Streptozotosin
T1D	: Type 1 Diabetes
TNF	: Tumor Necrosis Factor
TRAIL	: Tumor Necrosis Factor-related Apoptosis-Inducing Ligand
WHO	: World Health Organization

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GİRİŞ VE AMAÇ

Tip 1 diyabet, insülin üreten pankreatik beta hücrelerinin T-hücresi aracılı yıkımı sonucu ortaya çıktığından, otoreaktif T-hücrelerinin apoptozis yoluyla eliminasyonu, otoimmün hastalıkların önlenmesinde uygulanabilir bir stratejidir. Eğer Fas sinyal yolu aktive edilirken yardımcı stimülasyon engellenirse, periferel lenfositlerin in vitro apoptozisinde bir artış görülür. Halen Tip 1 Diyabetli hastaların tedavisi için, bu gibi stratejiler denenmektedir. Diyabet tedavisinde insan CTLA4-FasL geni taşıyan bir adenovirus (AdCTLA4-FasL) kullanılarak ümit verici sonuçlar elde edilmiş olsa da, FasL'in inflamasyonu tetiklemesi, immün tolerans oluşturmada kullanılma ihtimalini zayıflatmıştır. Örneğin, sıçan insülin promotorunun kontrolü altında FasL sentezleyen pankreatik adacıklar allojenik vericilere transplante edildiklerinde, polimorfonükleer hücrelerin ağır infiltrasyonu nedeniyle akut rejeksiyona sebep olmuşlardır. Öte yandan, DBA/1 farelerde kollajen ile indüklenen artrit (KİA) ve C57BL/6 deneysel otoimmün ensefalomyelitis fare modelinde TRAIL'in otoreaktif T hücresi aktivasyonunda bir inhibitor olarak görev aldığı gösterilmiştir. Ayrıca, TRAIL'in anti-CD3 antikoru ile aktive edilen T hücrelerinde DNA sentezini ve hücre döngüsünü bloke ettiği bilinmektedir.

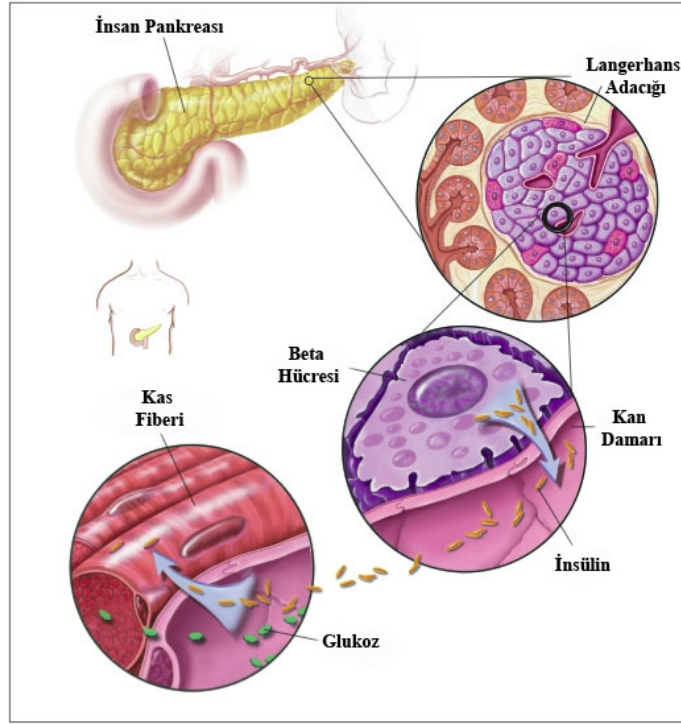
Bu sonuçlar, TRAIL'in T lenfositlerinin negatif seleksiyonunu etkilediğine işaret etmektedir. Bunun yanında TRAIL'in, adacık antijenlerini tanıyan otoreaktif T-hücrelerinin timik seleksiyonunda görev alıp almadığı bilinmemektedir. Buradan yola çıkarak, pankreatik adacıklarda TRAIL'in yüksek oranda sentezlenmesini sağlayacak olan Ad5hTRAIL gen tedavi metodunun etkinliği araştırıldı ve bu yolla nakil sonrası T-hücrelerini elimine/inhibe ederek diyabet tedavisi için terapötik bir yaklaşım geliştirilmesi planlandı. Bu amaçla, terapötik yaklaşımımızın etkinliğini belirlemek için Wistar sıçanlardan adacıklar izole edilip viral vektörlerle enfeksiyon sonrası STZ indüklemeli diyabetik Wistar sıçanlarına nakil yapıldı. Transplantasyon sonrasında adacıkların ne kadar süre tip 1 diyabetin baskılanmasında etkin oldukları kontrol gruplarıyla kıyaslanarak belirlendi.

GENEL BİLGİLER

2.1. Diyabet

Diyabet, genellikle aşırı miktarda idrara çıkma ve idrarda glukoz bulunması (glukozüri) ile karakterize bir hastalık olan Diabetes Mellitus'u (DM) tanımlamak için kullanılır. DM (*Yunanca diabetes* = idrara geçen ve *Latince mellis* = tatlı ya da bal), sıklıkla diyabet olarak adlandırılan, genellikle kalıtsal ve çevresel etkenlerin birleşimi ile oluşan ve kan glukoz seviyesinin aşırı derecede yükselmesiyle (hiperglisemi) sonuçlanan metabolik bir bozukluktur. Dünya Sağlık Örgütü'nün (WHO) verilerine göre her 10 -15 yılda mevcut hasta sayısının 2 katına çıktığı ve 2030 yılına gelindiğinde diyabet hasta sayısının yaklaşık 370 milyona ulaşacağı tahmin edilmektedir[1, 2]. Ayrıca, Uluslararası Diyabet Federasyonunun (IDF) verilerine göre Amerika'da görülen en yaygın üçüncü hastalık olmakla beraber, aynı zamanda ölüme sebebiyet veren hastalıklar arasında da dördüncü sırada yer alır[3]. Vücutta kan düzenlenmesi pek çok sayıda kimyasal madde ve hormonun karmaşık etkileşimi sonucunda sağlanır. Glukoz metabolizmasının düzenlenmesinde rol oynayan hormonlardan en önemlisi pankreasın beta hücrelerinden salgılanan insülin hormonudur (Şekil 2.1). Diyabet, ya insülin üretiminin azalması ya da insülinin etkisine karşı direnç gelişmesi ile oluşur[4]. Her iki durum da sonuçta kan glukozunun yükselmesine neden olur. Aşırı miktarda idrar üretimi diyabetin akut belirtilerinin başında gelir ve bu durumun başlıca sorumlusu hiperglisemidir. Susama ve sıvı tüketiminin artması ise aşırı idrarla sıvı kaybını dengeleme çabasının bir sonucudur. Görme fonksiyonunun bozulması, açıklanamayan kilo kayıpları, yorgunluk ve enerji metabolizmasındaki değişiklikler ise diyabetin diğer belirtileridir.

Diyabetin kendisi ve diyabette kullanılan tedavi yöntemleri pek çok komplikasyonlara yol açabilir. Eğer hastalık iyi kontrol edilmezse; hiperglisemi, ketoasidoz ya da nonketotik hiperozmolar koma gibi akut komplikasyonlar gelişebilir. Hastalığın uzun sürede ortaya çıkan kronik komplikasyonlarının başlıcaları ise; dolaşım sistemi (kardiyovasküler) hastalıkları (hipertansiyon, kalp yetmezliği ve ateroskleroz gibi), kronik böbrek yetmezliği (nefropati), körlüğe sebep olabilen retina hasarı (retinopati), çeşitli tiplerde sinir hasarları (periferik nöropati) ve yara iyileşmesini geciktiren ve erektil disfonksiyona sebep olan mikrovasküler bozukluklardır. Özellikle dolaşım bozukluklarının sonucu olarak ayaklarda ortaya çıkan yaraların geç iyileşmesi amputasyon ile sonuçlanabilir. Diyabetin uygun şekilde takip edilmesinin yanı sıra, kan basıncı kontrolüne yeterince önem verilmesi ve hayat tarzının iyileştirilmesi (sigara içmemek ve kilo kontrolü yapmak gibi) bu kronik komplikasyonların pek çoğunun oluşturduğu riskleri azaltabilir. Gelişmiş ülkelerde yapılan amputasyonların travma harici ana sorumlusu ve yaşa bağlı olmayan körlüklerin de en önemli sebebi diyabettir.



Şekil 2.1. İnsan pankreasında insülin üretimi[5]

2.2. Diyabetin Sınıflandırılması

Eskiden kullanılan "çocukluk çağı diyabeti", "juvenil diyabet" ve "insüline-bağımlı diyabet" terimleri yerine günümüzde tüm dünyada Tip 1 diyabet (T1D) terimi kullanılmaktadır. Benzer şekilde eskiden "yetişkin yaşta başlayan diyabet", "obeziteye bağlı diyabet" ve "insüline bağımlı olmayan diyabet" terimleri yerine de Tip 2 diyabet terimi kullanılmaktadır. Bu iki tip diyabetten farklı olarak, henüz isimlendirme konusunda genel bir anlaşmaya varılamamış değişik isimlendirmeler de vardır. Bunlara örnek olarak; insülin enjeksiyonunu gerektirecek kadar ilerlemiş tip 2 diyabet için kullanılan yetişkinlerde görülen gecikmiş otoimmün diyabet (Tip 1.5 diyabet[6, 7]) verilebilir. Ayrıca, "Gençlerde görülen erişkin tipli diyabet" olarak adlandırılan bir diyabet türü daha vardır. Bu hastaların ailelerinde diyabet hikayesi vardır ve tip 2 diyabete 30 yaşlarından önce yakalanırlar. Bu isim tek gen mutasyonu sonucunda oluşmuş (monogen) kalıtsal diyabeti tanımlamak için kullanılan genel bir terimdir.

2.2.1. Tip 1 Diyabet

T1D, pankreasın beta hücrelerinin ürettiği insülin miktarın ortadan kalkması ile meydana gelen ve sonuçta tam insülin yetersizliğinin oluşması ile karakterize bir hastalıktır. Bu tip diyabet "bağımsız-aracılı" ya da "idiyopatik" olarak ikiye

ayrılabilir. T1D hastalarının çoğunluğunu bağımsızlık-aracılı tip oluşturur. Bu tipte pankreasın beta hücrelerinde oluşan kayıpların temel sorumlusu T-hücre aracılı otoimmün yanıtıdır[4]. Tip 1 diyabetin oluşmasını engellemek için koruyucu bir önlem yoktur. Kuzey Amerika kıtası ve Avrupa'da görülen toplam DM vakalarının % 10 kadarını T1D hastaları oluşturmakla beraber Diyabet hasta sayısı ülkemizde yaklaşık 5-6 milyon kadardır. ve bu oran bazı bölgelerde daha da yüksektir. Hastalıktan etkilenen insanların çoğu hastalığa yakalandıklarında sağlıklı ve normal kilodadırlar. T1D çoğunlukla çocukları etkilemekle beraber yetişkinlerde de görülebilmektedir.

2.2.1.1. T1D hastalığının Tedavisi

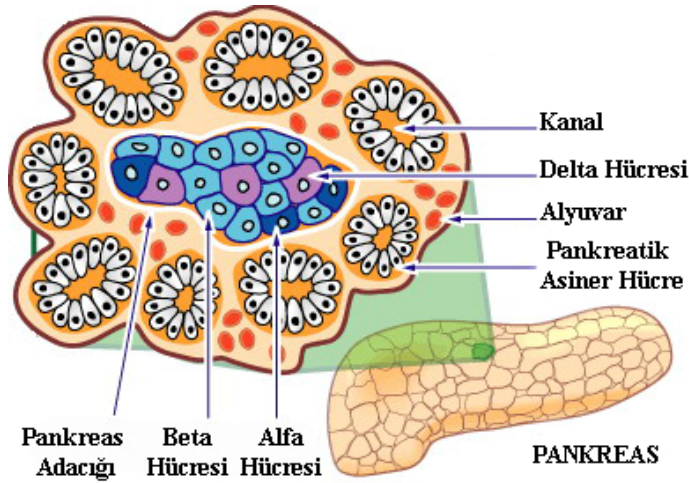
T1D'in başlıca tedavisi, henüz hastalığın başında bile olsa, sentetik insülinin vücuda enjekte edilmesi ve kan şekeri seviyelerinin sıkı bir şekilde kontrol edilmesidir. Eğer insülin olmazsa, sıklıkla diyabetik ketoasidoz durumu oluşur ve koma ile sonuçlanabilir. Günümüzde T1D'in tedavisinde, her ne kadar hastalığın seyrini geri çeviremeye de, hayat tarzı değişiklikleri de (diyet ve egzersiz) önem kazanmıştır. En çok kullanılan yöntem, subkütan insülin enjeksiyonundan farklı olarak, insülini bir pompa vasıtasıyla vücuda pompalamaktır. İnsülin pompaları, 24 saat boyunca belirli zamanlarda vücuda insülin verebilmelerinin yanında, yemek saatlerinde daha yüksek dozda insülini vererek normal şeker seviyesini düzenleyebilirler. İnsülinin solunum yoluyla (inhalasyon yoluyla) kullanılan bir formu Ocak 2006 tarihinde Amerikan Gıda ve İlaç Kuruluşu(FDA(Food and Drug Administration)) tarafından onaylanmış ve kullanıma girmiştir. Ancak bu ilaç, Ekim 2007 tarihinde ekonomik olmaması nedeniyle üretici firma tarafından piyasalardan çekilmiştir[8].

T1D hastalarına yaşam boyu insülin enjeksiyonu rutin bir tedavi biçimidir. Ayrıca, dışarıdan verilen insülin fizyolojik insülin salgısındaki kaybı istenildiği ölçüde karşılayamaz. T1D, pankreasın tek bir çeşit hücresinin (pankreasın Langerhans adacıklarında bulunan beta hücreleri) fonksiyonunun bozulması sebebiyle ortaya çıktığı için, çoğunlukla ya tüm pankreasın nakli ya da sadece beta hücrelerinin verilmesi şeklindeki kesin tedavi yaklaşımı pek çok kez denenmiştir[9]. Sadece pankreas ya da böbrek ile birlikte ve pankreas nakli yapılan ve dışarıdan insülin kullanma ihtiyacı duymayan T1D hastalarının kesin tedavi oldukları düşünülebilir. Diyabete çoğunlukla böbrek ile ilgili komplikasyonlar eşlik ettiği için (diyabetik nefropati), pankreas ve böbreğin birlikte nakledilmesi umut verici sonuçlar çıkarmaktadır. T1D hastalarında böbrekle birlikte pankreasında nakledildiği vakalardaki hayatta kalma oranı, sadece böbreğin nakledildiği vakalardaki hayatta kalma oranına benzer ya da bu orandan daha yüksektir[10]. Ancak, bu hastalar ciddi bir operasyon ile beraber nakilden sonra uzun süreli bağımsızlık baskılayıcı ilaçlar kullanmak zorundadırlar ve vücudun nakledilen yeni organı reddetme ihtimali vardır[9]. Bu nedenle, alternatif tedavi yaklaşımları için yoğun çalışmalar sürdürülmektedir. Bu yaklaşımlardan biri olan pankreas adacık nakli, geleneksel insülin enjeksiyonu ya da pankreas transplantasyonuna kıyasla ciddi bir operasyon gerektirmemesi ve hastaları yaşam boyu insülin kullanımı sonucu oluşabilecek komplikasyonlardan koruyabilmesi gibi avantajlarının olduğu tespit edilmiştir.

2.3. Pankreas Adacıkları ve Adacık Nakli

2.3.1. Pankreas Adacıkları

İnsan pankreası ortalama 80-90 gram ağırlığında olup karın boşluğunda midenin arka ve alt kısmında oniki parmak barsağı ve dalağın arasına yerleşiktir. Pankreas hem sindirim işlemi ile ilgili enzimler sentezler hem de kan glikozunun düzenlenmesinde görevli hormonları üretir. Bu hormonları üreten hücreler sindirim işlevi ile ilgili hücrelerin aralarında dağılmış olarak adacıklar halinde bulunurlar. Bu adacıklara, “Langerhans Adacıkları” denmektedir. Bu adacıkların sayısı bir milyon civarında olup pankreasın ağırlığının % 1 kadarını oluşturur. Adacıklar, pankreas’ın % 1-2 sini kaplamasına rağmen oksijene çok hassas yapılar olup pankreasa gelen temiz kanın % 15’ini alırlar. Langerhans adacıkları içinde üç önemli hücre grubu vardır (Şekil 2.2).



Şekil 2.2. Pankreas adacık hücreleri[11]

Alfa hücreleri: Glukagon hormonunu üretir. Bu hormonun kan şekerini yükseltici özelliği vardır.

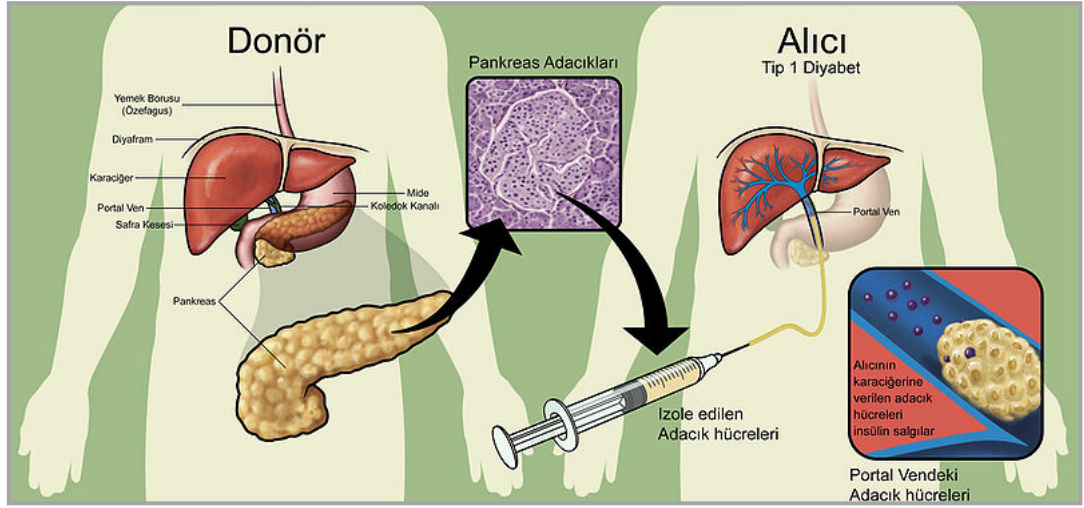
Beta Hücreleri: İnsülin hormonunun üretildiği yerdir. İnsülin kan şekerini düşüren hormondur.

Delta hücreleri: Somatostatin adlı hormonun yapıldığı hücrelerdir. Bu hormonun etkisi çok yönlüdür.

2.3.2. Pankreas Adacık Nakli

Pankreası bütün olarak nakletmek yerine donörlerden izole edilen pankreas adacık hücrelerinin T1D hastalarının karaciğerlerine enjekte edilmesiyle yapılan adacık nakillerinde umut vaat eden sonuçlar ortaya çıkmıştır[12]. Portal ven aracılığı ile enjekte edilen pankreas adacık hücreleri karaciğere yerleşmekte ve bir süre sonra insülin salgılamaya başlamaktadır (Şekil 2.3). Başlangıçta yapılan klinik adacık nakli çalışmalarında bir yıllık insülin bağımsızlık oranının % 8 oranında gerçekleşmesi

büyük bir hayal kırıklığı yaratmıştır. Ancak daha uygun adacık izolasyon ve saflaştırma protokollerinin geliştirilmesini takiben yapılan sonraki çalışmalarda; bir yıl süresince insülin bağımsızlığı sağlanan hasta sayısında yaklaşık %50 artış sağlanabilmiştir[13]. Steroid içermeyen sirolimus temelli immün baskılayıcı tedavi ile birlikte taze izole edilmiş adacık nakil işlemi diye tanımlanan Edmonton protokolünün uygulandığı öncül bir çalışmada ise, yedi hastanın yedisinde de nakil sonrası en az bir yıllık insülin bağımsızlığı sağlanmıştır[14]. Bu yöntemin başarısında; adacıkları rejeksiyondan ve otoimmüniteden koruyan sirolimus, takrolimus ve anti-interlökin 2 reseptör antikorlarıyla birlikte taze elde edilmiş iki adacık preparasyonunun (adacık implant kütlesi 13.000 IE/kg alıcı vücut ağırlığı) kullanımının rolü olduğu bildirilmiştir. Edmonton protokolünün uygulandığı ve 50 hastanın katıldığı sonraki bir çalışmada, insülin bağımsızlık oranının %80'e çıktığı belirtilmiştir[15].



Şekil 2.3. Pankreatik adacık nakli[12]. Pankreatik adacık naklinin temel prensibi, endokrin hormon sekresyonu yapan bezlerin tamamına yakınına yakınını donörden izole etmektir. İzole edilen adacıklar ince bir tüp yardımı ile portal ven aracılığı ile hastaya enjekte edilir. İnfüzyon sonrası kan akışı ile beraber adacıklar karaciğere taşınır, ve buraya yerleşerek kan şekeri kontrolünde gerekli olan insülin hormonunu salgılar.

Bütün bunlara rağmen beş yıllık hasta takip sonuçları, greft sağkalımının halen yüksek (%80) olmasına rağmen insülin bağımsızlığının %10'a düştüğünü göstermiştir[16]. Bu çalışmalar, uluslararası adacık nakli merkezlerini de içerecek şekilde genişletilmiş ve buralarda da benzer sonuçlar alınmıştır[17]. İntra-hepatik adacık nakli, her ne kadar T1D hastalarının tedavisi için umut vaat eden bir yaklaşım olsa da, bu yöntemin başarısı; fonksiyonel olmayan greft oranının yüksek olması ve nakil sonrasında gözlemlenen ikincil greft kaybı sonucu hastaların çoğunluğunun beş yıl içerisinde yeniden insülin alımına ihtiyaç duyması nedeniyle gölgelenmiş durumdadır[18].

2.4. Pankreas Adacık Naklinde Geliştirilen Stratejiler ve Gen Tedavisi

Pankreatik adacık naklinde temel hedef, hastaların immünsupresif ilaç kullanmadan diyabetik sendromların engellenmesi, buna bağlı olarak yaşam kalitesi ve süresinin artırılmasıdır. Uzun süreli adacık-greft fonksiyonu sağlanması, alloimmün ve otoimmün engellerin üstesinden gelinmesine bağlıdır. Bu nedenle, adacık naklinin ana temalarından biri, alıcıda greft toleransı oluşturabilmektir. Pankreatik adacık nakilleri sonrasında immün korumanın sağlanabilmesi amacıyla yapılan deneysel çalışmalarda öncelikle testis, beyin ya da timus gibi immün sistemden korunmuş bölgelere adacık nakli yapılmıştır. Yapılan çalışmalarda, gerçekte bu bölgelerin grefti immün sistemden uzak tutarak değil yüksek oranda Fas ligand (FasL) sentezi ile sağladığı belirlenmiştir[19]. Pankreatik adacıkların nakilden önce kapsülle kaplanması adacık sağkalımını uzatmak amacıyla denenen başka bir yaklaşım olarak karşımıza çıkmaktadır[20]. Ancak bu yöntemin kullanımı; kapsülasyon için kullanılan yabancı materyale karşı yoğun fibröz reaksiyonun gelişmesi ve nakledilen adacıkların en sonunda yıkımına neden olan sitokin aracılı immün cevabın gerçekleşmesi nedeniyle sınırlanmıştır. Tüm bu yöntemlere rağmen, şimdiye kadar nakledilen beta hücrelerinin kaybı geciktirilebilmiş, fakat sonuçta yıkım yine engellenememiştir. Bu nedenle, T1D hastalarının tedavisine alternatif olarak gen tedavi denemeleri gündeme gelmiştir.

Pankreatik adacıkların *in vitro* manipülasyonun kolaylıkla uygulanabilir olması nedeniyle, adacıklara çeşitli genler *in situ* olarak değişik vektörlerle transfer edilmektedir. Örneğin adacıkların akut yıkımdan korunması amacıyla anti-apoptotik gen transferi yapılmış, besin ve oksijen alışverişinde son derece önemli olan fakat adacık izolasyonu aşamasında yıkıma uğrayan mikrovaskülerize sistemin onarılmasına yönelik olarak farklı genler adacıklara aktarılmıştır.

Gen tedavisi terimi, 1970'lerden sonra birçok gen transfer teknolojisi için ortak bir yaklaşım olarak karşımıza çıkmaktadır. DNA'nın hücre ya da dokulara aktarımı viral veya viral olmayan araçlar ile sağlanabilmektedir. Liposom aracılı ya da elektroporatör ve balistik gen tabancaları gibi nükleik asitlerin direkt fiziksel olarak hücre içerisine aktarıldığı yöntemler viral olmayan gen aktarımına örnek verilebilir[21, 22]. Ancak, T1D tedavisinde kullanılmak için geliştirilen gen tedavi yaklaşımlarında (pankreatik adacıkların, beta hücre benzeri hücrelerin ya da immün hücrelerin modifikasyonunda) viral vektörlerin özellikle adenoviral vektörlerin çok daha başarılı olduğu belirtilmektedir[23].

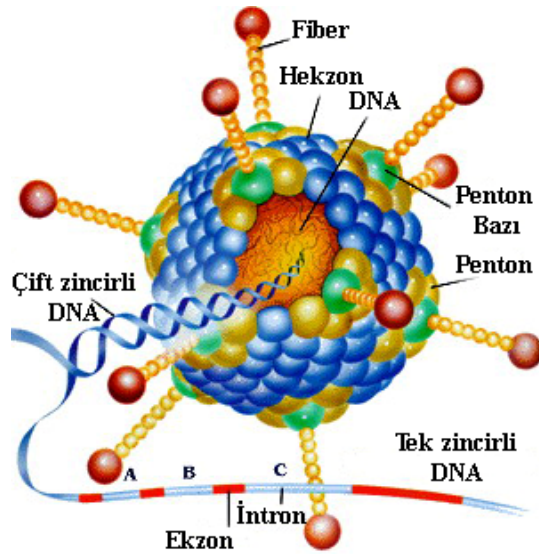
2.4.1. Adenoviral Vektörler

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[24].

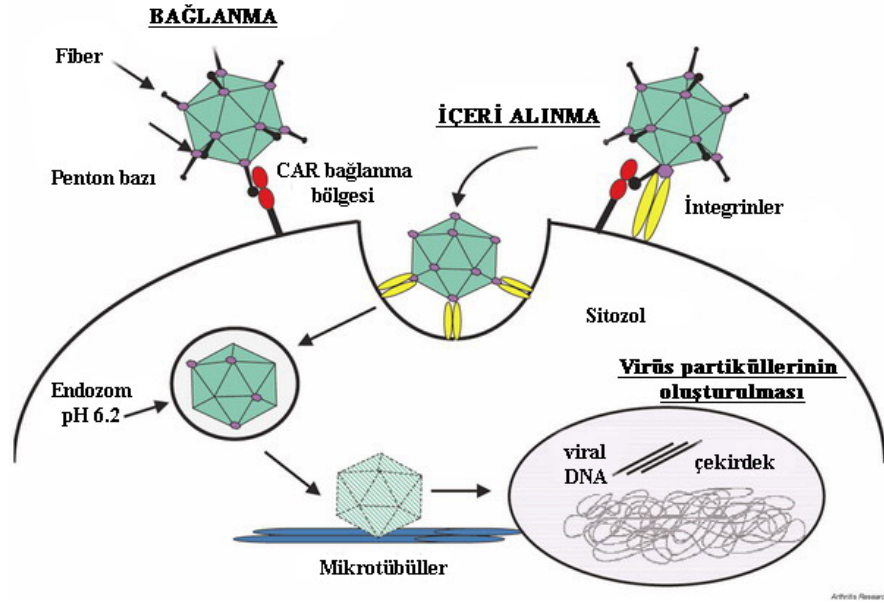
Adenovirüsler, yaklaşık 70-100 nm çaplı bir protein kapsitten, ve kapsid içinde yaklaşık 36 kb uzunluğunda tek kopya çift zincirli DNA'dan oluşur (Şekil 2.4). Adenovirüsler, konakçı hücre yüzeyine Cocksackie Adenovirüs Reseptor (CAR) reseptörleri aracılığıyla bağlanır. CAR proteinlerine bağlanan adenovirüsün, reseptör aracılı endositoz yoluyla hücre içine alınabilmesi için, aynı zamanda $\alpha\beta$ integrinlere bağlanması gerektiği gösterilmiştir (Şekil 2.5). Hücreye girdikten sonra dış kapsidin ayrışması ile virüs endozomdan dışarı çıkar 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 promotör yoluyla gerçekleştirilir. Tam virüs oluşumu enfeksiyondan sonra 20-24 saat içinde gerçekleşir, ve hücre 2 ila 3 gün içinde lizise uğrar[25].

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[24](Şekil 2.6).

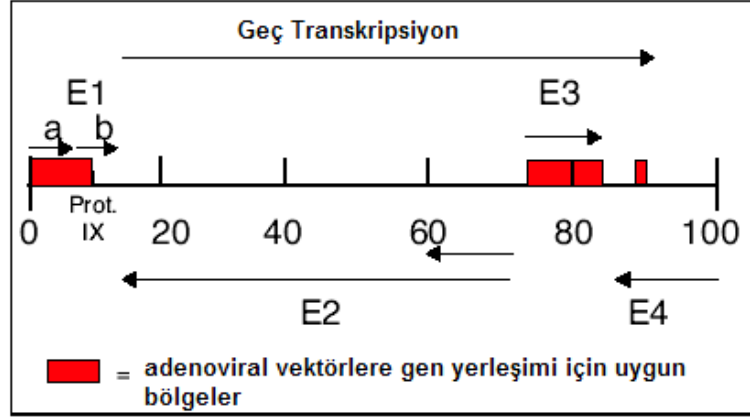


Şekil 2.4. Adenovirüsün şematize edilmiş görüntüsü[26]



Şekil 2.5. Adenovirüslerin hayat döngüsü[24]

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 hücrelerini 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[24].



Şekil 2.6. Adenoviral DNA'nın yapısı. Yaklaşık 8 kb'lık yabancı DNA adenoviral vektörlere yerleştirilebilir. Protein IX paketlenme için gereklidir, ve vektör üretiminde E1 bölgesine gen yerleştirilmesini kısıtlayıcı bir faktördür[24].

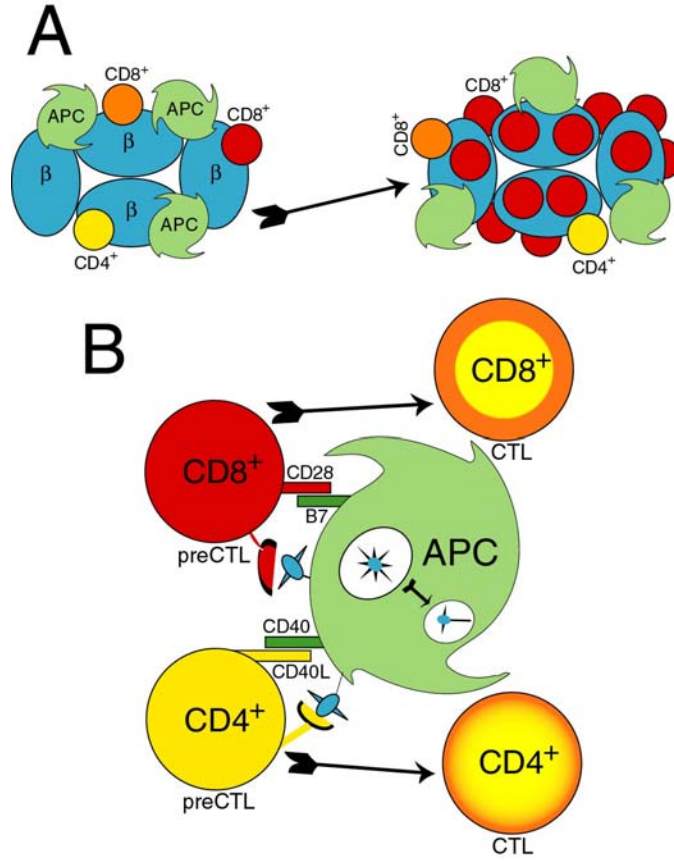
2.4.2. Pankreas Adacıklarında Gen Tedavisi ile İmmünmodülasyon

T1D, otoimmün bir reaksiyon nedeniyle pankreatik beta hücrelerinin yıkımı sonucu ortaya çıktığından[27, 28], bu immün reaksiyon, T1D ve aynı zamanda adacık-graft reddinde inflamatuvar araçlar tarafından tetiklenir[29, 30]. Nakilden sonra adacıkların kronik yıkımdan korunması amacıyla; immüno-regülatör gen tedavisi ya da inflamatuvar sitokinlerin ve sitokin aracılı hücre yolaklarının baskılanmasına yönelik gen tedavi stratejileri de geliştirilmektedir[31, 32]. Proinflamatuvar sitokinler üreten immün hücrelerin (özellikle lenfositler ve makrofajlar) zararlı etkisini engellemek amacıyla, TNF ailesi ölüm ligandlarının (TNF alfa, FasL) kullanıldığı apoptozis indükleyici yaklaşımlar bunlardan bazılarıdır. TNF ailesinin FasL ve TNF alfa gibi diğer üyeleri ile karşılaştırıldığında, TRAIL (Tumor Necrosis Factor-related Apoptotic-Inducing Ligand)'ın hücreler üzerinde farklı apoptozis indükleyici özellikleri bulunmaktadır. Örneğin, TNF alfa'nın[33] veya FasL'in[34] sentezi, pankreatik adacıklarda inflamasyondan sorumlu lenfosit infiltratları oluşturarak T1D gelişimini artırmıştır. Öte yandan, TRAIL etkinliğinin nötralizasyonu için saflaştırılmış çözünebilir TRAIL reseptörleri kullanıldığında veya TRAIL eksikliği bulunan hayvanlar ile çalışıldığında, TRAIL fonksiyonu yokluğunun otoimmün diyabeti hızlandırdığı ve pankreatik adacıklarda inflamasyon derecesini artırdığı görülmüştür[35]. Pankreatik adacıklara karşı gelişen immün yanıtı engelleyici ya da baskılayıcı gen tedavi stratejilerini uygulayabilmek için öncelikle T1D'in moleküler mekanizmasını iyi anlamak gerekmektedir.

2.5. T1D'in Moleküler Mekanizması

T1D'in etiyopatogenezinde genetik yatkınlık rol oynamaktadır. Ancak, araştırmacılar arasında kabul edilen ortak görüş, aktivasyon sürecinde meydana gelen hücre ve biyokimyasal olayların, viral enfeksiyon ve beslenme biçimi gibi çevresel faktörlerin etkisiyle tetiklendiğidir. Viral epitoplara, pankreatik beta hücrelerinde sentez edilen otoantijenler ile dizi benzerliğini kullanarak beta hücrelerine spesifik otoreaktif T-hücrelerinin aktivasyonuna yol açabilmektedir[36].

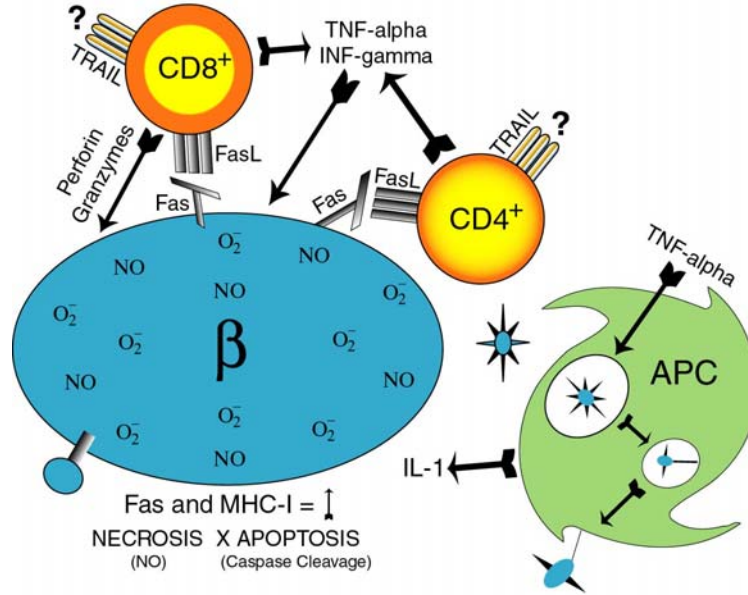
Beta hücre yıkımı, pankreatik Langerhans adacıklarında ve çevresinde meydana gelen kronik inflamasyonun son noktasıdır. T1D gelişiminin başlangıcında adacık inflamasyonu olarak tanımlanan pre-insülitis ve devamında insülitis gerçekleşir (Şekil 2.7A).



Şekil 2.7. T1D'in gelişim mekanizması[31]

T1D hastalığının çok iyi çalışıldığı iki kemirgen diyabet modelinde [(Diabetes-Prone BioBreeding (DP-BB) sıçan ve non-obese diabetic prone (NOD) fare)] immün saldırının akut fazında pankreatik adacıklar dendritik hücreler, makrofajlar gibi antijen sunucu hücreler (APC) ve CD4+ ve CD8+ T hücreleri tarafından yoğun bir infiltrasyona maruz kalmaktadırlar[37]. Zamanla T hücreleri insülitisin en önemli unsurları olarak, beta hücre hasarı ve yıkımından birinci derecede sorumlu hale gelirler. Bu süreçte yardımcı uyarıcı moleküllerin de rol aldığı düşünülmektedir (Şekil 2.7B). T hücreleri, beta hücrelerinde meydana getirdikleri apoptozu ya direkt olarak perforin/Granzyme B ve Fas/Fas Ligand aracılığıyla ya da dolaylı olarak Tumor Necrosis Factor-alpha (TNF- α) ve Interferon-gamma (IFN- γ)

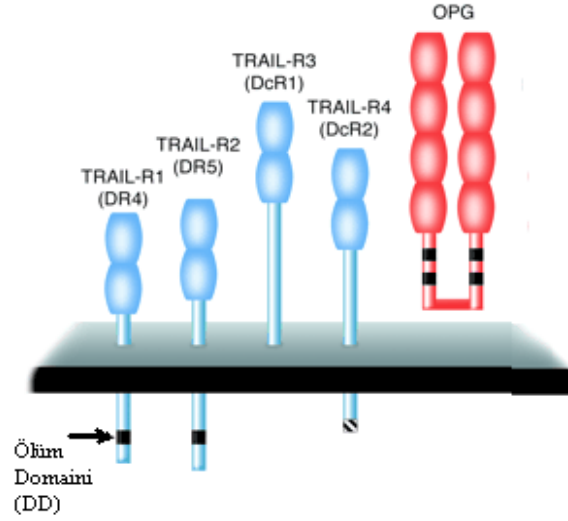
gibi sitokinler ile indüklerler (Şekil 2.8). B hücreleri ise, hastalık gelişiminin ilerleyen döneminde sürece katılırlar.



Şekil 2.8. T1D'in gelişiminde direkt ve dolaylı yoldan beta hücre yıkımı[31]

2.6. TRAIL Ölüm Ligandı ve TRAIL Sinyal Yolu

TNF- α süper ailesi üyeleri, yapısal olarak benzer proteinler içeren aynı zamanda hücre ölümü, immün yanıt ve inflamasyonun düzenlenmesinde önemli rol oynayan sitokinlerdir. Bu ailenin çok iyi tanımlanmış ilk üyesi TNF- α 'dır. TNF- α , T1D'te T-hücreleri ve makrofajlarca beta hücrelerine karşı oluşturulan immün yanıtta pro-apoptotik ve pro-nekrotik faktör olarak rol oynamaktadır. TNF- α dışında yine bu ailenin bir üyesi olan ve aynı zamanda APO-2L olarak da bilinen, TRAIL (Tumor Necrosis Factor-related Apoptotic-Inducing Ligand) bir tip II membran proteini'dir[38]. TRAIL'in hücre içi amino-terminal, transmembran ve hücre dışı karboksi-terminal bölgeleri mevcuttur. TNF- α üyeleri arasında en yüksek amino asit benzerliğini CD95L/FasL üyesi ile paylaşmaktadır. TRAIL, biyolojik etkisini hücre yüzeyinde bulunan TRAIL reseptörleri aracılığıyla gerçekleştirir. Bir veya iki reseptörü olan TNF- α ve FasL'in aksine, insanlarda TRAIL ile etkileşime girdiği belirlenen beş farklı TRAIL reseptörü tanımlanmıştır (Şekil 2.9).



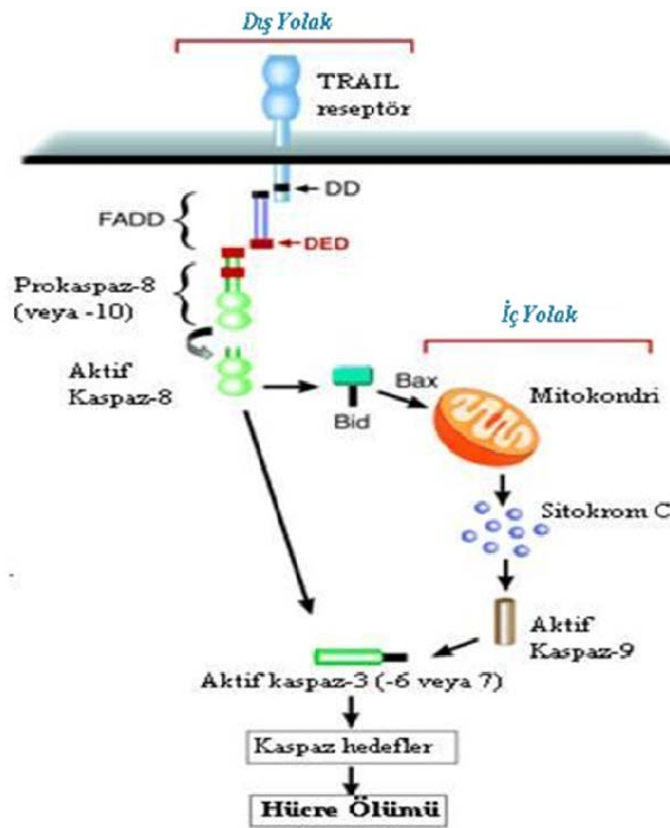
Şekil 2.9. TRAIL reseptörlerinin şematik gösterimi[39]

Ölüm reseptörleri olarak bilinen TRAIL-R1 (DR4) ve TRAIL-R2 (DR5), TRAIL ile etkileşim sonrası apoptotik sinyali hücre içine iletirken, TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) ve Osteoprotegrin (OPG) hücre içi ölüm bölgelerinden yoksun olduklarından dolayı, gelen sinyali aktaramazlar ve yalancı reseptörler olarak tanımlanırlar[40]. TRAIL ölüm reseptörlerine bağlandığında, iki ana sinyal yolu aracılığıyla hücrede apoptotik süreci başlatabilir (Şekil 2.10). Bunlar iç ve dış yollar olarak tanımlanır. Dış yolda, TRAIL'in ölüm reseptörlerine (DR4 ve DR5) bağlanması ve reseptör trimerizasyonunu takiben Fas-associated death domain (FADD) olarak adlandırılan adaptor moleküllerin bölgeye çekilmesi söz konusudur. Ölüm domainlerinin (DD) etkileşimi sonrası apoptozisi başlatıcı kaspaz aktivasyonu (kaspaz 8,10) gerçekleşir ve bu durum geri dönüşümsüz olarak apoptozis ile sonuçlanır. İç apoptotik yolak ise, TRAIL uyarımı sonrasında aktif kaspazların bir pro-apoptotik Bcl-2 ailesi üyesi olan Bid' i keserek aktive etmesiyle başlar. Aktive olan Bid molekülü, Bax ve Bak molekülleri ile etkileşime girerek mitokondriden sitokrom-c salınımını sağlar. Sitokrom-c, Apaf-1 kompleksinin oluşumunu tetikler ve kaspaz 9 aktivasyonunu sağlar. Bu aktivasyon diğer efektör kaspazların aktivasyonuna neden olur ve hücre yine geri dönüşümsüz olarak apoptoza gider.

2.7. TRAIL'in Otoimmün Hastalıklardaki Rolü

FasL ve TNF- α gibi TNF ailesinin diğer üyeleri ile karşılaştırıldığında, TRAIL'in hücreler üzerinde farklı etkileri olduğu görülmüştür. TRAIL' in büyümeyi durdurma ya da apoptozu indükleme yeteneği, p53, Bcl-2 ve MDR sentezinden bağımsız olarak gerçekleşmektedir[41]. TRAIL molekülü bu etkisini normal dokulara ya çok düşük seviyede etki ederek ya da hiç etki etmeyerek göstermektedir. Yapılan çalışmalarda TRAIL'in seçici ve etkili bir şekilde akciğer, meme, böbrek, kolon ve prostat tümör hücrelerini hem *in vitro* hem de *in vivo* olarak öldürdüğü gösterilmiştir. Bu işlevini, çok az düzeyde organ toksisitesine ya da inflamasyonuna

neden olarak ya da hiç olmayarak yerine getirmektedir[42, 43]. TRAIL ile kıyaslandığında, sistemik anti-kanser tedavilerinde TNF- α ve FasL kullanımı ise toksik yan etkiler ortaya çıkarmaktadır[41, 44]. Anti-kanser etkisinin dışında TRAIL' in, otoimmün cevabı başlatan ve şiddetlendiren TNF alfa'nın aksine, immün yanıtı azalttığı bildirilmiştir[45]. TRAIL'in lenfositler üzerindeki rolü, BALB/C farelerden izole edilen dalak hücrelerinin in vitro olarak aktive edilmesi ile araştırılmıştır. Song ve arkadaşlarının yaptığı bu çalışmada FasL aktive olmuş T hücrelerinde direkt apoptoz sağlarken TRAIL'in bu hücreleri apoptoza uğratmadığı ancak proliferasyonlarını engellediği gösterilmiştir. Aynı zamanda, TRAIL, DNA sentezini inhibe ederek lenfositlerin G1 fazından S fazına geçişlerini de bloke etmiştir. Bu nedenle, TNF veya FasL'm aksine, TRAIL'in lenfositlerin in vivo aktivasyonunu ve çoğalmasını engellediği, ancak bunları sistemden elimine etmediği ileri sürülmüştür.



Şekil 2.10. TRAIL aracılığı ile iç ve dış yolak sinyalizasyonu[40]

TRAIL'in transforme olmamış otoimmün hücrelerde inflamasyon ve hücre döngüsünün ilerlemesi bakımından rolü çok açık olmamakla beraber, taze izole edilmiş T lenfositlerinin tip 1 interferon veya CD3 ligasyonu ile muamele edilmedikçe TRAIL sentez etmedikleri bilinmektedir[46, 47]. Ayrıca, IFN- γ veya IFN- α uygulamasının, periferik kan monositlerinde ve dendritik hücrelerde TRAIL sentezini artırdığı görülmüştür[48]. Bu sonuçlar, TRAIL sentezinin T hücrelerinde, monosit ve makrofajlarda ancak stimülasyon ile gerçekleşebileceğini göstermektedir.

Dinlenme halindeki T hücrelerinin aksine, IL-2 ile indüklenmiş T hücreleri, TRAIL aracılı apoptozise duyarlıdır. Bu durum, TRAIL'in T hücrelerinin periferel eliminasyonunda rol oynadığını göstermektedir[49]. TRAIL sentezlemeyen farelerden izole edilen CD4⁺ ve CD8⁺ hücrelerinin anti-CD3 antikoru aracılı hücre ölümüne dirençlilik göstermesi, T hücrelerin timusta gerçekleşen eliminasyonlarının TRAIL aracılığıyla olduğuna işaret etmektedir[50].

2.8. T1D'te TRAIL'in Rolü

Elde edilen bilgiler doğrultusunda TRAIL'in diyabetin fizyopatolojisinde önemli bir rolü olduğu görülmektedir. T1D otoimmün bir hastalıktır ve TRAIL reseptörleri immün sistem hücrelerinde sentez edilmektedir. TRAIL' den yoksun farelerde yapılan çalışmalarda ileri derecede otoimmüitenin gösterilmesi, TRAIL'in immün yanıtın negatif regülatörü olduğu gerçeğini yansıtmaktadır[35, 50, 51]. Lamhamedi-Cherradi ve arkadaşları TRAIL'in T1D'teki potansiyel rolünü anlamak için iki hayvan modelinden yararlanmışlardır. İlk modelde TRAIL blokajının sağlanabilmesi için Obez olmayan diyabetik farelere (NOD) çözülebilir TRAIL reseptörü (sDR5) enjekte edilmiştir. Bu molekül insan DR5 reseptörünün güdük bir versiyonu olup, hem insanlarda hem de farelerde etkin bir şekilde TRAIL fonksiyonunu engellemektedir. Sonuç olarak, TRAIL fonksiyonunun blokajı, diyabet gelişimini önemli derecede hızlandırarak pankreatik adacıklarda otoimmün inflamasyonu artırmıştır. sDR5 kullanımı sonrasında benzer bir sonuç, diyabetojenik T hücre transferi yapılmış NOD/SCID farelerde de gözlenmiştir[52]. Diğer bir modelde ise TRAIL geninden yoksun (TRAIL^{-/-}) C57BL/6 fareler kullanılmıştır. Bu farelerde birden fazla ve düşük dozda çoklu STZ enjeksiyonu ile diyabet indüklenmiştir. Farelerde anatomik ve morfolojik olarak normal bir gelişim gözlenirken aynı zamanda yapısal olarak normal bir immün sistemin geliştiği belirlenmiştir. Fakat, TRAIL^{-/-} farelerde normal farelere (TRAIL^{+/+}) kıyasla diyabet gelişiminin hızlandığı ve diyabet sıklığının arttığı gösterilmiştir (Tablo 2.1).

Fare	Sıklık (%)	Başlangıç günü (ort ± SEM)	Ürün glukoz aralığı (mg/dl)	İnsülitis derecesi (ort ± SEM)
TRAIL ^{+/+}	7/13(53.8)	20.3 ±7.4	500-1,000	0.9 ±0.15
TRAIL ^{-/-}	12/13(92.3)	10 ±4.3	1,000-2,000	3.2 ±0.18

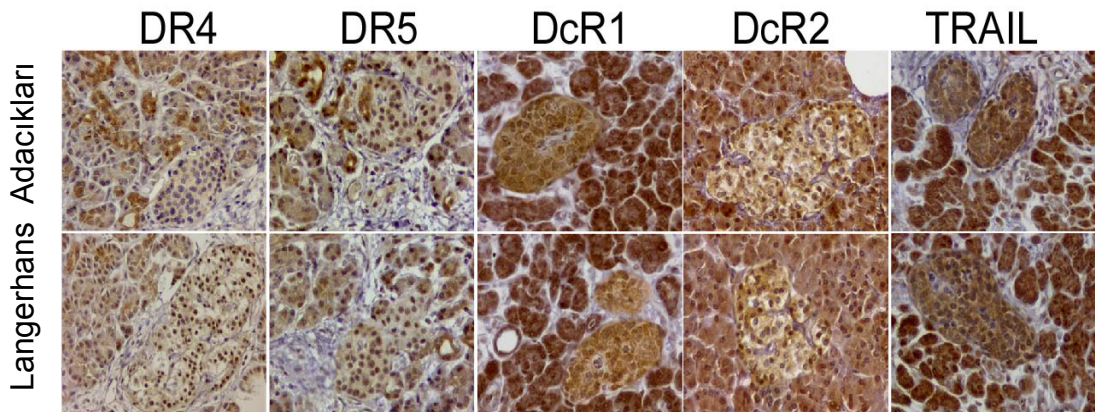
Tablo 2.1. TRAIL^{-/-} farelerde STZ ile indüklenmiş diyabet verileri[35]

Bu çalışmalar, TRAIL molekülünün hastalığın patogenezinde, apoptotik bir efektör olmaktan ziyade immün yanıtın bir regülatörü olarak fonksiyon gördüğünü göstermektedir. T1D gelişiminde, NOD farelerin pankreatik adacıklarında artan

miktarda TRAIL sentezi gösterilmiştir. Benzer şekilde, *in vitro* koşullarda TNF- α ve IFN- γ ile muamele edilmiş adacık beta hücrelerinde de artan miktarda TRAIL sentezi gösterilmiştir. Burada görülüyor ki TRAIL sentezinin uyarılması apoptozu artırmamakta, aksine diyabetojenik T-hücrelerinde hücre döngüsü bloke edilerek bu hücrelerin enerjik duruma gelmesi sağlanmaktadır. TRAIL'in *in vivo* blokajı ya da TRAIL^{-/-} farelerde düşük doz STZ uygulaması ile diyabetin indüklenmesi spesifik olarak diyabetojenik yanıtın, diyabet gelişiminin ve adacık inflamasyonun artmasına neden olmaktadır[35]. Bu nedenle, otoimmün diyabet açısından hayvan modelleri üzerindeki çalışmalar, TRAIL'in T1D'de koruyucu bir etkisinin olduğuna ve potansiyel bir immün regülatör olarak görev aldığına işaret etmektedir.

2.9. Pankreas Adacıklarında TRAIL Ligand ve Reseptör Kompozisyonu

TRAIL molekülünün T1D'te immünolojik rolünün incelenmesi açısından Ou D. ve arkadaşları, apoptozisin TRAIL sinyal yolu ile ilişkisini araştırmışlardır[53]. Bu çalışmada beta hücrelerinin çoğunun tüm TRAIL reseptörlerini ve/veya TRAIL'i sentez ettiği gösterilmiştir. Aynı çalışmada kullanılan pankreatik adacık hücrelerinin TRAIL'e dirençli olduğu görülmüştür. Bu hücreler yalancı reseptör olarak bilinen DcR1' in adacık hücre membranında elimine olmasını sağlayan bir enzim (PI-PLC) ile muamele edildiğinde, bu hücrelerin TRAIL'e duyarlı hale geldiği gösterilmiştir. Bu sonuçlara göre, TRAIL sinyal yolağı insan beta hücrelerinde mevcuttur ve fonksiyoneldir. Hücre yüzeyindeki TRAIL yalancı reseptör DcR1 sentezi bu dirençliliğin sebebi olarak görülmektedir. Grubumuzun insan pankreas dokusu üzerinde TRAIL ve reseptörlerinin sentez profili açısından yaptığı yeni bir çalışmada asiner hücrelerin yüksek oranda TRAIL ve ölüm reseptörü DR4 sentez ettiği, düşük düzeyde DR5 sentez ettiği gösterilmiştir. Kanal hücrelerinde ise TRAIL sentezi ile beraber yalancı reseptörler DcR1 ve DcR2 sentezi görülmüştür. Benzer şekilde Langerhans adacıklarında da (Şekil 2.11) sadece TRAIL ve yalancı reseptör sentezinin olduğu görülmüştür[32].



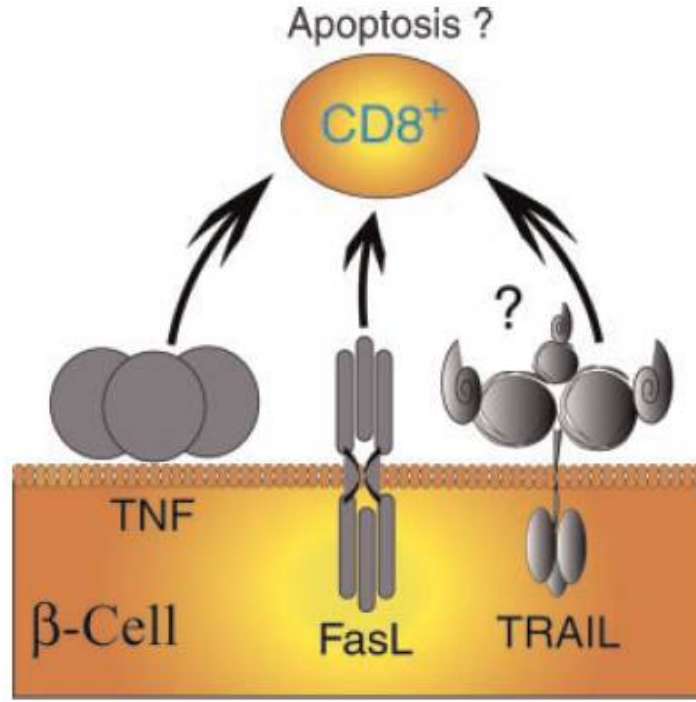
Şekil 2.11. Langerhans adacıklarında TRAIL ve TRAIL reseptörlerinin sentez profili [32]

Başka bir çalışmada TRAIL ve reseptörlerinin lokalizasyonu fetal pankreas dokusunda konfokal floresans immünohistokimya yöntemi ile incelenmiştir[54]. Bu yöntem ile bazı hücrelerde DcR1 ve DcR2 sentezi saptanırken, DR4 ve DR5 sentezi hiçbir hücrede saptanmamıştır. Bu hücrelerin TRAIL ve yalancı reseptörlerini sentezlemesi, hücrelerin apoptoza dirençli olabileceğini düşündürmektedir. Bunun yanında, başka bir çalışmada NOD farelerde TNF- α ve IFN- γ muamelesi sonucunda TRAIL gen sentezinin arttığı gözlenmiştir[52]. Aynı çalışmada yeni izole edilen adacıklarda TRAIL'in apoptozu indükleyemediği görülmüştür. Tüm bu sonuçlar değerlendirildiğinde pankreatik adacıklarda TRAIL dirençliliğinin nedeni TRAIL ölüm reseptörlerinin yalancı reseptörlere oranındaki değişiklikler olabilir.

2.10. Pankreas Adacıklarında Ölümcül Ligand Sunumunun Sonuçları

T1D, pankreatik beta hücrelerinin T-hücre aracılı kalıcı yıkımı sonucu ortaya çıktığından[55], adacıklara-spesifik otoreaktif T-hücrelerinin baskılanması, otoimmün diyabetten korunmada uygulanabilir bir stratejidir. Yapılan çalışmalarda Fas sinyal yolağı aktive edildiğinde yardımcı stimülasyon engellendiğinde, periferel lenfositlerin *in vitro* apoptozisinde bir artış görüldüğü belirtilmiştir[56]. Diyabet tedavisi için insan CTLA4(B7/CD28 blokörü)-FasL geni taşıyan bir adenovirus (AdCTLA4-FasL) kullanılarak ümit verici sonuçlar elde edilmiş olsa da, FasL'in inflamasyonu tetiklemesi, immün tolerans oluşturmada kullanılma ihtimalini azaltmıştır. Lin B.ve arkadaşlarının yaptığı bir çalışmada STZ ile indüklenmiş T1D'in patogeneğinde Fas-FasL yolağının etkisini araştırmak için transgenik CMV-hFasL fareler oluşturulmuştur[57]. İlginç olarak bu transgenik farelerin kontrollere oranla diyabet gelişimine karşı daha hassas oldukları bildirilmiştir. Buna neden olarak, aşırı FasL sentezinin interlökin-1 (IL-1) üretimini indüklemesi ve nötrofil infiltrasyonunu kolaylaştırması gösterilmiştir[58]. Bu sonuçlar, FasL'in beta hücrelerindeki transgenik sentezinin, daha erken dönemde T1D gelişimine neden olduğunu gösteren çalışmalar ile uyumludur[34, 59, 60].

Tip 1 diyabetli hastalarda pankreatik beta hücrelerine spesifik T-hücrelerinde aşırı miktarda TRAIL sentezinin gözlemlenmesi, T-hücrelerinin TRAIL aracılığıyla beta hücre yıkımı gerçekleştirebileceğini düşündürmüştür[61]. Son yaptığımız çalışmalarımızdan birinde de pankreasdaki TRAIL sentezi ile hücre ölümleri arasında pozitif bir korelasyonun olması bize TRAIL in insan pankreasında apoptozisi tetikleyici bir ajan olabileceğini gösterdi[32]. Bu durumda biz de nakledilecek pankreatik adacıklarda adenovirüs aracılı TRAIL sentezinin bu adacıkları alloimmün T-hücre saldırısına karşı koruyabileceği savını ileri sürdük (Şekil 2.12). Yukarıda bahsedilen tüm literatür bilgileri değerlendirildiğinde, T1D tedavisi için nakledilecek pankreatik adacıklarda adenovirüs aracılı TRAIL sentezinin bu adacıkları alloimmün T-hücre saldırısına karşı koruyup koruyamayacağı araştırıldı. Projemizde, terapötik yaklaşımımızın etkinliğini belirlemek için Wistar ratlarından pankreatik adacıklar izole edilip viral vektörler ile enfeksiyon sonrası STZ indüklemeli diyabetik Wistar sıçanlarına nakil yapıldı. Nakil sonrasında bu işlemin adacık-graft sağkalım süresi ve fonksiyonu üzerindeki etkileri kontrol gruplarıyla kıyaslanarak belirlendi.



Şekil 2.12. Pankreas adacıklarında ölümcül ligand sentezi[31]

MATERYAL ve METODLAR

3.1. Sıçan Pankreas Adacık İzolasyonu

Kullanılan Solüsyonlar:

Serumsuz DMEM Besiyeri:

9,6 g toz DMEM (Sigma) tartıldı ve 930 ml distile su içinde çözüldü. 121°C'de 15 dakika otoklavlandı. Besiyeri oda sıcaklığına geldiğinde, içerisine 49.3 ml steril %7.5 sodyum bikarbonat, 20 ml 200 mM steril L-glutamin, 2 ml gentamisin eklendi. +4°C'de saklandı.

%10 Serumlu DMEM Besiyeri:

1 lt serumsuz DMEM besiyerine, 110 ml inaktif yenidoğan dana serumu (Biochrom AG) eklendi. +4°C'de saklandı.

Liberase RI Enzim(Roche):

Ticari olarak alınan 100 mg liyofilize Liberase RI enzimi 4 ml enjeksiyonluk suda çözüldü ve 2 ml'lik ependorflara dörde bölündü (25 mg/ml). 1 ml enzim 9 ml enjeksiyonluk suda seyreltilerek 1'er ml olarak ependorflara aktarılarak kullanıma hazır hale getirildi (2,5 mg/ml).

Enzim solüsyonu:

Tek kullanımlık hazırlanan enzim (2.5mg/ml) 9ml serumsuz besi yerinde çözüldü ve kullanım süresine kadar buzda bekletildi.

Hazır Solüsyonlar:

Ksilazin (Alfazyne)
Ketamin (Alfamine)
Histopaque 1077(Sigma)

3.1.1. Pankreasın Cerrahi Olarak Çıkarılması:

6-8 haftalık, 200-250 g ağırlığındaki, Wistar türü dişi sıçanlara, intra-peritoneal yolla ksilazin (40-80 mg/kg) ve ketamin (5-10mg/kg) karışımı verilerek uyutuldu. Sıçan, çalışma tablasına sırt üstü yatırıldı ve abdomen % 80'lik alkol ile temizlendi. V-insizyon ile karın boşluğuna ulaşıldı. Karaciğer kraniyele çekildi ve üzerine bir gazlı bez yerleştirildi. Barsaklar, hayvanın soluna çıkartılarak pankreas ortaya çıkarıldı. Ortak safra kanalının karaciğer ucuna ve duodenuma birleştiği uca hemostat klempler yerleştirildi. Ortak safra kanalının karaciğer ucuna bir kesik atıldı ve bu kesikten PE50 tüpü (Biopac) ile ortak safra kanalına girildi. PE50 tüpünün diğer ucuna enzim solüsyonu (0.25 mg/ml) çekilmiş enjektör yerleştirildi ve PE50 aracılığıyla 10 ml soğuk enzim solüsyonu enjekte edilerek pankreasın tamamı şişirildi. Kalbe bir kesik atılarak kansızlaştırma işlemi uygulandı. Pensetler yardımıyla, pankreas çevre doku organlardan ayrıldı ve temiz bir tüp içine alındı.

3.1.2. Pankreasın Enzimatik Sindirimi

Tüp, 37°C su banyosunda 17-19 dakika enzimatik inkübasyona bırakıldı. Süre sonunda, tüpün üzeri buzda bekletilmiş serumlu DMEM ile 35-40 ml'ye tamamlandı ve 20 saniye kuvvetlice çalkandı. 1600 rpm 75 sn 4°C santrifüj edildi ve dökelti atıldı. Çökelti üzerine 20 ml serumlu DMEM eklendi ve homojenize edildi oluşan süspansiyon 400 µm çapında porları olan çelik filtreden (Thomas scientific) geçirilerek temiz bir tüpe toplandı. Çelik filtrede kalan dokuların toplanması için, 30 ml serumlu DMEM filtreden geçirilerek aynı tüpte toplandı. Tüp, 1600 rpm 75 sn 4°C santrifüj edildi ve dökeltinin tamamı atıldı.

3.1.3. Pankreas Adacıklarının Saflaştırılması

Çökelti üzerine 20 ml soğuk Histopak-1077 (Sigma) eklendi ve çökeltinin tamamen çözünmesi sağlandı. Üzerine yavaşça 10 ml serumsuz DMEM eklenerek, histopak ile besiyeri arasında keskin bir hat oluşturuldu. Tüp, 2500 rpm 17 dakika 4°C, yavaş hızlanmalı ve yavaşlanmalı olarak santrifüj edildi. Santrifüj sonrası orta hatta oluşan adacık tabakası, 14 Gauge (G) şırınga ile çekilerek temiz bir tüpe aktarıldı. Tüpün üzeri serumlu DMEM ile 45 ml'ye tamamlandı. 1600 rpm 2 dakika 4°C santrifüj edildi ve dökelti atıldı. Çökelti üzerine, 25 ml serumlu DMEM eklendi ve 4 dakika buz üzerinde bekletildi. Süre sonunda tüpün üzerinden 10 ml çekilerek atıldı, tekrar 10 ml serumlu DMEM eklendi. Karıştırılarak tekrar 4 dakika buz üzerinde bekletildi. Bu işlem 4 kez yapıldı.

3.1.4. İzole Edilen Pankreas Adacıklarının Saflık ve Canlılık Tayini

3.1.4.1 Pankreas Adacıklarının Saflık Oranının Belirlenmesi

Kullanılan Solüsyonlar:

Ditizon solüsyonu (DTZ):

5 mg toz ditizone (Fluka) tartıldı ve 2 ml dimetil sülfoksit (DMSO, Sigma) içinde çözüldü. Hazırlanan solüsyon, HBSS (Sigma) ile 20 ml'ye tamamlandı. -20°C'de saklandı.

Hank's Buffered Salt Solution (HBSS): Ticari olarak hazır kullanıldı.

PBS (Phosphate Buffer Saline) Solüsyonu:

8 g NaCl (Sigma), 0.2 g KCl (Sigma), 1.44 g NaH₂PO₄ (Sigma), 0.24 g KH₂PO₄ (Sigma) tartılarak, 800 ml bidistile su içinde çözüldü. NaOH ile pH 7.4'e ayarlandı ve toplam hacim bidistile su ile 1 litreye tamamlandı. Solüsyon 121°C'de 15 dakika otoklavlandı ve +4°C'de saklandı.

İşlemler:

35 mm doku kültür kabına saflık testi için yaklaşık 50 adet pankreatik adacık ayrıldı. Adacıkların üzerine 1 ml PBS eklendi. Saflık tayini için 350 µl DTZ solüsyonu eklendi. 5 dakika sonra mikroskop altında görüntüleri alındı ve pankreatik adacıkların saflık oranları analiz edildi.

3.1.4.2. Pankreas Adacıklarının Canlılık Oranının Belirlenmesi

Kullanılan Solüsyonlar:

PBS: 3.1.4.1' de anlatıldığı gibi hazırlandı.

Floresan Diasetat Solüsyonu (FDA):

5 mg toz floresan diasetat (Sigma) tartıldı ve 1 ml aseton içinde çözüldü. +4°C'de karanlıkta saklandı.

Propidyum İyodit solüsyonu (PI):

1 mg toz propidyum iyodit (Sigma) tartıldı ve 1 ml distile su içinde çözüldü. +4°C'de karanlıkta saklandı.

İşlemler:

35 mm doku kültür kabına canlılık testi için yaklaşık 50 adet pankreatik adacık ayrıldı. Adacıkların üzerine 1 ml PBS eklendi. Canlılık tayini için 2 µl FDA, 2 µl PI solüsyonu eklendi. 5 dakika sonra floresan mikroskop altında görüntüleri alındı ve pankreatik adacıkların canlılık oranları analiz edildi.

3.2. Birinci Jenerasyon Adenoviral Vektörlerin Çoğaltılması ve Saflaştırılması

Kullanılan Solüsyonlar:

Serumsuz RPMI-1640 Besiyeri:

10,26 g toz RPMI-1640 besiyeri (Sigma), 962 ml bidistile su içinde çözüldü ve pH'sı HCl asit ile 4.0 olarak ayarlandı. Hazırlanan besiyeri 121°C'de 15 dakika otoklavlandı. Solüsyon oda sıcaklığına geldiğinde, üzerine 26.7 ml %7.5 steril sodyum bikarbonat (Sigma), 10,25 ml 200 mM steril L-glutamin (Biological Industries), 2 ml 80 mg gentamisin (Genta) eklendi. Hazırlanan besiyeri +4°C'de saklandı.

Serumlu RPMI-1640 Besiyeri:

1 litre serumsuz RPMI-1640 besiyerine 110 ml inaktif fetal sığır serumu (Biochrom AG) eklenerek %10 serumlu, 220 ml eklenerek %20 serumlu besiyeri hazırlandı. Hazırlanan besiyerleri +4°C'de saklandı.

2XTripsin-EDTA:

20 ml 10X Tripsin-EDTA stok solüsyonu (Biological Industries), 80 ml serumsuz RPMI-1640 ile karıştırılarak 2X Tripsin-EDTA hazırlandı.

PBS Solüsyonu: 3.1.4.1' de anlatıldığı gibi hazırlandı.

Transfeksiyon Solüsyonu:

410 ml serumsuz besiyerine, 200 virüs partikülü/hücre (MOI, multiplicities of infection) yoğunlukta adenoviral vektör eklenerek buz üzerinde tutuldu.

1 M Tris-Cl (Amresco):

24,2 g Tris 160 ml distile su içinde çözüldü. HCl ile pH 8.1'e ayarlandı ve toplam hacim distile su ile 200 ml'ye tamamlandı. Solüsyon 121°C'de 15 dakika otoklavlandı ve +4°C'de saklandı.

10 mM Tris-Cl (Amresco):

1 M Tris-Cl'den 5 ml alınarak, distile su ile 500 ml'ye tamamlandı. 121°C'de 15 dakika otoklavlandı ve +4°C'de saklandı.

Ağır CsCl Solüsyonu (Roche):

42,33 g CsCl tartıldı ve 57,77 ml 10 mM Tris-Cl içinde çözüldü. HCl ile pH 8.1'e ayarlandı. 121°C'de 15 dakika otoklavlandı ve +4°C'de saklandı

Hafif CsCl Solüsyonu (Roche):

22,39 g CsCl tartıldı ve 77,61 ml 10 mM Tris-Cl içinde çözüldü. HCl ile pH 8.1'e ayarlandı. 121°C'de 15 dakika otoklavlandı ve +4°C'de saklandı.

3.2.1. Adenoviral Vektörlerin HEK293 Hücrelerinde Çoğaltılması

HEK 293 hücre hattı, %10 serumlu RPMI-1640 besiyeri kullanılarak 150 mm hücre kültürü kaplarında (Sarstedt), 37°C sıcaklık ve %5 CO₂'li nemli ortamda üretildi. HEK 293 hücreleri, 40 adet 150 mm'lik doku kültür kabını yaklaşık %80 yoğunlukta kaplayacak şekilde çoğaldığında, adenoviral vektörlerle transdükte edildi. Bunun için, doku kültür kaplarındaki besiyeri uzaklaştırıldı ve hücrelerin üzerine 10'ar ml transfeksiyon solüsyonu eklenerek inkübatöre kaldırıldı. 2 saat sonra doku kültür kaplarına %20 serumlu besiyeri eklenerek, mevcut besiyerinin serum içeriği %10'a çıkarıldı. Transdüksiyonun 30-54. saat aralığında hücreler kontrol edildi. Sitopatik etkinin belirgin hale geldiği anda, yani hücrelerin yuvarlaklaşarak yüzeyden ayrılma eğiliminde oldukları anda, hücreler 25 ml'lik pipetler aracılığıyla toplanarak 250 ml'lik 2 adet steril tüpe aktarıldı. Tüpler, 4°C 4000 rpm 10 dakika santrifüj edildi. Santrifüj sonrası dökelti atıldı ve çökelti 10 mM Tris-Cl ile (her 150 mm'lik kap için 0,5 ml 10mM Tris-Cl) çözülerek 15 ml'lik tüplere aktarıldı.

3.2.2. Adenoviral Vektörlerin Saflaştırılması

Tüpler 3 kez etil alkol-kuru buz banyosunda hızlı donma-çözünme işleminden geçirildi. Daha sonra, 4°C 5000 rpm 10 dakika santifüj edildi ve dökelti toplanarak 10 mM Tris-Cl ile 20 ml'ye tamamlandı. Şeffaf 40 ml'lik ultrasantrifüj tüplerine (Beckman), 10 ml ağır CsCl, üzerine 10 ml hafif CsCl ve en son viral dökelti eklendi ve tüpler ultrasantrifüj rotoruna (Beckman SW28) yerleştirilerek 4°C 20000 rpm gece boyunca santifüj edildi. Santrifüj sonrasında oluşan viral bantlar şırınga yardımı ile çekilerek, 10 mM'lık Tris-Cl ile toplam hacim 4 ml'ye tamamlandı. Şeffaf 20 ml'lik ultrasantrifüj tüplerine (Beckman), 4 ml ağır CsCl, üzerine 4 ml hafif CsCl ve en son viral bant eklendi ve tüpler ultrasantrifüj rotoruna (Beckman SW41) yerleştirilerek 4°C 20000 rpm 6 saat santrifüj edildi. Santifüj sonrası oluşan viral bantlar şırınga ile çekilerek 15 ml'lik tüpe aktarıldı. Spektrofotometrede ölçüm yapılarak, OD₂₆₀ değeri belirlendi. Ad5EGFP [62, 63], AdCMVLacZ [64, 65] ve Ad5hTRAIL[66] adenoviral stokları, yukarıda tanımlandığı şekilde hazırlandı. Elde edilen virüs hacminin ¼'ü hacimde gliserol (Sigma) eklendi ve virüs stokları -86°C'de saklandı.

3.3.1 İzole Edilen Pankreas Adacıklarının Birinci Jenerasyon Adenoviral Vektörler ile Transdüksiyona Uğratılması

İzole edilen pankreatik adacıklar süspense halde 100 mm doku kültür kabına aktarıldı. Mikroskop altında, 200 µl pipet aracılığıyla, adacıklar tek tek toplandı ve 5 ml serumsuz RPMI besiyeri içeren, steril 100 mm doku kültür kabında aktarıldı. Toplanan adacık sayısına göre (her adacık 1×10^3 hücre), verilecek adenoviral vektör miktarı hesaplandı (MOI =10, 50, 200 ve 1000). Hesaplanan miktardaki viral vektör partikülü, doku kültür kabında toplanan adacıkların üzerine eklendi. Doku kültür kapları, 37°C sıcaklık ve %5 CO₂'li nemli ortamda 2 saat tutuldu. Süre sonunda kaplara, 5ml %20 serumlu RPMI eklenerek mevcut serum miktarı %10'a çıkarıldı. Doku kültür kapları, 37°C sıcaklık ve %5 CO₂'li nemli ortamda 48 saat tutuldu. Transdüksiyon etkinliği hem floresan mikroskop hem de florometrik ölçümler ile belirlendi.

3.3.2. Transdüksiyona Uğratılan Adacıklarda Transgen Sentezinin Belirlenmesi

Ad5hTRAIL ile enfekte edilmiş pankreatik adacıklar besiyeri ile birlikte 200-500 µl olacak şekilde ependorf tüplere alındı. Adacık süspansiyonu Cytospin Collection Fluid (CCF) ile 1 ml tamamlanarak Cytofunnel içerisinde 800 rpm de 3 dakika Cytospinde (Shandon Cytospin® Cyto centrifuge) santrifüj edildi. Süre sonunda lam üzerinde bulunan pankreatik adacıklar, aseton/metanol (1:1) karışımı uygulanarak fiske edildi. Fiksasyon sonrasında preparatlar, immünohistokimyasal boyamalar için 20 dakika kurumaya bırakıldı.

3.3.2.1 İmmünohistokimyasal boyama

Kullanılan Solüsyonlar:

PBS: 3.1.4.1' de anlatıldığı gibi hazırlandı.

Hazır Solüsyonlar:

Hidrojen Peroksit (H₂O₂): (ScyTec)
Ultra V Block: (Lab Vision)
TRAIL primer antikoru (Alexis)
Biotin işaretli Goat antipolivalent (Lab Vision)
Streptavidin peroksidaz (Lab Vision)
Hematoksilen

Kromojenik substratın hazırlanması:

40 µl DAB Plus Chromogene (Lab Vision), 2 ml DAB Plus Substrat (Lab Vision) ile karıştırıldı ve 10 saniye vorteks ile homojen hale getirildi.

İşlemler:

Cytospin sonucu lam üzerine fiske edilen pankreatik adacıkların çevresi PAP-pen ile çizilerek 2 x 5 dakika PBS ile yıkandı. Endojen peroksidaz aktivitesine bağlı olarak oluşabilecek spesifik olmayan boyamaları önlemek için hazırlanan preparatlar H₂O₂ içerisinde oda sıcaklığında 10 dakika inkübe edildi. Ardından preparatlar 2 x 5

dakika PBS ile yıkandı. Yine spesifik olmayan boyanmaları engellemek için preparatlar Ultra V Block içerisinde oda sıcaklığında 5 dakika tutuldu. Ultra V blok ile muamele sonunda yıkama yapılmadan 1/200 oranında dilüe edilen TRAIL primer antikoru ile 2 saat inkübe edildi. Süre sonunda 2 x 5 dakika PBS ile yıkama yapıldı. Hemen ardından Biotin işaretli Goat antipolivalent sekonder antikoru ile muamele edildi. Süre sonunda 2 x 5 dakika PBS ile yıkama yapıldı. Yıkamayı takiben Streptavidin peroksidaz ile 20 dakika inkübasyon uygulandı. Daha sonra enzim çekilip atılarak 2 x 5 dakika PBS ile tekrar yıkama yapıldı. Daha önce hazırlanmış olan kromojenik substrat ile oda sıcaklığında mikroskop altında renk değişim reaksiyonu görülünceye kadar muamele edildi. Reaksiyonun oluştuğu gözlemlendiğinde preparat musluk suyu ile yıkandı. Zıt boyama yapılması için preparat işaretli alan üzerine Hematoksilin damlatıldı ve boyanma kalitesine bağlı olarak 3-8 saniye bekletildi. Hazır hale gelen preparatlar Kaiser's Glycerol Gelatine (Merck) ile kapatılarak gece boyu kurumaya bırakıldı.

3.4. Annexin V-FITC boyama

Apoptozis olarak bilinen programlı hücre ölümü, bir dizi hücre 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. Annexin V, Ca^{2+} bağımlı bir fosfolipid bağlayıcı proteindir. Annexin V, FS için yüksek afinitesi olması nedeniyle, hücre yüzeyinde FS sunulan apoptotik hücrelere bağlanır. Çalışmamızda, apoptotik hücrelerin belirlenmesinde Annexin V proteinine karşı FITC takılı bir kit kullanıldı.

Kullanılan Solüsyonlar:

Annexin V-FITC Florasan Mikroskopi Kiti (BD Pharmingen):

Annexin V-FITC
10X Annexin V bağlanma tamponu
PBS

İşlemler:

Kültüre edilen pankreatik adacık hücreleri ependorf tüplere alındı ve 30 saniye 800 rpmde santrifüj edildi. Çökelti 100 µl 1X Annexin V bağlanma solüsyonu ile homojenize edildi. Daha sonra, hücreler üzerine 10 µl AnnexinV-FITC eklenip oda sıcaklığında ve karanlık bir ortamda 15 dakika bekletildi. Süre sonunda adacık hücreleri 96 kuyucuklu kültür kaplarına alınarak floresan mikroskopi ile görüntülendi. Pozitif kontrol olarak, pankreatik adacık hücreleri Annexin V-FITC boyaması öncesinde 1 saat 1mM H₂O₂ ile muamele edildi.

3.5. Diyabetik Hayvan Modellerinin Oluşturulması

Kullanılan Solüsyonlar:

Sodyum Sitrat Çözeltisi:

14.705 g Sodyum Sitrat (Merck) tartıldı. 500 ml bidistile su içinde çözüldü. +4°C'de saklandı.

Sitrik Asit Çözeltisi:

10.507 g Sitrik Asit (Merck) tartıldı. 500 ml bidistile su içinde çözüldü. +4°C’de saklandı.

Sitrat Tamponu:

Hazırlanan sodyum sitrattan 100 ml alındı ve pH’sı sitrik asit ile 4.5’e ayarlandı. +4°C’de saklandı.

Streptozotosin Solüsyonu (STZ):

Toz streptozotosin (Sigma), Wistar sıçanlar için 30, 40 ve 50 mg/kg olacak şekilde tartıldı. 500 µl sitrat tamponu içinde hesaplanan miktarda STZ çözüldü. STZ, enjeksiyona kadar buz üzerinde, karanlıkta saklandı.

Kullanılan Deney Hayvanları:

Deneylerde kullanılan hayvanlar, Akdeniz Üniversitesi Deney Hayvanları Ünitesinden temin edildi. Diyabetik sıçan modeli olarak, yaklaşık 250-300 g ağırlığında, dişi, Wistar sıçan türleri kullanıldı. Deney süresince, Wistar sıçanlara kobay yemi ve musluk suyu verildi. Hayvanlar, 22±2°C sıcaklıkta ve %50-60 nemli koşullarda tutuldu ve 12 saat aydınlık/12 saat karanlık periyod uygulandı.

Hazırlanan STZ solüsyonu, hayvanlara intraperitoneal olarak enjekte edildi. STZ enjeksiyonundan sonra ilk 10 gün her gün sonrasında 1 hafta aralıklarla kan şekeri ölçüm cihazı (glukometre) (Accu-Check Go, Roche) kullanılarak kan şekerleri ölçüldü. Bunun için, hayvanların kuyruklarından bir damla kan örneği kullanıldı. Ard arda iki ölçümde 300 mg/dl’nin üzerinde kan şekeri değeri ölçüldüğünde hayvan diyabet kabul edildi.

3.6. Pankreas Adacıklarının Nakli**3.6.1 Pankreas Adacıklarının Nakil için Hazırlanması**

Doku kültür kabındaki pankreatik adacıklar, transfeksiyonun 48. saatinde toplanarak temiz 50 ml’lik tüp içine alındı. 4°C 1200 rpm 1 dakika santrifüj edildi ve dökelti dibinde biraz kalacak şekilde atıldı. Adacık çökeltisi, 750 adacık/sıçan olacak şekilde yaklaşık 100 µl besiyeri içinde temiz mikrosantrifüj tüplerine aktarıldı. Pankreatik adacıklar mikrosantrifüj tüpünden, PE50 tüpüne aktarıldı ve 4°C 1250 rpm 1 dakika santrifüj edilerek, adacıklar PE50 tüpünün içerisinde yoğunlaştırıldı.

3.6.2. Diyabetik Hayvanların Adacık Nakli için Hazırlanması

STZ enjeksiyonundan sonra kan şekeri ölçümleri ile diyabet olarak kabul edilen hayvanlar ketamin/ksilazin karışımı ile uyutuldu. Hayvanların sol karın bölgeleri traş edildi ve antiseptik solüsyon ile temizlendi. Sol böbrek üzerinden küçük bir kesikle peritona ulaşıldı. Peritondan böbreğin geçebileceği büyüklükte ikinci kesik açıldı. Kesikin kenarlarından baskı uygulanarak böbrek vücut dışına çekildi. Operasyon süresince böbrek serum fizyolojik solüsyonu ile sık sık nemlendirildi.

3.6.3. Pankreas Adacıklarının Sıçanlara Nakledilmesi

Böbrek kapsülüne küçük bir kesik atıldı ve kapsülün altından, içerisinde

pankreatik adacıkların bulunduğu PE50 tüpü sokularak ilerletildi. PE50 tüpünün diğer ucundan Hamilton şırıngası (Hamilton) yardımıyla basınç uygulanarak, adacıkların PE50 içerisinde hareket etmesi ve kapsül altına yerleşmesi sağlandı. Nakilden sonra PE50 yavaşça kapsülün altından çıkartıldı ve kapsül koterize edildi. Böbrek yavaşça peritona yerleştirildi. Periton 3.0 eriyebilir katgüt (Boz) ile kapatıldı. Dış deri ise 3.0 ipek iplik (Medeks) ile kapatıldı. İntraperitonel yolla 10 mg/ml ağrı kesici (Tramadol Hidroklorür) verildi. Hayvanlar anestezinin etkisi geçinceye dek 37°C küvezde tutuldu. Uyanan hayvanlar kafeslerine yerleştirildi ve postoperatif bakıma alındı.

3.7. Adacık İnflamasyonunun belirlenmesi

Kullanılan Solüsyonlar:

% 10 formalin

%100, 90, 80, 70 alkol

Ksilol (Merck): Ticari olarak hazır alındı.

Hematoksilen: Ticari olarak hazır alındı.

Eozin: Ticari olarak hazır alındı.

Nakledilen pankreatik adacık graftalarında mononükleer hücre infiltrasyonunun H&E boyama yöntemi ile belirlenebilmesi için Ad5hTRAIL, AdCMVLacZ ile enfekte ve enfekte edilmemiş adacıkların nakledildiği sıçanlar 15, 30, 60 ve 90'ncü günlerinde sakrifiye edildi. Her üç gruptan her bir zaman diliminde 3'er hayvanın böbrekleri cerrahi olarak çıkarıldı. Çıkarılan böbrekler % 10 formaline alınarak etiketlendi. 1 günlük formalin fiksasyonu sonrasında böbrek üzerinde adacık graftının bulunduğu bölge kesilerek formalin etkisinin giderilmesi için 3 saatlik akan su altında tutuldu. Süre sonunda böbrek kesitleri sırasıyla 1'er gün % 70, 80, 90 ve 3 saat süre ile % 100 alkol serisine alındı. Bu işlemin ardından böbrek kesitleri 1-2 dakika ksilol de tutularak mikrotomda kesit alınabilecek pozisyonda parafine gömüldü. Daha sonra parafine gömülü dokulardan 5 µm kalınlıkta seri kesitler alınarak adacık graft preparatları hazırlandı. Preparatlar deparafinize edildikten sonra önce 5 dakika ksilol sonra sırasıyla 5'er dakika % 100, 90, 80, 70 alkol serisine alındı. Hemen ardından su ile yıkanan preparatlar 10 saniye Hematoksilen ile boyanıp tekrar yıkandı. Yıkamanın ardından 5 saniye eozinle boyanan preparatlar yıkanıp tekrar alkol serisinde (%70, 80, 90, 100) 3'er dakika bekletildi. Ksilolden geçirilen preparatlar entellan ile lamel kapatılarak oda sıcaklığında kurumaya bırakıldı.

3.8. İstatistiksel analiz

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

BULGULAR

4.1. Pankreas Adacık İzolasyon Protokolünün Modifiye Edilmesi Adacık Sayı ve Kalitesini Artırdı

Öncelikle, daha yüksek sayıda ve daha kaliteli pankreatik adacık elde etmek amacıyla literatürde var olan sıçan pankreatik adacık izolasyon protokolünde bazı değişiklikler yapıldı. Pankreatik kanalın on iki parmak barsağına açıldığı noktanın (Vater Ampulası) her iki yanı hemostat klemplerle kapatıldı ve *Liberase RI* enziminin tüm pankreas dokusuna yayılımı sağlandı. Bu şekilde bir pankreastan elde edilen adacık sayısında belirgin artış sağlandı. Daha sonra izole edilen pankreatik adacıklar hücre canlılığı ve saflığı açısından değerlendirildi (Şekil 4.1). Taze izole edilen ve pipet yardımı ile toplanan pankreatik adacıkların düzenli bir morfolojik yapısı olmakla birlikte, adacıklar ortalama 100-200µm çapına sahip idi. DTZ boyaması sonrasında adacıkların saflığı yaklaşık % 90 olarak belirlendi. PI/FDA boyaması ile izole edilen pankreatik adacıkların bazı apoptotik hücreler dışında tamamen canlı olduğu saptandı. Sonuçlarımız, yüksek sayıda ve kalitede adacık elde edilmesinde enzimin pankreas içerisine tamamen dağılımının önemli olduğunu gösterdi.

4.2. Pankreas Adacıkları Adenoviral Vektörler ile Etkin Bir Şekilde Transdüksiyona Uğratıldı

Sıçan pankreasından izole edilen pankreatik adacıklar birinci jenerasyon adenoviral vektörler ile transdükte edildi. Transdüksiyon etkinliğinin belirlenmesi için adacıklar, artan dozlarda (Multiplicity of Infection (MOI))'de yeşil floresan protein (Enhanced Green Fluorescent Protein (EGFP)) kodlayan adenoviral vektörler (AdEGFP) ile enfekte edildi. Enfeksiyondan 48 saat sonra hem floresan mikroskopi (Şekil 4.2A) hem de florometrik ölçümler ile transdüksiyon etkinliği belirlendi (Şekil 4.2B). Enfekte edilmemiş pankreatik adacıklarda EGFP sinyali gözlenmezken, AdEGFP ile enfekte edilmiş örneklerde, kullanılan viral vektör dozuna bağlı olarak önemli düzeyde EGFP sinyali saptandı. 200 MOI'de ölçülebilir düzeyde transdüksiyon sağlanırken, optimum transdüksiyon etkinliği 1000 MOI' de elde edildi. Bu sonuçlar, pankreatik adacıkların rekombinant adenoviral vektörler ile başarılı bir şekilde enfekte edilebildiğini, ve bu transdüksiyon etkinliğinin kullanılan viral vektör dozuna bağlı olduğunu gösterdi.

Yukarıdaki sonuçlara dayanarak, izole edilen pankreatik adacıklarda aşırı TRAIL sentezi sağlayabilmek amacı ile sıçan adacıkları 48 saat süre ile 200 ve 1000 MOI'de Ad5hTRAIL ile enfekte edildi. Sonrasında TRAIL transgen ifadesi immünohistokimyasal yöntemler ile belirlendi. Şekil 4.3'de görüldüğü üzere hem enfekte edilmemiş hem de AdCMVLacZ ile enfekte edilmiş pankreatik adacıklarda ölçülebilir derecede TRAIL sentezi gözlenmedi. Buna rağmen, Ad5hTRAIL ile enfekte edilen adacıklarda kullanılan viral vektörün dozuna bağlı olarak yüksek düzeyde TRAIL sentezi belirlendi.

4.3. Pankreas Adacıklarında Aşırı TRAIL Sentezinin Toksik Bir Etki Oluşturmadığı Belirlendi

TRAIL, tümör hücreleri için sitotoksik bir etki oluştururken, normal hücre ve dokulara karşı herhangi bir toksisitesi bulunmamaktadır. Buradan yola çıkarak, adacıklarda Ad5hTRAIL aracılı aşırı TRAIL sentezinin adacık canlılığı üzerine olan etkisi araştırıldı. İzole edilen pankreatik adacıklar, farklı 48 saatliğine farklı dozlarda AdCMVLacZ ya da Ad5hTRAIL ile enfekte edildi. Kullanılan vektörlerin adacıklar üzerinde toksik bir etki oluşturup oluşturmadığı Annexin V-FITC boyaması ile belirlendi (Şekil 4.4). 1000 MOI'de, ne AdCMVLacZ ne de Ad5hTRAIL ile enfekte edilen adacıklarda apoptoz gözlenmedi. Bununla birlikte, enfeksiyon 5000 DNA partikülü/hücre oranına çıkarıldığında her iki viral vektör ile enfekte olan pankreatik adacıklarda apoptoz oranının arttığı belirlendi. Sonuçlarımız, adacıklardaki apoptotik oranın artışının transgen (TRAIL ya da LacZ) sentezine bağlı olmadığını, aksine kullanılan viral vektörün dozuna bağlı olduğunu gösterdi.

4.4. STZ ile İndüklenen Tip 1 Diyabetik Sıçan Modellerinde Optimum STZ Dozu 40 mg/kg Olarak Belirlendi

STZ, seçici olarak pankreatik beta hücrelerini yıkıma uğratan diyabetojenik bir ajan olup deneysel olarak T1D modeli oluşturmak için yaygın olarak kullanılmaktadır. Sıçanlarda T1D modeli oluşturmak için her grupta 10-12 hayvan olacak şekilde 3 farklı hayvan grubuna tek seferde intraperitoneal enjeksiyon oluyla 30, 40 ve 50 mg/kg STZ verildi. Enjeksiyon sonrası hayvanların sağlık durumu 100 gün süre ile takip edildi (Şekil 4.5). 50 mg/kg STZ uygulanan grupta enjeksiyonun 4. gününde sağkalım oranının % 16.6'ya kadar düştüğü saptandı. Bu grup STZ'nin olumsuz etkilerinden dolayı 35. günde sonlandırıldı. Diğer taraftan, 40 mg/kg STZ uygulanan grupta sağkalım oranı 4. günde % 70 olarak gözlemlendi ve takip süresinin sonuna kadar başka ölüm gözlenmedi. En yüksek sağkalım oranı ise % 91 ile 30 mg/kg STZ uygulanan grupta saptandı.

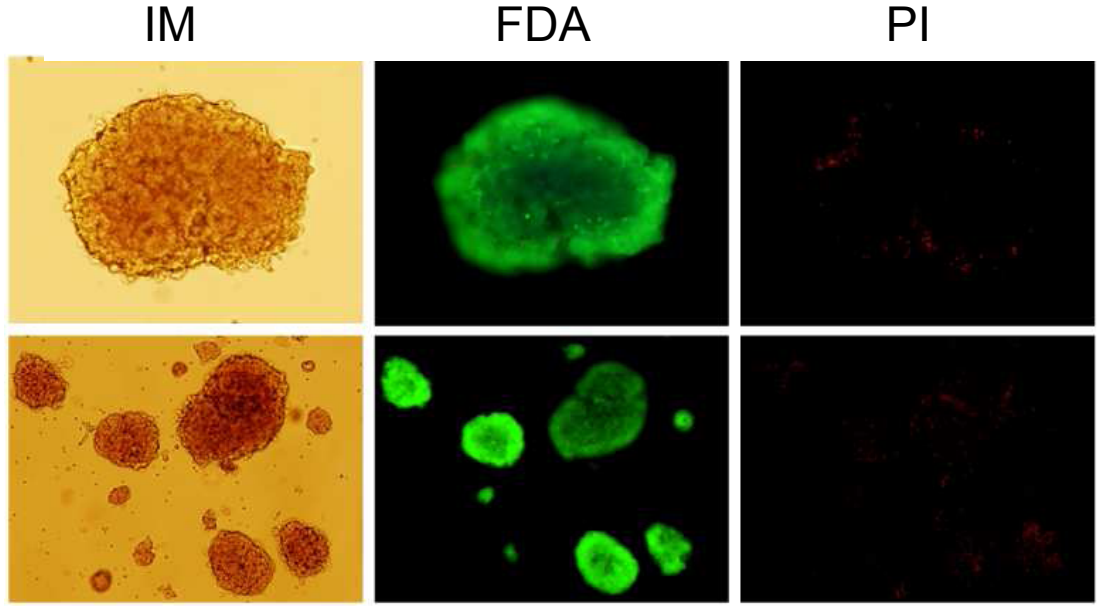
Deney gruplarındaki hayvanlarda sağkalım oranları ile eş zamanlı olarak kan şeker düzeyleri de takip edildi. Şekil 4.6'da da görüldüğü gibi STZ enjeksiyonunu takiben 24 saat sonra her üç grupta da kan şeker düzeyi 200 mg/dl'nin üzerinde ölçüldü. Değerlendirme süresince 40 ve 50 mg/kg STZ uygulanan sıçanların kan şeker düzeyleri yüksek seyrederken, 30 mg/kg STZ uygulanan sıçanların kan şekeri düzeyinin 3. günde azalmaya başladığı ve 10. günde normoglisemi seviyesine (<200 mg/dl) düştüğü saptandı. Bu hayvanların kan şeker düzeyi geriye kalan takip süresince 200 mg/dl seviyesinin altında kaldığı için normoglisemik olarak kabul edildi. Takip süresince her üç grupta bulunan sıçanların aynı zamanda vücut ağırlıkları da ölçüldü (Şekil 4.7). 30 ve 40 mg/kg STZ enjeksiyonu uygulanan sıçanlarda 10. güne kadar yaklaşık % 10 oranında ağırlık kaybı gözlemlendi. 10. günden sonra 30 mg/kg STZ uygulanan gruptaki hayvanlar kilo almaya başlarken 40 mg/kg STZ uygulanan grubun 14. güne kadar ağırlık kaybetmeye devam ettiği, sonrasında almaya başladığı gözlemlendi. Her iki grup arasındaki bu farka rağmen sıçanların enjeksiyon öncesindeki ağırlıklarına ulaştığı saptandı. Diğer taraftan 50 mg/kg STZ uygulanan gruptaki hayvanlarda enjeksiyonun ilk 3 gününde yaklaşık % 25 oranında ağırlık kaybı olduğu gözlemlendi. Bu kaybın daha fazla artmadığı ve kalıcı olduğu görüldü. Sonuç olarak, hayvanların sağkalımı, kan şekeri düzeyi ve vücut ağırlıklarındaki değişim değerlendirildiğinde sıçanlarda T1D hastalık modelinin oluşturulması açısından 40 mg/kg STZ optimum doz olarak belirlendi.

4.5. Ad5hTRAIL ile Transdüksiyona Uğratılan Pankreas Adacıkları STZ - İndüklü T1D Sıçanlarda Normoglisemi Süresini Artırdı

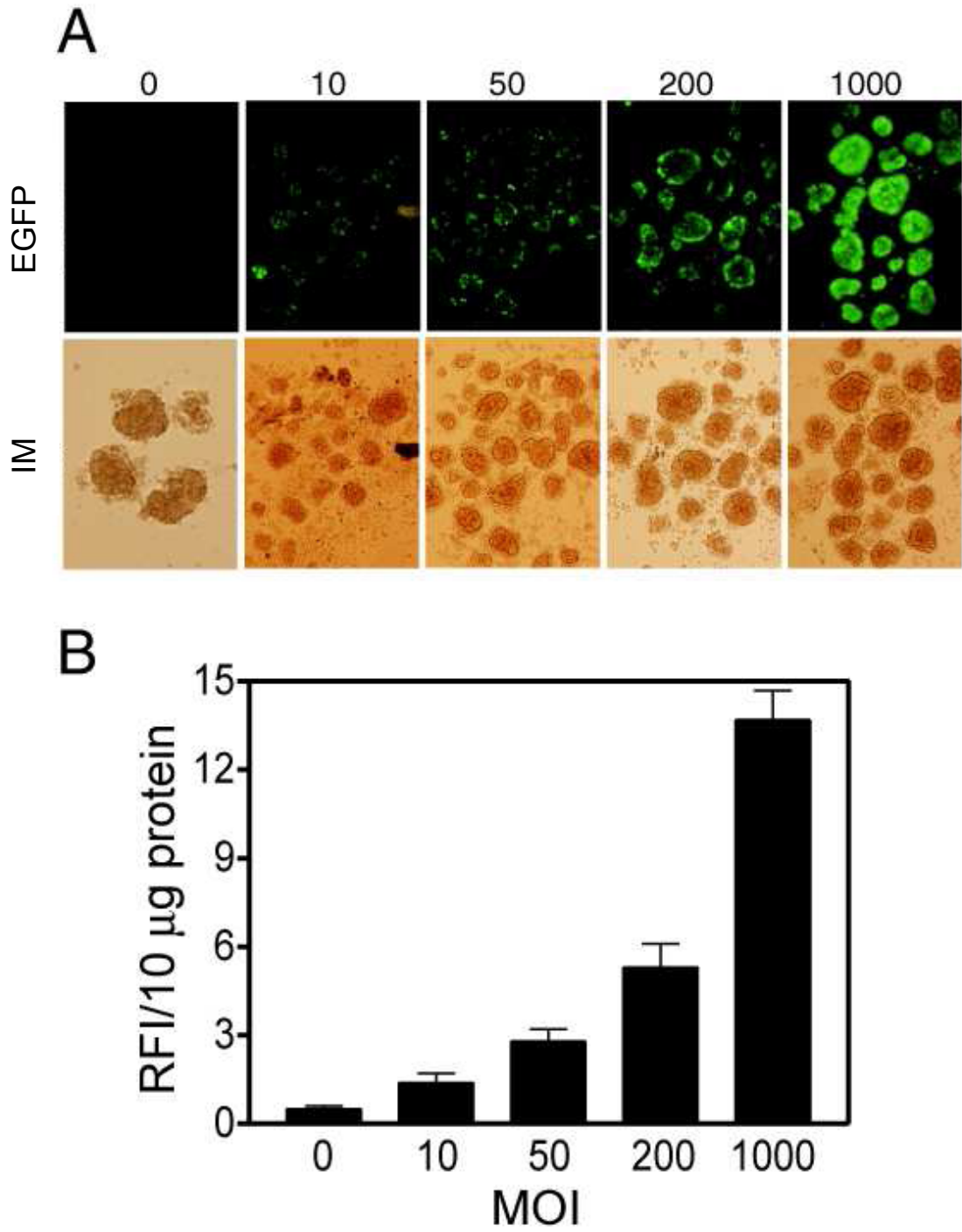
Deneysel koşullarımıza bağlı olarak STZ ile indüklenen diyabetik sıçanlarda normogliseminin sağlanması için en az 750 adacığa (Islet Equivalent (IE)) gerek duyulmaktadır. Bu nedenle pankreatik adacık nakillerinde 750 IE kullanıldı. Sıçan pankreatik adacıkları AdCMVLacZ ya da Ad5hTRAIL (1000 MOI) ile 48 saatliğine enfekte edilerek STZ ile indüklenen diyabetik Wistar sıçanların sol böbrek kapsülünün altına nakledildi (kan şekeri ≥ 300 mg/dl). Nakil sonrasında kan şekeri düzeyleri ilk 10 günde günlük, daha sonra haftalık olarak ölçüldü. Enfekte edilmemiş adacıkların nakledilmesinden 14 gün sonra hayvanlarda normoglisemi sağlanırken bu korumanın kısa süreli olduğu ve sıçanların 32 gün sonra tekrar hiperglisemik olduğu belirlendi (Şekil 4.8). AdCMVLacZ ile enfekte edilen adacıkların nakledildiği diyabetik sıçanların deney süresince hiç normoglisemiye ulaşmadığı gözlemlendi. Buna karşın, Ad5hTRAIL ile transdüksiyona uğratılan adacıkların nakledildiği sıçanların 7 gün sonra normoglisemik olduğu ve bu korumanın 60 gün sürdüğü belirlendi. Normogliseminin nakledilen adacıkların fonksiyonuna bağlı olup olmadığını teyit etmek amacıyla ile sıçanlara 32. günde nefrektomi uygulandı ve 24 saat içerisinde tekrar hiperglisemik (kan şekeri ≥ 300 mg/dl) oldukları saptandı.

4.6. Diyabetik Sıçanlara Nakledilen Pankreas Adacıklarında Ekzojen TRAIL Sentezi Mononükleer Hücre İnfiltrasyonunu Azalttı

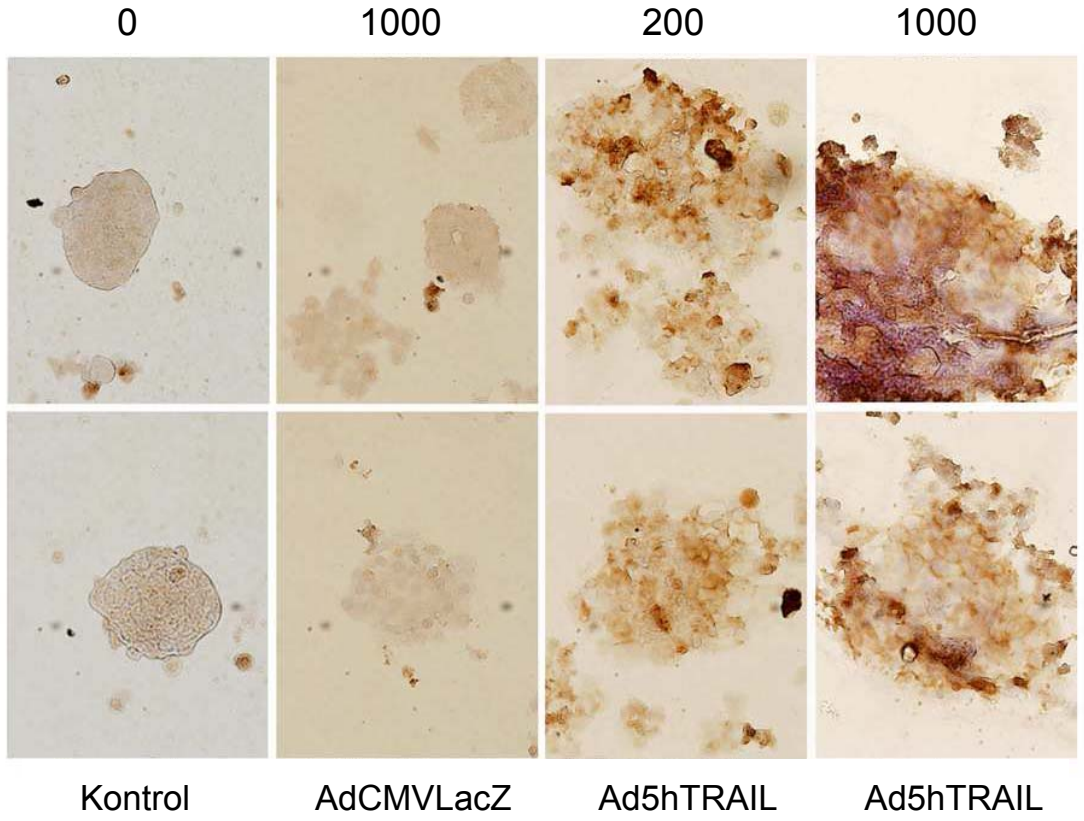
Ad5hTRAIL ile enfekte edilen pankreatik adacık allografları STZ ile indüklenen diyabetik sıçanlarda normoglisemi süresinin artmasını sağladı. Bu sonuçlar ışığında adacık graflarındaki inflamasyon derecesinin belirlenmesi planlandı. Bu amaçla, viral vektörler ile enfekte edilmiş adacıkların naklinden sonra farklı zaman aralıklarında diyabetik sıçanların böbrekleri çıkarılarak immünohistokimyasal analizler yapıldı. Şekil 4.9'da da görüldüğü gibi enfekte edilmemiş ya da AdCMVLacZ ile enfekte edilmiş adacıkların naklinden 15 gün sonra mononükleer hücre infiltrasyonunun başladığı gözlemlendi. 30. güne gelindiğinde her iki grupta da ileri derecede infiltrasyonun olduğu belirlendi. Buna karşın, Ad5hTRAIL ile enfekte edilen adacıkları alan sıçanlarda 15. günde belirlenebilir düzeyde infiltrasyonun olmadığı saptandı. Bunun yanı sıra enfekte edilmemiş ya da AdCMVLacZ ile enfekte edilmiş adacık graflarında mononükleer hücreler yoğun bir şekilde gözlemlendi. Aynı zamanda her üç grup için inflamasyon miktarı da belirlendi. Ad5hTRAIL ile enfekte edilen adacık grafları ile kıyaslandığında, enfekte edilmemiş ya da AdCMVLacZ ile enfekte edilmiş adacık graflarında yüksek derecede insülitis saptandı (Şekil 4.10). Tüm bu sonuçlar değerlendirildiğinde, nakledilen adacıklarda TRAIL sentezinin, STZ ile indüklenen diyabetik sıçanlarda inflamasyon oranını düşürerek normoglisemi süresini arttırdığı ve belirlendi.



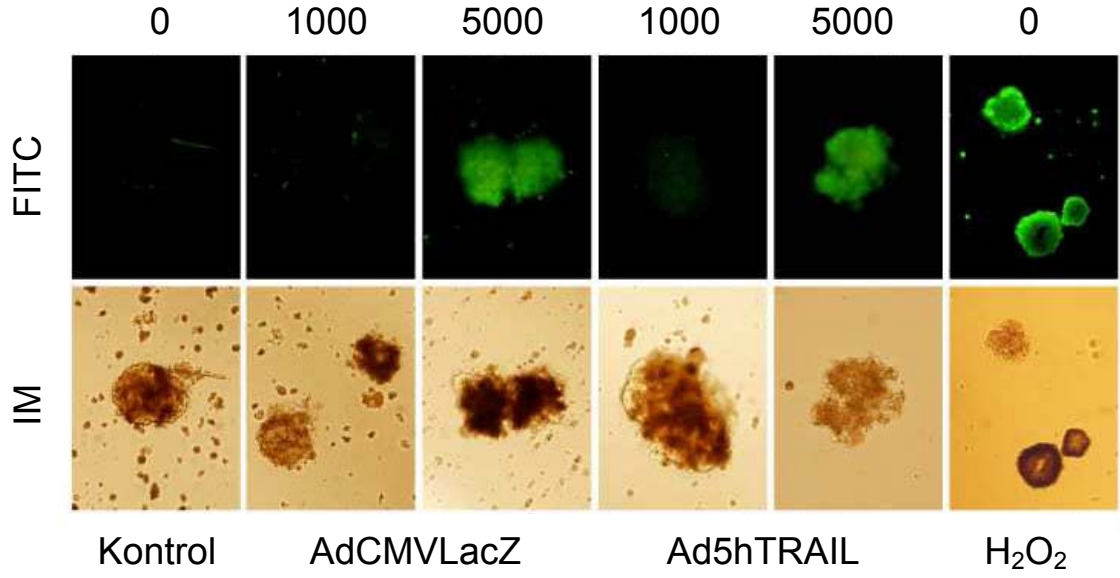
Şekil 4.1. İzole edilen pankreas adacıklarının hücre canlılığı. Pankreatik adacıklarda PI/FDA boyaması gerçekleştirildi (üstte 400x, altta 200x büyütme). Normal mikroskopi (sol), FDA (orta) ve PI (sağ) floresan mikroskopi görüntüleri gösterilmekte. IM= Işık Mikroskobu



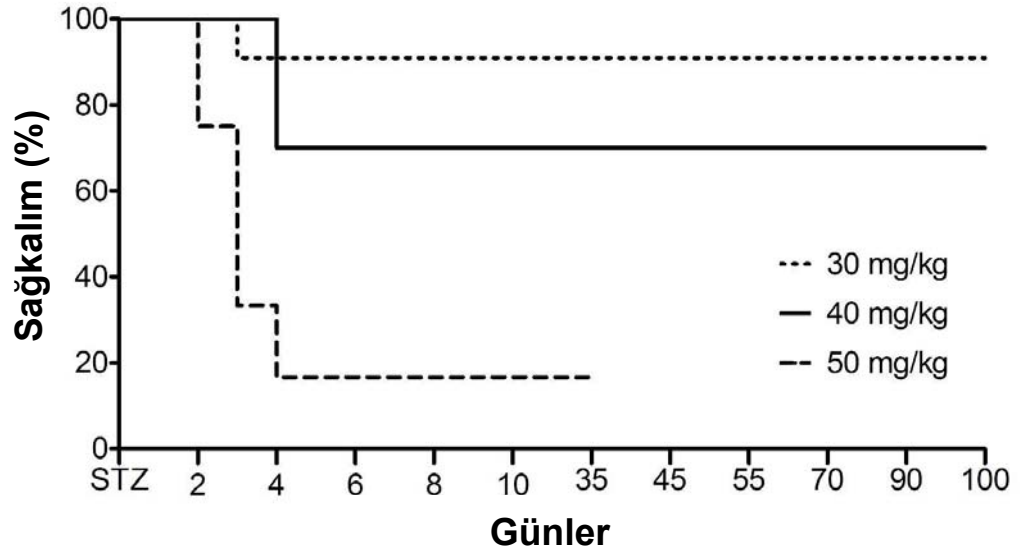
Şekil 4.2. Pankreas adacıklarının AdEGFP ile transdüksiyona uğratılması. Taze izole edilmiş sıçan pankreatik adacıkları AdEGFP vektörü (MOI: 0, 10, 50, 200 ve 1000) ile enfekte edildi. (A) Florasan mikroskopi görüntüleri enfeksiyondan 48 saat sonra elde edilirken, (B) florometrik ölçümler (RFI/10 μ g protein) transdüksiyon seviyesini belirlemek için kullanıldı. Rakamlar DNA partikülü/hücre olarak MOI oranlarını belirtmektedir. IM= Işık Mikroskobu



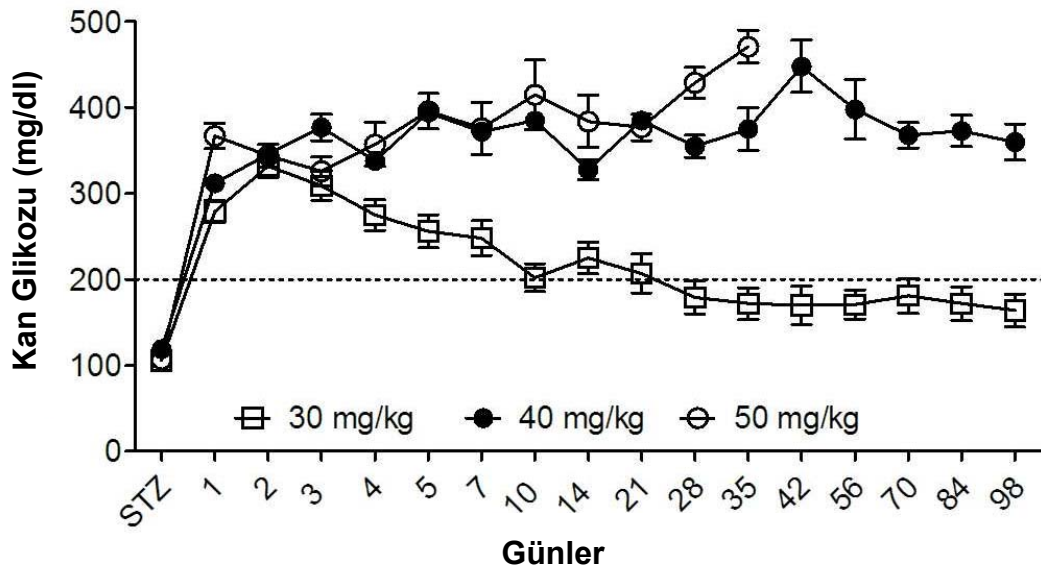
Şekil 4.3. Pankreas adacıklarında TRAIL sentezinin immünohistokimyasal yöntemler ile analizleri. Enfekte edilmemiş (kontrol), AdCMVLacZ (1000 MOI) ve Ad5hTRAIL (1000 MOI) ile enfekte edilen adacıklar enfeksiyonu takiben 48 saat sonra TRAIL sentezi için boyandı. Görüntüler 200x büyütmede alındı.



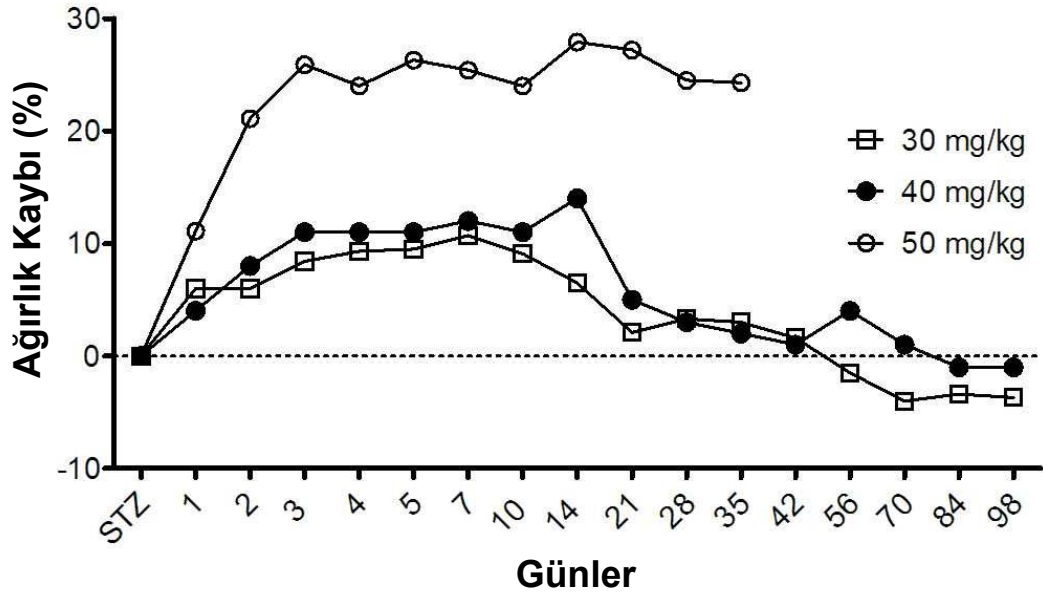
Şekil 4.4. Adenoviral transdüksiyonunun pankreas adacıkları üzerindeki sitotoksik etkisi. Enfekte edilmemiş, AdCMVLacZ ve Ad5hTRAIL ile enfekte edilen adacıklar (1000 ve 5000 MOI) enfeksiyonu takiben 48 saat sonra Annexin V-FITC ile boyandı ve floresan mikroskopi ile analiz edildi. Pozitif kontrol olarak pankreatik adacıklar 1mM H₂O₂ ile muamele edildi. Görüntüler 200x büyütmede alındı. IM= Işık Mikroskobu



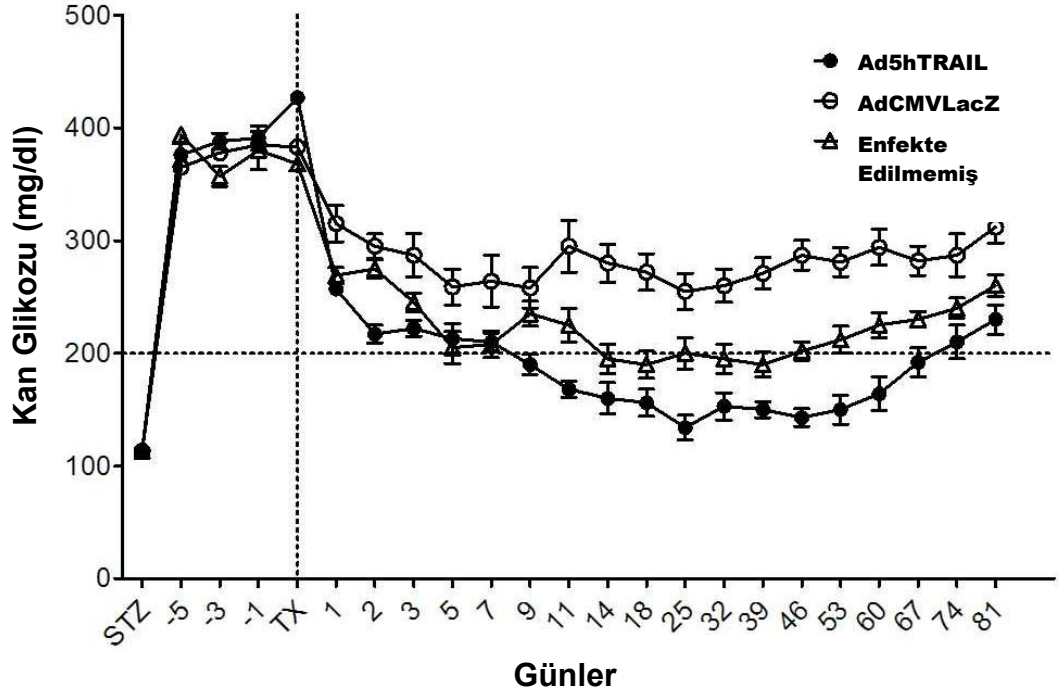
Şekil 4.5. STZ enjeksiyonu sonrası sağkalım oranları. İntraperitonal olarak 30 (n=11), 40 (n=10) ve 50 (n=12) mg/kg STZ enjekte edilen Wistar sıçanları sağkalım oranlarının belirlenmesi için 100 gün süre ile takip edildi.



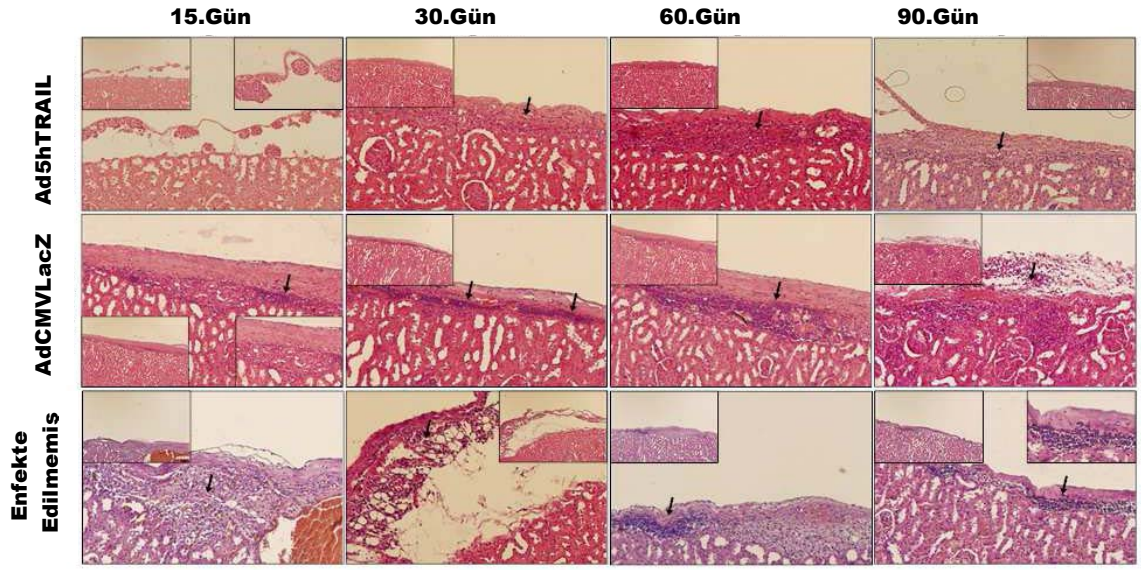
Şekil 4.6. STZ ile indüklenen diyabetik sıçanlarda kan şekeri değişimlerinin gösterilmesi. 30, 40 ve 50 mg/kg STZ enjekte edilen sıçanlarda kan şekeri düzeyleri (mg/dl) glukometre kullanılarak ölçüldü. Değerler, bağımsız verilerin ortalamasını (\pm S.E.M) temsil etmektedir. * $p < 0.05$



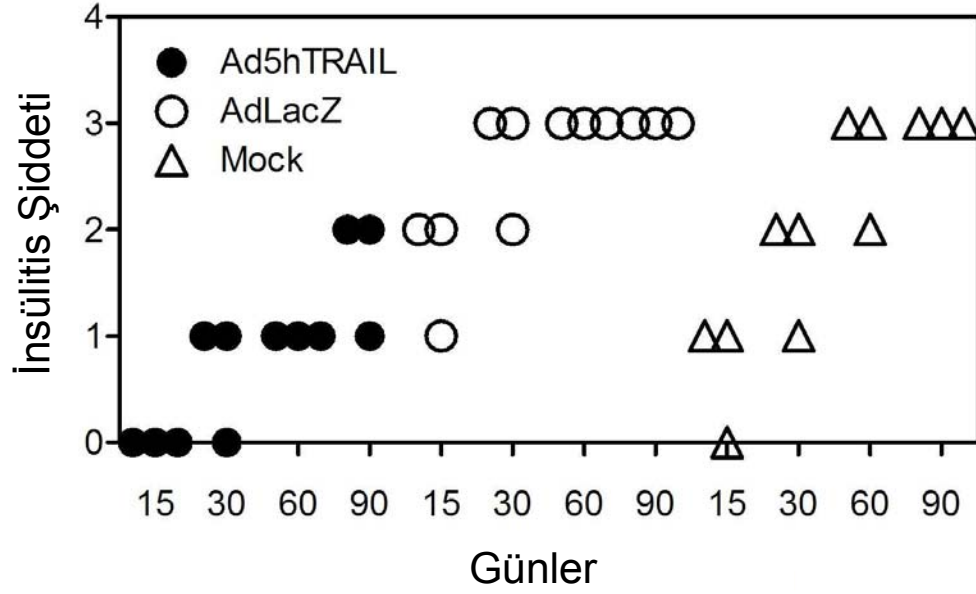
Şekil 4.7. STZ enjeksiyonu sonrası ağırlık kayıpları. 30 ve 40 mg/kg STZ enjekte edilen diyabetik sıçanların ağırlık değişimleri 100 gün süre ile takip edildi. 50 mg/kg STZ enjekte edilen sıçanlar yüksek ölüm oranları nedeniyle sadece 35 gün takip edildi. Değerler, bağımsız verilerin ortalamasını (\pm S.E.M) temsil etmektedir. * $p < 0.05$



Şekil 4.8. Genetik olarak modifiye edilmiş adacık nakillerinin diyabetik sıçanlardaki terapötik sonucu. STZ enjeksiyonundan 7 gün sonra diyabetik sıçanlara AdCMVLacZ (n=10) ve Ad5hTRAIL (n=10) ile enfekte edilmiş ve enfekte edilmemiş 750 adacık nakli gerçekleştirildi. X eksenindeki çizgili hat adacıkların nakledildiği günü, Y eksenindeki çizgili hat ise normoglisemi sınırını göstermektedir (üst üste iki gün kan şekeri düzeyinin ≤ 200 mg/dl olduğu ölçümler). Değerler, en az iki farklı verinin ortalamasını (\pm S.E.M) temsil etmektedir.



Şekil 4.9. Genetik olarak modifiye edilmiş adacık nakilleri sonrasında adacık inflamasyonu. AdCMVLacZ, Ad5hTRAIL ile enfekte edilmiş ve enfekte edilmemiş adacıklar STZ ile indüklenen diyabetik sıçanlara nakledildi. Adacık greftleri nakledilen 15, 30, 60 ve 90 gün sonra mononükleer hücre infiltrasyonları açısından H&E boyaması ile değerlendirildi. Oklar mononükleer hücre birikimlerini işaret etmektedir. Büyük görüntüler 100x büyütmede alınırken, resim içerisindeki küçük görüntüler 200x büyütmede alınmıştır.



Şekil 4.10. İnsülitis skorlamasında dot-plot analizi. Skorlar histokimyasal analizler sonucunda Ad5hTRAIL (koyu daire), AdCMVLacZ (açık daire) ile enfekte edilmiş ve enfekte edilmemiş adacıklar (üçgen) ile nakil edilen sıçanlardan alınmıştır. Her üç grup için belirlenen zamanlarda (15, 30, 60 ve 90) üçer sıçan kurban edildi.

TARTIŞMA ve SONUÇLAR

Tüm diyabetik hastaların % 10'unu oluşturan T1D'li hastaların tedavisi için rutin olarak insülin enjeksiyonu uygulanmaktadır. Fakat bu tedavi yaklaşımı hastaları nefropati, nöropati ve retinopatiden koruyabilirken; hastaların hipoglisemik sendromlara, zaman zaman nöbet ve komaya girmelerini engelleyemez. Pankreas nakli ile hastaların yaşam kalitesini ve yaşam süresini artırsa da, hastaların ciddi bir operasyona maruz bırakılmaları ve uzun süreli immünsüpresif kullanmaları zorunluluğu nedeniyle alternatif tedavi yaklaşımlarına ihtiyaç vardır. Bu alternatif yaklaşımlardan biri olan pankreatik adacık nakli, daha az girişimsel olması ve hastaları rutin insülin kullanımına bağlı olarak gelişen komplikasyonlara karşı koruyabilmesinden dolayı önemli bir avantaj sağlamaktadır.

Pankreatik adacık naklinin ana hedefi, kronik immünsüpresif ilaç tedavisine ihtiyaç duyulmaksızın diyabetik sendromun tamamen iyileştirilmesidir. Fakat uzun süreli adacık-graft fonksiyonu sağlayabilmek için, hem otoimmün hem de alloimmün engellerin üstesinden gelerek graft toleransı sağlanmalıdır. [67]. Adacıkları immün sistemden korumayı başarabilmek amacıyla yapılan deneysel çalışmalarda, adacık nakil bölgesi olarak immün sistemden korunmuş olduğu bilinen bölgeler seçilmiştir. Yapılan çalışmalarda, gerçekte bu bölgelerin, graftı immün sistemden uzak tutarak değil, yüksek oranda FasL sentezleyerek grafta karşı koruma sağlandığı belirlenmiştir[19]. Adacık sağkalımını uzatmak amacıyla denenen başka bir yaklaşım da adacıkların nakilden önce kapsülle kaplanmasıdır[20]. Ancak bu yöntem, kullanılan yabancı materyale karşı yoğun fibröz reaksiyonun gelişmesi ve nakledilen adacıkların en sonunda yıkımına neden olan sitokin aracılı immün cevabın gerçekleşmesi nedeniyle kısıtlanmıştır. Beta hücrelerini immün aracılı yıkımdan korumak için geliştirilen tüm bu yöntemlere rağmen, şimdiye kadar nakledilen beta hücrelerinin kaybı geciktirilebilmiş, fakat sonuçta yıkım yine engellenememiştir. Bu nedenle, T1D hastalarının tedavisine alternatif olarak gen tedavi denemeleri gündeme gelmiştir[68].

T1D tedavisinde kullanılabilir gen tedavisi stratejilerinde temel yaklaşım; tedavi edici genin adacıklara transferi ile adacık graflarının sitotoksik T-hücre saldırısından korunması ve böylece adacık graflarının daha uzun süre ile fonksiyon görmesidir[31]. Bu amaçla, TNF[69, 70] ve FasL[71] gibi TNF ailesi üyelerinin viral vektörlerle pankreatik adacıklara transfer edilmesini içeren birçok yaklaşım test edilmiştir. Örneğin, pankreatik adacıklarda adenovirüs aracılı FasL sentezinin, adacıkları immün saldırılara karşı koruyamadığı, tersine hızlı bir şekilde yıkıma uğrattığı gösterilmiştir[72, 73].

TRAIL, tümör hücrelerinde apoptozu indüklemesine rağmen, normal hücre ve dokulara karşı toksik değildir[38, 43]. Hücrelerin sağkalımı ve ölümü üzerindeki bu farklı etkisi, TRAIL'i diğer TNF ailesi üyelerinden ayırmaktadır. Ayrıca, tümöre karşı olan fonksiyonu dışında TRAIL, immünolojik mekanizmalarda TRAIL aracılı ölümden de önemli bir rol oynamaktadır. Yapılan bir çalışmada, TRAIL sentez ya da fonksiyon yokluğunun, otoimmün diyabet sıklığını ve adacıklarda inflamasyon şiddetini artırdığı gösterilmiştir[35]. Yine başka bir çalışmada, TRAIL ile transdüksiyona uğratan dendritik hücrelerin, antijen spesifik T-hücrelerini baskıladığı ve fareleri akut GvHD'den [Graft versus Host Disease (graftın alıcısı reddi)] ve lösemi relapsından koruduğu bildirilmiştir[74]. Bu bilgiler ışığında, pankreatik adacıklarda TRAIL sentezinin artırılması ile adacık greftlerinin , sitotoksik T-lenfosit saldırısından TRAIL aracılı olarak korunabileceği fikri, bu çalışmanın hipotezini oluşturmaktadır. Hipotezimizi test etmek için, Ad5hTRAIL transdüksiyonu ile sıçan pankreatik adacıklarında TRAIL'in aşırı sentezi sağlandı ve bu adacıklar STZ ile indüklenen diyabetik sıçanların böbrek kapsülleri altına nakledildi. AdCMVLacZ ile enfekte edilen adacıkların nakledildiği sıçanlar normoglisemi sağlamazken, Ad5hTRAIL ile enfekte edilen adacıklara sahip sıçanlarda normoglisemi süresinin arttığı gözlemlendi. Bu sıçanlardaki adacık greftleri değerlendirildiğinde, TRAIL sentezleyen adacıkların nakledildiği sıçanlarda, AdCMVLacZ ile enfekte edilen ya da hiç enfekte edilmemiş adacıkların nakledildiği sıçanlara kıyasla daha az sayıda mononükleer hücre infiltrasyonu olduğu belirlendi. Daha önceki çalışmalarda, TRAIL'in bir immün modülatör olarak rol aldığı iddia edilmesinden sonra, bu modelde TRAIL'in mononükleer hücre infiltrasyonunu baskıladığını ya da geciktirdiğini ileri sürmek oldukça mantıklıdır. Bununla birlikte, NOD farelerde yapılan bir çalışmada, pankreatik adacıklarda transgenik TNF sentezinin, bu adacıklarda şiddetli insülitise neden olduğu ancak diyabet oluşturmadığı belirtilmiştir[75, 76]. Bu sonuçlar, diyabetin indüklenmesinde insülitisin kaçınılmaz olduğunu, ancak tek başına yeterli olamayacağını göstermektedir. Ayrıca söz konusu senaryoda, adacık spesifik T-hücre inhibisyonu, bu fenotipin gözlenmesinden sorumlu tutulmaktadır[77]. Öte yandan, NOD farelerde yapılan bir çalışmada, neonatal dönemde adacık spesifik TNF- α sentezinin, adacık antijen sunumunu artırarak diyabeti tetiklediği bildirilmiştir[78]. Bu çelişkili sonuçlar üzerine, neonatal dönemde değil de, geç dönemdeki adacık spesifik TNF- α sentezinin T1D'teki otoimmün süreci engelleyebildiği düşünülmüştür[79]. Sonuç olarak, T1D gelişim süreci, adacık spesifik TNF- α sentezinin zamanlamasına bağlı olarak değişiklik göstermektedir. Tersine TRAIL'in, lenfositlerin aktivasyonunu ve yayılımını engellediği ve bunu lenfositleri sistemden elimine etmeden gerçekleştirdiği belirtilmiştir[45]. Bu nedenle, TNF ve FasL'in aksine TRAIL, otoimmün yanıtın engellenmesinde çok önemli bir rol üstlenmiş olabilir. Sonuçlarımız aynı zamanda pankreatik adacıklarda adenovirüs aracılı İnterlökin Reseptör Antagonist Protein 1 (IRAP1) aşırı sentezinin, adacık greftlerinde lenfosit

infiltrasyonunu azalttığını belirten bir çalışmayı doğrulamaktadır[80].

Adacık allograftlarının reddedilme süreci her ne kadar hala çözümlenmesi gereken bir problem olarak görünse de, çalışmamızda *ex vivo* adenoviral TRAIL gen transferinin adacık allograft sağkalım süresini artırdığı açık bir şekilde gösterilmektedir. Tüm bu sonuçlar değerlendirildiğinde, gutless (=yardımcı bağımlı) adenoviral ya da Adeno Associated Viral (AAV) vektörler kullanıldığında, adacık sağkalımı ve fonksiyonun da daha uzun süreli terapötik bir etki beklenmektedir[81, 82].

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ÖZGEÇMİŞ

1977 yılında Almanya'nın Bremen kentinde doğan Ercüment DİRİCE, ilköğrenimine Almanya'da başlamış ve Sinop Cumhuriyet İlkokulu'nda tamamlamıştır. 1989-1996 yılları arasında Sinop Anadolu Lisesi'nde ortaokul ve lise öğrenimi görmüştür. 1996 yılında başladığı Akdeniz Üniversitesi Fen-Edebiyat Fakültesi Biyoloji Bölümü'nden, 2000 yılında bölüm birincisi ve fakülte ikincisi olarak mezun olmuştur. Mezuniyetinin hemen arkasından 2000 yılında Akdeniz Üniversitesi Sağlık Bilimleri Enstitüsü 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 Tıbbi Genetik yüksek lisans programına kayıt olmuştur. 2003 yılında yüksek lisans programından mezun olmuş ve aynı Enstitü'nün aynı Anabilim Dalı'nda Tıbbi Genetik doktora programına başlamıştır.

2001-2002 yılları arasında Tubitak Bilim Adamı Yetiştirme Grubu (BAYG) bursuna layık görülmüş, 2003 yılında Schering-Alman Uroonkoloji kongresinde poster sunum 3.lüğü elde etmiştir. 2004 yılında 19. Ulusal Hematoloji Kongresi'nde Genç Bilim Adamı ödülü almış, Mayıs 2007'de yapılan 43. Ulusal Diyabet Kongresi'nde ise poster sunum 2.liğini kazanmıştır. 2009 yılına Ocak ayında Diyabet, Obezite ve Beslenme Derneği tarafından layık görüldüğü Diyabet araştırmaları desteği ile başlayan Ercüment Dirice, Nisan ayında 11. Avrupa Endokrinoloji Kongresi'nde Genç Bilim Adamı Ödülü'ne layık görülmüş, Novartis Bilim Ödülü'nü ve 6. Metabolik Sendrom Sempozyumunda Poster sunum 1.liğini kazanmıştır. Doktora sonrası için pankreas adacıklarında hücre rejenerasyonu ile ilişkili yazdığı proje 2009 Ocak ayında Harvard Üniversitesi'nce kabul edilmiş ve araştırmalarına Harvard Üniversitesi, Joslin Diyabet Merkezi'nde devam edecektir.

Ekler

High TRAIL Death Receptor 4 and Decoy Receptor 2 Expression Correlates With Significant Cell Death in Pancreatic Ductal Adenocarcinoma Patients

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Objectives: The importance of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) and TRAIL receptor expression in pancreatic carcinoma development is not known. To reveal the putative connection of TRAIL and TRAIL receptor expression profile to this process, we analyzed and compared the expression profile of TRAIL and its receptors in pancreatic tissues of both noncancer patients and patients with pancreatic ductal adenocarcinoma (PDAC).

Methods: Thirty-one noncancer patients and 34 PDAC patients were included in the study. TRAIL and TRAIL receptor expression profiles were determined by immunohistochemistry. Annexin V binding revealed the apoptotic index in pancreas. Lastly, the tumor grade, tumor stage, tumor diameter, perineural invasion, and number of lymph node metastasis were used for comparison purposes.

Results: TRAIL decoy receptor 2 (DcR2) and death receptor 4 expression were up-regulated in PDAC patients compared with noncancer patients, and the ductal cells of PDAC patients displayed significant levels of apoptosis. In addition, acinar cells from PDAC patients had higher DcR2 expression but lower death receptor 4 expression. Increased DcR2 expression was also observed in Langerhans islets of PDAC patients.

Conclusions: Differential alteration of TRAIL and TRAIL receptor expression profiles in PDAC patients suggest that the TRAIL/TRAIL receptor system may play a pivotal role during pancreatic carcinoma development.

Key Words: TRAIL, pancreas, ductal adenocarcinoma, cell death

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Pancreatic cancer (PC) is the fourth leading cause of cancer-related death, with an overall 5-year survival rate of 4% after diagnosis.¹ Of 31,860 newly diagnosed PCs, 31,270 patients died in 2004 as reported by the American Cancer Society. Pancreatic ductal adenocarcinoma (PDAC) is a highly malignant and invasive form of PC,² as the median survival rate of PDAC patients is less than 6 months.³ At the time of

diagnosis, ~40% of patients with PC have metastatic disease, 40% to 50% have locally advanced stage disease that is not amenable to surgery, and only ~10% of patients can go through a potentially curative resection.⁴ Unfortunately, the result of surgery alone is relatively poor, with an ~80% rate of local or distant recurrence, and the 5-year survival rate for cases involving total resection is only 10% to 24%.^{5–8}

Because of the high mortality rate in PDAC, novel treatment modalities, such as gene therapy, are being explored to develop effective alternative treatments for PDAC patients. One of the therapeutic genes, which has currently been evaluated in the context of gene therapy, is tumor necrosis factor–related apoptosis-inducing ligand (TRAIL).⁹ TRAIL is a type II membrane protein that can bind to 5 different receptors: TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR1), TRAIL-R4 (DcR2), and osteoprotegerin.¹⁰ DR4 and DR5 are the death receptors that signal for apoptosis, whereas DcR1, DcR2, and osteoprotegerin are considered antagonistic because they are unable to induce such signaling because of the lack of intracellular death domain or are secreted molecules.¹¹ In comparison to the other death-inducing members of the tumor necrosis factor family (Fas ligand and tumor necrosis factor), TRAIL has discrete apoptosis-inducing properties on cancer cells. In particular, TRAIL is a potent inducer of tumor cell apoptosis¹² but is nontoxic to normal cells and tissues.^{13,14} Interestingly, an adenovirus vector encoding the TRAIL cDNA (Ad-TRAIL) efficiently killed pancreatic tumors in vitro and in vivo.¹⁵ Furthermore, systemic administration of Ad-TRAIL suppressed pancreatic tumor growth in the liver.¹⁶ Despite these encouraging results, the mechanism(s) that regulate the TRAIL-mediated signaling cascade is not well understood,^{17,18} and there is significant effort investigating why more than 50% of human tumors are TRAIL resistant.¹⁹ For example, analysis of PDAC cell lines revealed variable degrees of TRAIL sensitivity due to TRAIL decoy receptor gene expression.²⁰

Resistance to TRAIL-induced apoptosis can occur at various levels in the TRAIL signaling cascade. High DcR2 expression was recently correlated with TRAIL resistance in breast, prostate, and lung cancer cell lines,^{21–23} and siRNA strategy targeting DcR2 sensitized both lung and prostate cancer cells to TRAIL.^{23,24} Intriguingly, PC cells differentially express DcR1 and DcR2,²⁵ and DcR2 overexpression mediates TRAIL resistance in PC cells.²⁶ In accordance with this, the lack of TRAIL death receptor (DR4 or DR5) gene expression was also implicated in TRAIL resistance in PC cell lines.²⁷ Thus, TRAIL and TRAIL receptor expression profiles in PC patients may predict the feasibility of using TRAIL gene therapy as a treatment option.

In addition, the lack of early detection systems and inherent resistance of PDAC to all known conventional treatment modalities have contributed to the high mortality rate observed in PDAC.²⁸ Because the overexpression of transforming growth

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factor β type II receptor in PDAC patients has been associated with decreased survival,²⁹ the evaluation of novel markers was suggested for an early detection and follow-up of PC patients.³⁰ One of the potential markers useful for the follow-up of PDAC patients may be TRAIL.³¹ However, it is unclear how the TRAIL/TRAIL receptor system contributes to carcinogenesis. Benign and malignant prostate cancer cells differentially display TRAIL and its receptors, and this profile was connected to prostate carcinogenesis.³² In addition, high DcR2 expression is correlated with high Gleason scores, prostate-specific antigen recurrence, and decreased survival in patients with prostate carcinoma.³³ Lastly, DR4 expression positively correlated with tumor grade in breast cancer patients with invasive ductal carcinoma.³⁴ These studies suggest that the expression of TRAIL and its receptor in non-PDAC versus PDAC patients can provide useful information on the development of PDAC. Ultimately, we believe that understanding the expression profile of TRAIL and the TRAIL receptors in PDAC patients may elucidate a potential mechanism of pancreatic carcinoma.

MATERIALS AND METHODS

Immunohistochemistry Procedure Using Antibodies Developed Against TRAIL/TRAIL Receptors on Pancreas

Hematoxylin counterstaining was performed on all pancreatic tissue sections as described previously.^{23,32,34} The following primary antibodies (Alexis Biochemicals, Lausen, Switzerland) were used at 1:300 dilution for the staining of pancreatic tissues: anti-human TRAIL (III6F; ALX-804-326-C100), anti-human DR4 (HS101; ALX-804-297A-C100),

anti-human DR5 (ALX-210-743-C200), anti-human DcR1 (ALX-210-744-C200), and anti-human DcR2 (HS402; ALX-804-299A-C100). Pancreatic tissue samples that were stained only with the secondary antibody were used as negative controls.

Quantitative Assessment of TRAIL and TRAIL Death-Decoy Receptor Expressions for Immunohistochemical Scoring

Tissue sections were analyzed by a single pathologist (O.E.) with no prior knowledge of the patient status or antibodies used. The calculation of the final immunohistochemical staining scores in pancreatic tissues included both intensity and marker distribution (percentage of the positively stained epithelial cells). The intensity of the pancreatic tissue staining was assessed as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. Moreover, marker distribution was calculated as 0, less than 10%; 1, 10% to 40%; 2, 40% to 70%; and 3, more than 70% of the epithelial cells stained on the sections. Summing the scores of both the intensity and the marker distribution for a given patient resulted in the final immunostaining score.

Detection of Apoptotic Cells With Annexin V

Paraffin-embedded tissues were sectioned at 4- μ m thickness. Antigen retrieval was accomplished by boiling samples in a solution containing 0.01 M citrate buffer for 20 minutes after the deparaffinization and dehydration processes. The samples were then treated with proteinase K for 10 minutes. An annexin V fluorescent microscopy kit (BD Pharmingen, San Diego, Calif) was used to identify apoptotic cells in the pancreas. Pancreas sections were washed with 1 \times phosphate-buffered saline, followed by 1 \times annexin V-binding buffer (BD Pharmingen). Pancreatic tissue sections were then treated with annexin

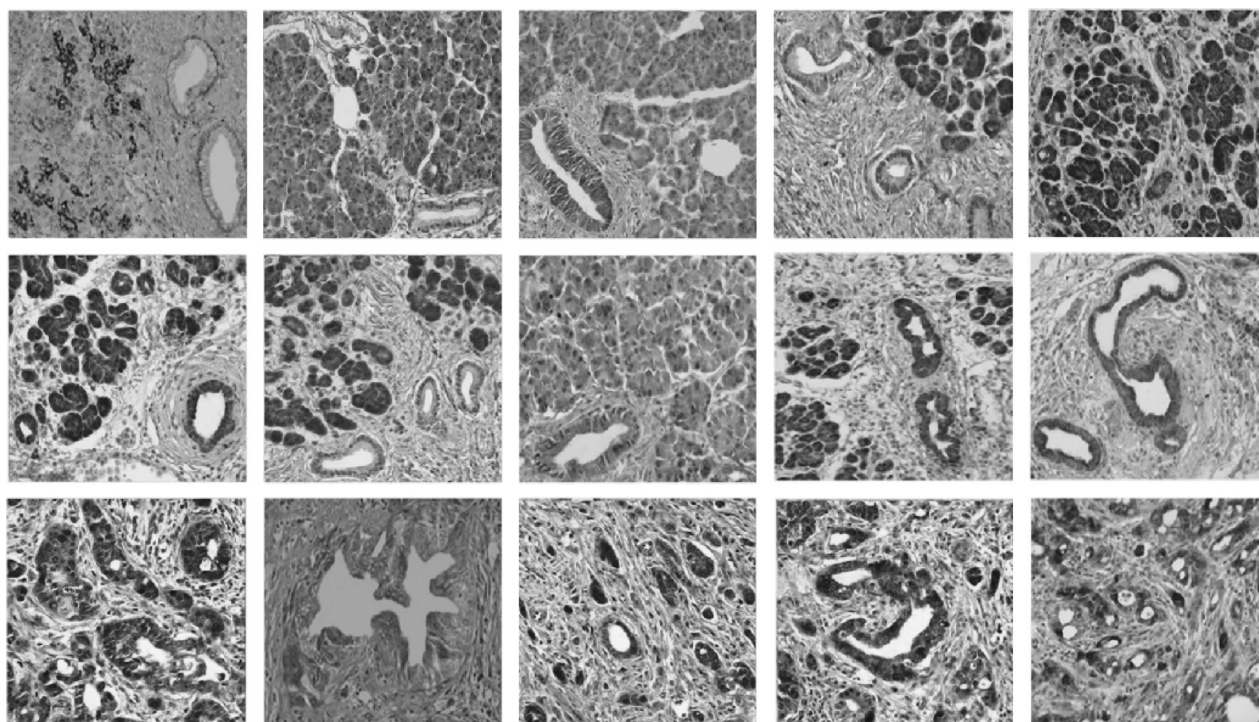


FIGURE 1. Immunohistochemical staining of TRAIL and TRAIL receptors in noncancer patients ($n = 31$) versus patients with PDAC ($n = 34$). Representative images are provided from pancreatic ductal region of noncancer patients (upper panels), normal ductal region of cancer patients (middle panels), and from PDAC tissues (lower panels). TRAIL and TRAIL receptor subtypes are listed above each image, and each image represents a single patient.

V-fluorescein isothiocyanate (FITC) diluted 1:10 in 1× annexin V-binding buffer for 15 minutes at room temperature. Annexin V-FITC-stained cells were analyzed under fluorescent microscopy after washing samples in annexin V-binding buffer. To determine the extent of apoptosis in each section, positive and negative cells were counted in randomly selected high-power fields of acinar, ductal, or islet cells (area of each field is ~0.06 mm²). The apoptotic index was calculated as the percentage of annexin V-positive cells based on the ratio of annexin V-stained cells to the total number cells counted.

Statistical Analysis

Statistical analyses were performed using SPSS 13.0 software for Windows (SPSS Inc, Chicago, Ill). Statistical significance was considered at 5% probability level ($P < 0.05$). The SEM is displayed as error bars for all data points in all of the figures.

RESULTS

Clinical Assessment of Patients With PDAC

Pancreatic tissue samples from 34 patients with pancreatic ductal carcinoma and 31 patients without PC (as a control group) were evaluated. The median age of PC patients was 55 years, ranging from 1 to 80 years, whereas the median age of patients without the PC was 54 years, ranging from 36 to 74 years. All of the patients were clinically staged according to the American Joint Committee on Cancer guidelines. Based on this staging system, 3 cases (8.8%) were T1, 10 cases (29.4%) were T2, 11 cases (32.4%) were T3, and 10 cases (29.4%) were T4. Thirteen patients (38%) had well-differentiated tumors, whereas 17 (50%) had moderate levels of differentiation. Only 4 cases (12%) had poorly differentiated pancreatic tumors. Perineural invasion was observed in 13 patients (38%), and 8 (24%) of the PC patients displayed lymph node metastasis. Whipple operation (R0/R1) was performed in 18 patients. Biopsies (R2) were taken from 16 patients. Both definitive and adjuvant external radiotherapy at a median dose of 50.4 Gy in 28 fractions was delivered to the primary region and to the lymphatics. Twenty-nine patients concurrently received 5-fluorouracil-based che-

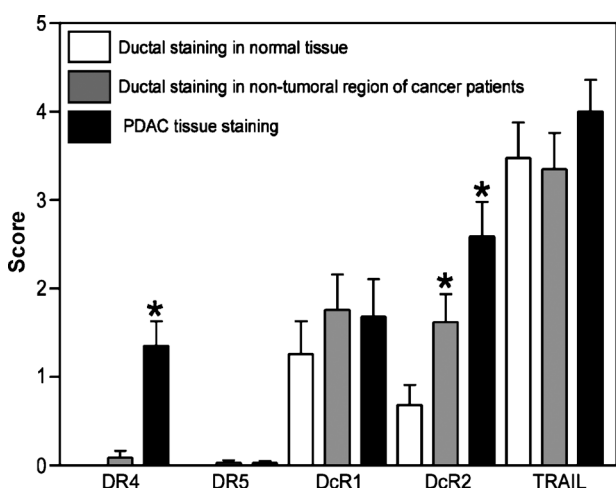


FIGURE 2. Quantitative analysis of the immunohistochemical ductal staining of non-PDAC versus PDAC patients. Immunohistochemical scoring (mean ± SEM) was performed as described in the Materials and Methods using the indicated antibodies. Asterisk indicates statistically significant differences among the 3 groups.

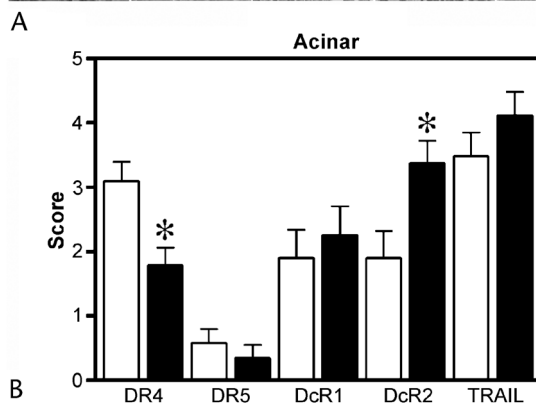
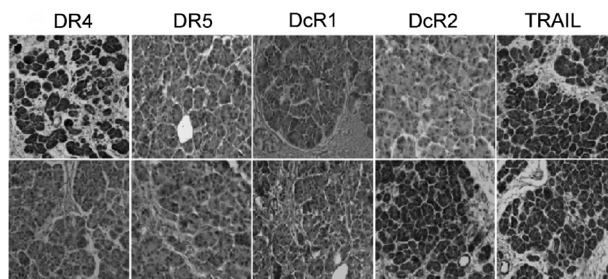


FIGURE 3. Acinar staining patterns of TRAIL and TRAIL receptors in pancreatic tissues of non-PDAC (upper panels) versus PDAC patients (lower panels). A, Representative images of immunohistochemical staining, where each image is taken from a single patient. B, Quantitative analyses of the immunohistochemical staining (mean ± SEM) of pancreata dissected from non-PDAC patients are displayed in open bars, whereas those for pancreata of PDAC patients are shown in solid bars. Asterisk indicates statistically significant differences between the 2 groups.

motherapy. Neoadjuvant treatment was used only in 11 of these patients.

High DR4 and DcR2 Expressions Were Detected in Patients With Pancreatic Adenocarcinoma

Patients were divided into 2 groups based on the presence or absence of tumor in the pancreas (PDAC). In addition, the PDAC patients were further subdivided into 2 groups based on immunohistochemical staining of the tumor itself or the surrounding nontumoral pancreatic ductal tissue. Representative images of pancreatic ductal staining from different patient groups are shown in Figure 1. Whereas TRAIL, DcR1, and DcR2 expressions were clearly detectable in the pancreatic ductal tissue of non-PC patients and the nontumoral ductal region of the PC patients, DR4 and DR5 expressions were not readily detectable in these cases. In contrast, tumor tissues of PDAC patients expressed TRAIL and all 4 TRAIL receptors. Furthermore, TRAIL was the highest marker expressed in PDAC tissue sections. Kolmogorov-Smirnov test ($n = 99$) was used to determine whether the patient groups were normally distributed. Because a Gaussian distribution was not detected, the statistical difference among the groups was determined using the Kruskal-Wallis test. The groups were then paired for comparison using the Mann-Whitney U test. Quantitative analysis of the immunohistochemical staining suggested that both DR4 and DcR2 expressions were up-regulated in PDAC patients compared with nonneoplastic pancreatic ductal tissues of the same patients or noncancer patients (Fig. 2). It is

interesting to note that nonneoplastic pancreatic ductal tissues of cancer patients displayed intermediate levels of DR4 and DcR2 expressions compared with pancreatic ductal tissues of non-cancer patients or PDAC sections.

Patients With PDAC Displayed Increased DcR2 Expression in the Acinar Cells Compared With Nontumor Patients

Acinar cell immunohistochemical staining for TRAIL and the TRAIL receptors were also compared between patients with or without PDAC. As shown in Figure 3A, the death and decoy TRAIL receptors were expressed in the acinar cells of the pancreas in patients with or without PDAC. The quantitative expression profiles of the each molecule are shown in Figure 3B. First, the normality of the groups was tested by Shapiro-Wilk method. Because neither group displayed a Gaussian distribution, the Mann-Whitney *U* test was used to determine the statistical significance between the 2 groups. Whereas lower DR4 expression and higher DcR2 expression were detected in the acinar cells of PDAC patients compared with noncancer patients, TRAIL was expressed the highest in acinar cells of patients with or without PDAC.

High DcR2 Expression Was Observed in Langerhans Islets of the Patients With Pancreatic Adenocarcinoma Versus Noncancer Patients

We next measured TRAIL and TRAIL receptors expression on the Langerhans islets. Although DR4 and DR5 were not

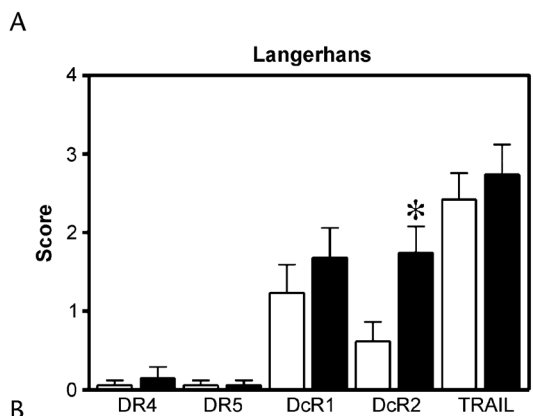
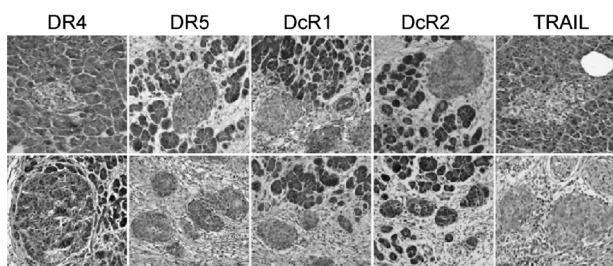


FIGURE 4. Langerhans islet staining of TRAIL and TRAIL receptors in pancreatic tissues of non-PDAC (upper panels) versus PDAC patients (lower panels). A, Representative images of immunohistochemical staining, where each image is taken from a single patient. B, Quantitative analysis of immunohistochemical staining (mean ± SEM), with open bars representing non-PDAC patients and solid bars representing patients with PDAC. Asterisk indicates a significant difference between the 2 groups of patients.

TABLE 1. Correlation Between TRAIL and TRAIL Receptor Expression in PDAC Patients as Determined by Spearman ρ Correlation Test

Spearman ρ Correlations		DR4	DR5	DcR1	DcR2	TRAIL
DR4	Correlation coefficient	1.000	-0.154	-0.130	0.065	0.069
	<i>P</i> (2-tailed)	—	0.384	0.465	0.714	0.698
	n	34	34	34	34	34
DR5	Correlation coefficient	-0.154	1.000	0.311	0.284	0.228
	<i>P</i> (2-tailed)	0.384	—	0.074	0.103	0.194
	n	34	34	34	34	34
DcR1	Correlation coefficient	-0.130	0.311	1.000	0.170	0.427*
	<i>P</i> (2-tailed)	0.465	0.074	—	0.335	0.012
	n	34	34	34	34	34
DcR2	Correlation coefficient	0.065	0.284	0.170	1.000	0.090
	<i>P</i> (2-tailed)	0.714	0.103	0.335	—	0.613
	n	34	34	34	34	34
TRAIL	Correlation coefficient	0.069	0.228	0.427*	0.090	1.000
	<i>P</i> (2-tailed)	0.698	0.194	0.012	0.613	—
	n	34	34	34	34	34

*Correlation is significant at the 0.05 level (2-tailed).

clearly detectable because of low expression, both DcR1 and DcR2, as well as TRAIL, were readily detectable in the pancreatic islets of both patient groups (PC patients vs non-PC patients; Fig. 4A). In addition, TRAIL was expressed the highest compared with the TRAIL receptors in Langerhans islets. Because neither of the patient groups exhibited a Gaussian distribution as tested by the Shapiro-Wilk method, the Mann-Whitney *U* test was performed to determine statistical significance between the 2 groups. Patients with PDAC expressed statistically higher amounts of DcR2 on Langerhans islets compared with patients without the tumor.

DcR1 and TRAIL Expression Were Positively Correlated in Patients With Pancreatic Adenocarcinoma

The Spearman ρ correlation test was next used to test any correlation among the TRAIL markers in PDAC patients. Table 1 shows that only DcR1 and TRAIL expressions were positively correlated in these patients. No such correlation was detected when tumor grade, tumor stage, tumor diameter, perineural invasion, number of lymph node metastasis were taken into account (data not shown).

PDAC Tissues Showed Increased Apoptosis Compared With Pancreata of Noncancer Patients

The presence of apoptotic cells in the pancreas and their correlation to TRAIL and TRAIL receptor expression were analyzed using FITC-conjugated annexin V. Although the fluorescent microscopic images in Figure 5 display annexin V-FITC-stained cells, a quantitative assessment of cell death is provided in Figure 6. Among the tissue sections analyzed, both nontumoral and tumoral ductal region of pancreas in PDAC patients exhibited increased apoptosis compared with pancreata of noncancer patients. However, no correlation was detected between TRAIL marker expression and the amount of cell death

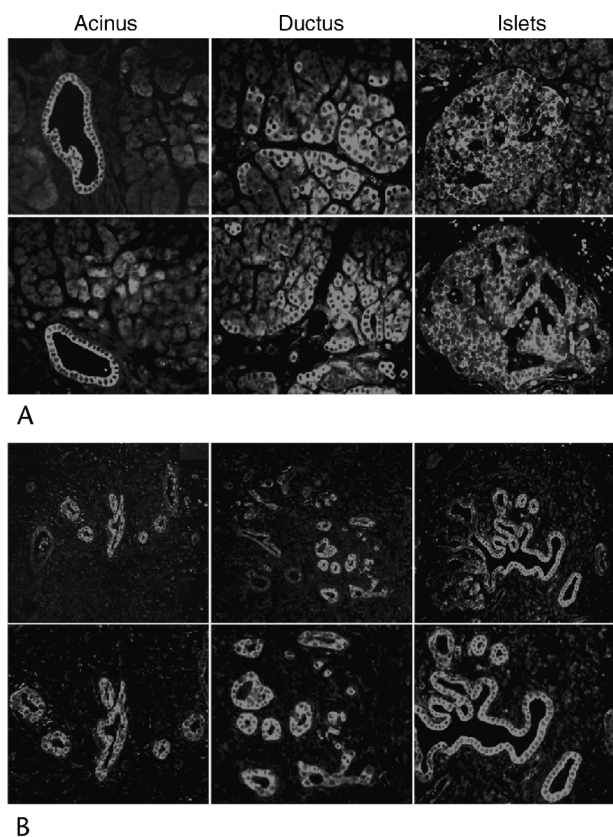


FIGURE 5. Assess of apoptosis in the pancreas by annexin V-FITC binding. Quantification of apoptotic cells was performed as described in Materials and Methods. A, Representative images (original magnification $\times 200$) of acinar (left), ductal (middle), and islet cells (right). Normal pancreas tissue sections are in the upper row, whereas nontumoral regions of pancreatic tissue dissected from PC patients are in the lower row. B, Annexin V-FITC staining of 3 different PDAC cases (original magnification $\times 200$). Images in the lower panels are taken at high-power magnification (original magnification $\times 400$) of the same fields.

observed in pancreatic ductal cells of PDAC patients (data not shown). Conversely, islet, but not ductal or acinar, cell death correlated with high TRAIL expression as revealed by Spearman ρ correlation test in PDAC patients (Table 2). Annexin V-binding assay results were also confirmed using TUNEL assay (data not shown).

DISCUSSION

Because PDAC is resistant to conventional treatment methods and exhibits high mortality rates, novel treatment modalities are needed to improve survival rates of PDAC patients. Furthermore, there is also a necessity to discover new PC tumor markers for both diagnostic and prognostic purposes. Although adenovirus delivery of TRAIL effectively kills pancreatic tumor cell lines in vitro and in vivo,¹⁵ high TRAIL decoy receptor expression²⁰ and TRAIL-mediated nuclear factor κ B activity³⁵ were implicated for TRAIL resistance in PDAC. Thus, it is important to know the in vivo expression profiles of TRAIL and the TRAIL receptors in patients with PDAC. Previously, the expression of TRAIL and the 4 TRAIL receptors were determined by reverse transcriptase-polymerase chain reaction in 17 cases of PDAC and 5 cases of normal pancreatic

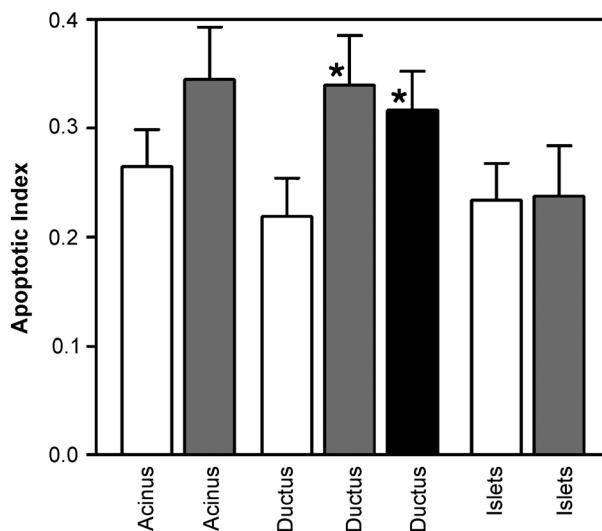


FIGURE 6. Quantitative analysis of apoptotic index. Apoptotic scores (mean \pm SEM) of pancreas sections of noncancer patients (open bars), nontumoral regions of PC patients (shadowed bar), and PDAC tissues (solid bars) were determined. * $P < 0.05$.

tissues.³⁶ Both normal pancreata and pancreata of PDAC patients displayed varying degrees of TRAIL and TRAIL receptor expression. Although reverse transcriptase-polymerase chain reaction is a useful method to detect the presence or absence of gene expression, mRNA expression does not necessarily correlate with the protein expression in the cell or on the cell surface. The expressions of TRAIL, DR4, and DR5 were analyzed in another study involving 10 non-PC and 11 PC patients.³⁷ It was found that despite the expression of TRAIL, DR4, and DR5, the PC cells displayed low sensitivity to TRAIL-mediated apoptosis compared with Jurkat T-lymphoma cells. However, this study excluded the analysis of TRAIL decoy receptor gene expression.

In the data described herein, immunohistochemistry analyses were used to determine the tissue distribution pattern

TABLE 2. Correlation of Apoptotic Islet Cells With TRAIL and TRAIL Receptor Profiles in Pancreas

Annexin V Staining		Islets
DR4	Correlation coefficient	0.228
	<i>P</i> (2-tailed)	0.194
	<i>n</i>	34
DR5	Correlation coefficient	0.283
	<i>P</i> (2-tailed)	0.105
	<i>n</i>	34
DcR1	Correlation coefficient	0.317
	<i>P</i> (2-tailed)	0.068
	<i>n</i>	34
DcR2	Correlation coefficient	0.139
	<i>P</i> (2-tailed)	0.434
	<i>n</i>	34
TRAIL	Correlation coefficient	0.389*
	<i>P</i> (2-tailed)	0.023
	<i>n</i>	34

*Correlation is significant at the 0.05 level (2-tailed).

and expression of TRAIL and the 4 TRAIL receptors as a complete set in both non-PDAC ($n = 31$) and PDAC patients ($n = 34$). Our results indicate that TRAIL expression was highest on average compared with the expression profiles of TRAIL death and decoy receptors particularly in PDAC tissues. As recently suggested, high TRAIL expression may be important for the protection of tumor cells from attacking inflammatory cells.^{20,37} In accordance with this observation, TRAIL enhanced the metastatic spread of orthotopically transplanted human PDAC cells in severe combined immunodeficient mice.³⁸ Thus, high TRAIL expression on cancer cells might be beneficial for metastasis considering immune-protection and invasion scenarios. In addition, high DcR2 expression in PDAC tissues compared with nonneoplastic ductal cells may also allow these cells to escape from TRAIL-mediated apoptosis. Although the functional consequence of increased DR4 expression in PDAC tissues is currently not known, PDAC cells displayed resistance to TRAIL-mediated apoptosis despite high levels of death receptor expression.³⁷

In addition, acinar cells of PDAC patients expressed lower DR4, but higher DcR2 expression, suggesting that not only PDAC cells but also acinar cells of PDAC patients have developed a mechanism to escape apoptosis compared with patients without cancer. Apart from the exocrine constituent, Langerhans islets are the discrete units of endocrine compartment of the pancreas. The failure of pancreatic beta cell function due to autoimmune destruction mediated by islet-reactive T cells results in type 1 diabetes. The expression profiles of TRAIL and the TRAIL receptors were also analyzed in Langerhans islets of non-PDAC versus PDAC patients. The Langerhans islets from both non-PDAC and PDAC patients expressed considerable TRAIL, DcR1, and DcR2, but only the low expressions of DR4 and DR5 were detected in both cases. Interestingly, increased TRAIL expression was observed in the infiltrating immune cells of pancreatic islets in patients with type 1 diabetes.³⁹ Thus, under normal circumstances, Langerhans islets are expected to be protected from the immune-mediated attacks through TRAIL expression and from death ligand-mediated apoptosis by way of decoy receptor expression.⁴⁰ Because Langerhans islets of PDAC patients exhibited higher DcR2 expression compared with noncancer patients, it would be interesting to see if these patients are more resistant to developing type 1 diabetes compared with noncancer patients.

Spearman ρ correlation test suggested the existence of a positive correlation between TRAIL and DcR1 expression in PDAC patients. Although increasing the level of TRAIL decoy receptor expression might be necessary for protection from T cell-mediated attacks, TRAIL overexpression might endanger tumor cell survival because it would activate apoptotic pathways. One of the ways to counteract the action of TRAIL is to up-regulate decoy receptor expression on surface. DcR1 expression blocks TRAIL-mediated apoptosis by acting as a decoy receptor.⁴¹ Thus, PDAC cells might be protected from the side effects of TRAIL overexpression through up-regulation of TRAIL decoy receptor expression.

Annexin V-binding indicated that pancreatic ductal tissues of PDAC patients exhibited increased apoptosis compared with pancreata of noncancer patients. It is interesting to note that PDAC tissues also displayed increased DR4 expression. Although these cells also had increased DcR2 expression, the up-regulation of DcR2 was not sufficient to protect PDAC cells from apoptosis. In addition, although there was lower DR4 expression and higher DcR2 expression seen in acinar cells of PDAC patients, there was no difference in the apoptotic

index detected between the 2 groups. Similarly, although there was higher DcR2 expression detected in islet cells of PDAC patients compared with noncancer patients, annexin V-FITC staining did not indicate any difference in apoptosis between the 2 patient groups. Islets of PDAC patients exhibited less apoptosis compared with acinar cells of the same patient group. This can be explained by the DR4 expressed on acinar cells of PDAC patients, which was expressed higher than that seen on islets. Nevertheless, similar to the observation reported before in noncancer patients,³¹ TRAIL expression displayed a positive correlation with increased cell death in PDAC patients.

In conclusion, the TRAIL and TRAIL receptor expression profile may play critical roles during pancreatic carcinoma development by way of modulating apoptotic cell death.

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High Levels of Endogenous Tumor Necrosis Factor–Related Apoptosis–Inducing Ligand Expression Correlate With Increased Cell Death in Human Pancreas

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Objectives: Type 1 diabetes (T1D) has been characterized by the T cell–mediated destruction of pancreatic β cells. Although various members of the tumor necrosis factor (TNF) family, such as Fas ligand or TNF, have recently been implicated in the development of T1D, the lack of TNF-related apoptosis–inducing ligand (TRAIL) expression or function facilitates the onset of T1D. Thus, the goal of the present study was to investigate the expression profiles of TRAIL and its receptors in human pancreas.

Methods: Pancreata of 31 patients were analyzed by immunohistochemistry using antibodies developed against TRAIL and its receptors. Apoptosis was confirmed by Annexin V–fluorescein isothiocyanate binding and terminal deoxynucleotidyl transferase–mediated 2′-deoxyuridine 5′-triphosphate nick end labeling assays.

Results: Acinar cells displayed high levels of TRAIL and death receptor 4, but only low levels of death receptor 5. In contrast, only TRAIL and TRAIL decoy receptors (DcR1, DcR2) were detected in ductal cells. Similarly, Langerhans islets expressed only TRAIL and TRAIL decoy receptor. High levels of TRAIL expression in pancreas correlated with increased number of apoptotic cells.

Conclusions: Although the expression of TRAIL decoy receptors might be necessary for defense from TRAIL-induced apoptosis, high levels of TRAIL may provide protection for Langerhans islets from the immunological attack of cytotoxic T cells.

Key Words: TRAIL, death-decoy receptors, pancreas, Langerhans islets

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The pancreas consists of both exocrine and endocrine tissues that operate independently to regulate carbohydrate metabolism and digestion in the gastrointestinal tract. Langerhans islets are the discrete units of the endocrine compartment involved in the secretion of insulin and glucagon to control glucose homeostasis. The exocrine pancreas, by contrast, holds clusters of acinar cells full of zymogens (inactive pancreatic digestive enzymes), including trypsin, chymotrypsin, carboxypeptidase, amylase, and lipase. Zymogen activation typically takes place in the duodenum after its secretion through the pancreatic duct.

Type 1 diabetes (T1D) occurs as a result of the pancreatic β -cell destruction induced by an autoimmune reaction.^{1,2} The inflammatory mediators that trigger the immune reaction in T1D are also responsible for type 2 diabetes and islet graft failure.^{3,4} Various members of the tumor necrosis family, such as tumor necrosis factor (TNF) and Fas ligand (FasL), have recently been implicated in the development of T1D (Sanlioglu AD, PhD, unpublished data). Invading immune cells (activated mononuclear cells) release proinflammatory cytokines such as TNF, interleukin 1 β (IL-1 β), and interferon- γ to induce pancreatic β -cell death.⁵ In addition, these inflammatory cytokines increase the vulnerability of islet cells to autoimmune destruction.⁶ For example, TNF production by human islets induces postisolation cell death.⁷ Similarly, patients with T1D demonstrate elevated levels of TNF production from islet-infiltrating macrophages and dendritic cells.⁸ In addition, up-regulation of Fas in pancreatic islets via macrophage production of inflammatory cytokines is another means of causing β -cell death.⁹ Fas expression can be up-regulated by streptozotocin (STZ), a diabetogenic agent which induces β -cell death.¹⁰ To further explore the functional role of the Fas-FasL pathway in the pathogenesis of T1D, Lin et al¹¹ created cytomegalovirus–human FasL transgenic mice. Interestingly, transgenic mice were more sensitive to low doses of STZ-induced T1D than the control wild-type mice. In similar studies, Miwa et al¹² observed that FasL expression in β cells in rat insulin promoter–FasL–nonobese diabetic (NOD) mice resulted in the earlier onset of T1D because the FasL stimulated IL-1 β production that facilitated neutrophil infiltration.^{13,14}

The TNF-related apoptosis–inducing ligand (TRAIL) is another TNF family member that interacts with 4 different receptors: TRAIL-R1 (death receptor 4 [DR4]), TRAIL-R2 (death receptor 5 [DR5]), TRAIL-R3 (decoy receptor DcR1), and TRAIL-R4 (decoy receptor DcR2).¹⁵ The DR4 and DR5 are the TRAIL receptors that signal for apoptosis, whereas DcR1

and DcR2 are unable to induce such signaling because they lack the intracellular death domain.¹⁶ In comparison with other members of the TNF family, TRAIL has distinct cytotoxic and immunologic properties. For example, unlike TNF, which initiates and exacerbates autoimmune diseases, TRAIL can down-regulate immune responses. The potential outcome of TRAIL blockade or TRAIL deficiency has been analyzed in 2 different animal models of autoimmune diabetes.¹⁷ First, TRAIL function was counteracted by an injection of soluble TRAIL receptor into NOD mice, which enhanced the degree of autoimmune inflammation in pancreatic islets and facilitated the onset of diabetes. Second, the delivery of multiple, low doses of STZ into TRAIL-deficient mice resulted in a higher degree of islet inflammation and an earlier onset of diabetes. Although TNF and IFN- γ treatments also up-regulate TRAIL gene expression in pancreatic islets of NOD mice,¹⁸ TRAIL does not induce apoptosis in freshly isolated pancreatic islets. Because of its connection to T1D, this study investigated the endogenous expression profile of TRAIL and its receptors in human pancreas.

MATERIALS AND METHODS

Patient Assessment

Pancreata of 31 patients admitted to the Akdeniz University Hospitals and Clinics were sectioned and immunostained as described below. Patients' written consents (Helsinki Declaration) were obtained before the operation, and the procedures were approved by the Akdeniz University Hospital Committee on Ethics. Retrospective analyses and immunostaining procedures were performed in the Pathology department. Patients' ages ranged from 36 to 74 years, with a median age of 54 years. Fourteen patients were men and 17 were women. The normal tissues were obtained from 19 Whipple resections of duodenal adenocarcinoma. In addition, 12 specimens were derived from patients who underwent resection for gastric adenocarcinoma in which the tumor extended close to the pancreas. Moreover, spleen sections prepared from 6 different patients ($n = 6$) were also stained using antibodies developed against TRAIL and its receptors. Formalin-fixed and paraffin-embedded tissue blocks from these cases were retrieved from the database of the Department of Pathology. Hematoxylin and eosin-stained sections were prepared from each block, and slides were reevaluated. In each case, there was no evidence of tumor involvement.

TRAIL/TRAIL Receptor Immunohistochemistry on Normal Pancreas

All pancreatic tissue sections were initially stained with hematoxylin and eosin. Immunohistochemistry for TRAIL and TRAIL receptors was carried out as described previously.^{19,20} All primary antibodies were obtained from Alexis Biochemicals (Switzerland). The following primary antibodies were used at 1:300 dilution in the staining of pancreatic tissues: antihuman TRAIL monoclonal antibody (mAb) (III6F; ALX-804-326-C100), antihuman TRAIL-R1 mAb (HS101; ALX-804-297A-C100), antihuman TRAIL-R2 polyclonal antibody (ALX-210-743-C200), antihuman TRAIL-R3 polyclonal antibody (ALX-210-744-C200), and antihuman

TRAIL-R4 mAb (HS402; ALX-804-299A-C100). Whereas lymph node staining was performed as a positive control, negative controls included pancreatic tissue samples that were stained only with the appropriate secondary Ab. All sections were counterstained with hematoxylin in Figures 1–4, 5B, and 6.

Immunohistochemical Scoring of TRAIL and TRAIL Death-Decoy Receptor Expression

One pathologist (O.E.) with no prior knowledge of the data and blinded to the names of the antibodies was charged with specimen analysis. Both intensity and marker distribution (percentage of the positively stained epithelial cells) were considered for the calculation of the final immunohistochemical staining scores in pancreatic tissues. The intensity of the pancreatic tissue staining was scored as: 0, negative; 1, weak; 2, moderate; and 3, strong. Similarly, the marker distribution was scored as 0, less than 10%; 1, 10% to 40%; 2, 40% to 70%; and 3, greater than 70% of the epithelial cells stained on the sections. The final immunostaining score was assigned by summing the scores of both the intensity and the marker distribution for a given patient.

Annexin V Binding Assay and the Quantification of Apoptosis

Paraffin-embedded blocks were sectioned at 4- μ m thickness. After deparaffinization and dehydration, the antigen retrieval process was carried out by boiling samples in a solution containing 0.01 M of citrate buffer for 20 minutes. Proteinase K treatment was then performed for an additional 10 minutes. An Annexin V fluorescent microscopy kit (BD Pharmingen, cat no. 550911) was used for the detection of apoptotic cells in pancreas. To do this, pancreatic sections were washed with 1 \times phosphate-buffered solution (BD Pharmingen, cat no. 51-6635KC) then with 1 \times Annexin V binding buffer (BD Pharmingen, cat no. 51-66121E). Sections were then stained with Annexin V–fluorescein isothiocyanate (FITC) (BD Pharmingen, cat no. 51-8074KC) diluted 1:10 in 1 \times Annexin V binding buffer for 15 minutes at room temperature. After washing in Annexin V binding buffer, Annexin V–FITC–stained cells were analyzed under fluorescent microscopy. In each section, positive and negative cells were counted in randomly selected 200 \times high-power fields of acinar, ductal, or islet cells (area of each field, 0.06 mm²). The apoptotic index refers to the percentage of Annexin V–positive cells and calculated from the ratio of Annexin V–stained cells to the total number of cells counted.

Terminal Deoxynucleotidyl Transferase–Mediated 2'-Deoxyuridine 5'-Triphosphate Nick End Labeling Assay

The in situ cell death detection kit POD (Cat no. 11 684 817 910) was used to confirm apoptosis according to the protocol described by the manufacturer (Roche Applied Science, Philadelphia, Pa). The kit is specifically designed for the immunohistochemical detection and quantification of apoptosis at single-cell level based on labeling of DNA strand breaks. In this assay, labeling of DNA strand breaks is accomplished via terminal deoxynucleotidyl transferase enzyme, which catalyzes polymerization of labeled nucleotides to free 3'-OH DNA ends in a template-independent manner.

Then, incorporated fluorescein is detected by antifuorescein antibody Fab fragments from sheep, conjugated with horseradish peroxidase (POD). After substrate reaction, stained cells were analyzed under light microscope. The apoptotic index was calculated as described previously.

Statistical Analysis

The Statistical Package for the Social Sciences 13.0 software for Windows (SPSS Inc, Chicago, Ill) was used to perform the statistical analyses as specifically stated in the results. Statistical significance was considered at 5% probability level ($P < 0.05$). Error bars for all data points in all figures display the \pm SEM.

RESULTS

TRAIL and TRAIL Receptor Expression Profiles in Pancreatic Acinar Cells

Before the analyses of TRAIL and its receptor expression profiles in pancreas, specificities of these primary antibodies were confirmed on lymph node sections. As shown in Figure 1,

primary antibodies developed against TRAIL and its receptors generate a strong staining pattern on lymph node sections. Conversely, incubation of lymph node sections with the secondary antibody alone (negative control) did not produce any detectable staining.

Normal acinar expression profiles of TRAIL and its receptors in 31 pancreata were revealed using immunohistochemistry as described in "Materials and Methods," and representative images are depicted in Figure 2A. Normality of the group was tested by the Shapiro-Wilk method. Because a Gaussian distribution was not observed, the Friedman test followed by the Wilcoxon signed rank test was applied to reveal the statistical differences in the group. Although DR4 and TRAIL expression levels were the highest in acinar cells compared with other death and decoy receptor expressions, no statistical difference in the expression levels was observed between DR4 and TRAIL (Fig. 2B). As both decoy receptors DcR1 and DcR2 exhibited equivalent levels of expression, DR5 expression was statistically the lowest. Spearman ρ correlation test was administered to determine a possible correlation among the markers. A

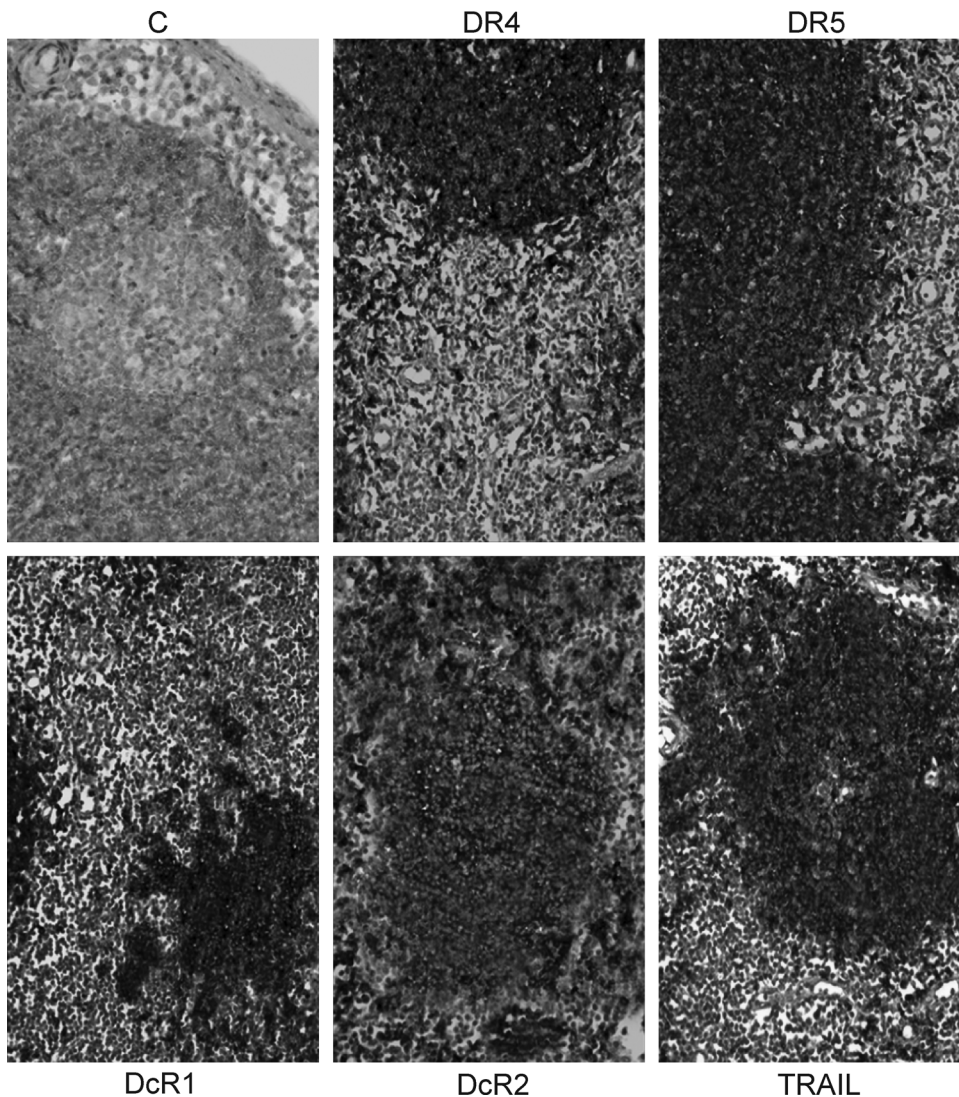


FIGURE 1. Lymph node staining of TRAIL and its receptors. All representative images are provided only from a single patient. C represents negative control staining using secondary antibody alone. Original magnification $\times 200$.

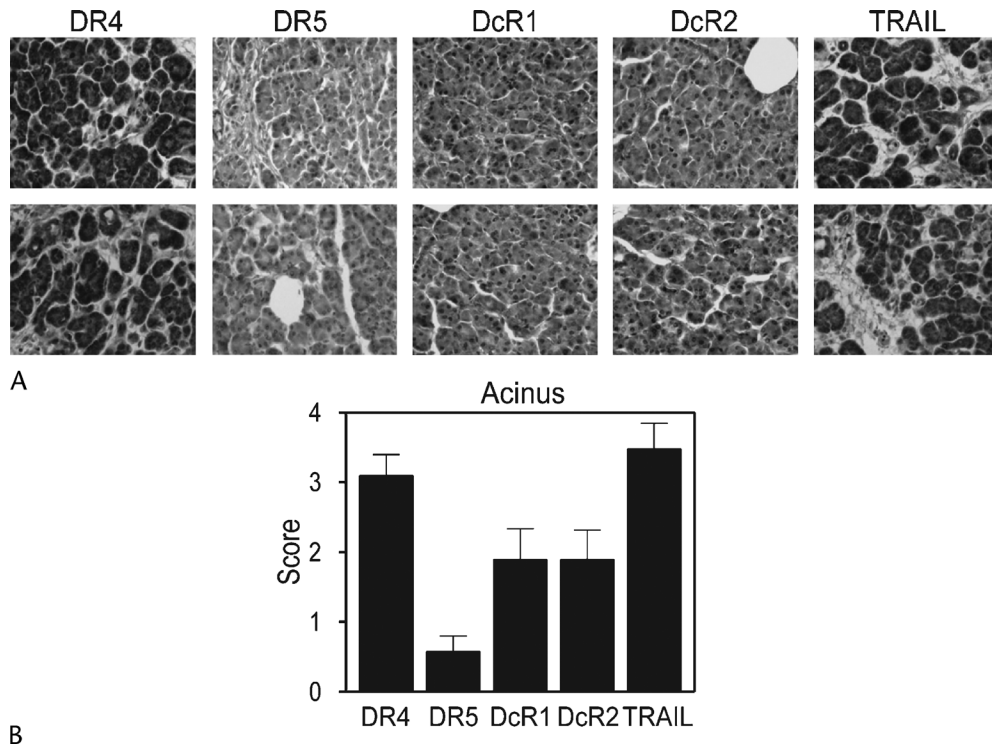


FIGURE 2. Acinar expression profile of TRAIL and TRAIL receptors in pancreas (n = 31). A, Representative images of immunohistochemical staining, with duplicate samples representing 2 different patients are provided. Original magnification ×200. B, Quantitative immunohistochemical scoring results. Error bars represent ±SEM.

positive correlation was detected between the decoy receptors DcR1 and DcR2, as well as between DcR1 and TRAIL (Table 1).

Pancreatic Ductal Staining Profiles of TRAIL and Its Receptors

Immunohistochemical staining of ductal region of pancreas was analyzed microscopically, and representative

images from 2 patients are displayed in Figure 3A. Although both decoy receptors and TRAIL were readily detectable, no TRAIL death receptor (DR4 or DR5) expression was observed in ductal cells. As equivalent levels of expression from both decoy receptors were measured, TRAIL expression was statistically the highest (Fig. 3B). In addition, Spearman ρ correlation test demonstrated the presence of a positive correlation between DcR1 and TRAIL (Table 2).

TABLE 1. Spearman ρ Correlation Test Indicating Putative Correlations Detected Among Acinar Staining Profiles of TRAIL and TRAIL Receptors

Spearman ρ Correlation (Acinus)		DR4	DR5	DcR1	DcR2	TRAIL
DR4	Correlation coefficient	1.000	-0.098	-0.197	0.039	0.005
	Significance (2-tailed)		0.601	0.287	0.837	0.980
	N	31	31	31	31	31
DR5	Correlation coefficient	-0.098	1.000	0.182	0.133	0.269
	Significance (2-tailed)	0.601		0.327	0.477	0.143
	N	31	31	31	31	31
DcR1	Correlation coefficient	-0.197	0.182	1.000	0.465*	0.566*
	Significance (2-tailed)	0.287	0.327		0.008	0.001
	N	31	31	31	31	31
DcR2	Correlation coefficient	0.039	0.133	0.465*	1.000	0.240
	Significance (2-tailed)	0.837	0.477	0.008		0.193
	N	31	31	31	31	31
TRAIL	Correlation coefficient	.005	.269	.566*	.240	1.000
	Significance (2-tailed)	0.980	0.143	0.001	0.193	
	N	31	31	31	31	31

*Correlation is significant at the 0.01 level (2-tailed).

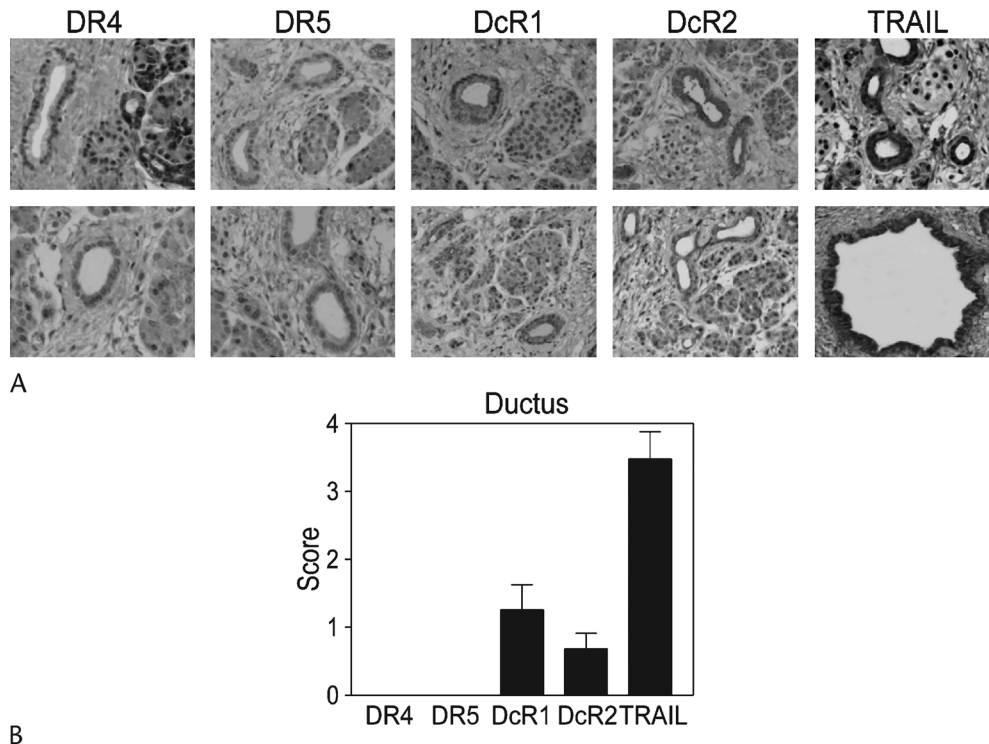


FIGURE 3. The TRAIL and TRAIL receptor expression profiles in ductal cells. A, Representative images of immunohistochemical staining. Original magnification $\times 200$. B, Quantitative assessment of ductal staining patterns of 31 pancreata. Error bars represent \pm SEM.

Correlation coefficients for DR4 and DR5 were not assessable because of the lack of expression in ductal cells.

Distinctive Expression Profiles of TRAIL and Its Receptors in Langerhans Islets

Representative images of the immunohistochemical staining of the Langerhans islets using specific antibodies against TRAIL and the TRAIL receptors are provided in Figure 4A. Similar to the finding in ductal cells, the Langerhans islets primarily expressed the TRAIL decoy receptors DcR1 and DcR2 and TRAIL. Although very low

levels of TRAIL death receptors were detectable, TRAIL expression was statistically the highest (Fig. 4B). Langerhans islets displayed equivalent levels of DcR1 and DcR2 expressions. Only DcR1 displayed a positive correlation with TRAIL as shown by Spearman ρ correlation test (Table 3).

Presence of Apoptotic Cells in Pancreas and the Connection to TRAIL and TRAIL Receptor Expression Profiles

Because acinar, ductal, and islet cells in the pancreas displayed differential amounts of TRAIL and TRAIL receptor expression, an Annexin V binding assay was performed on pancreatic sections to determine the level of apoptosis. Whereas fluorescent microscopic views display Annexin V-FITC-stained cells (Fig. 5A), quantitative assessments regarding the apoptotic index are provided below each panel. No difference in the degree of apoptosis was observed among acinar, ductal, and islet cells in pancreas. These results were further confirmed by terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick end labeling (TUNEL) assay on the same sections (Fig. 5B). Quantitative analyses of TUNEL assay did not reveal any difference in the degree of apoptosis among the cell types tested, as shown below each panel in Figure 5B. Moreover, both Annexin V binding assay and TUNEL method detected equivalent levels of cell death for each cell type. However, high levels of TRAIL expression did correlate with increased cell death as revealed by Spearman ρ correlation test (Table 4). Intriguingly, some degree of correlation was also evident

TABLE 2. Correlation Coefficients Among TRAIL and TRAIL Receptors in Pancreatic Ducts as Revealed by Spearman ρ Correlation Test

Spearman ρ Correlation (Ductus)		DcR1	DcR2	TRAIL
DcR1	Correlation coefficient	1.000	0.151	0.550*
	Significance (2-tailed)		.417	0.001
	N	31	31	31
DcR2	Correlation coefficient	0.151	1.000	-0.060
	Significance (2-tailed)	0.417		0.747
	N	31	31	31
TRAIL	Correlation coefficient	0.550*	-0.060	1.000
	Significance (2-tailed)	0.001	0.747	
	N	31	31	31

*Correlation is significant at the 0.01 level (2-tailed).

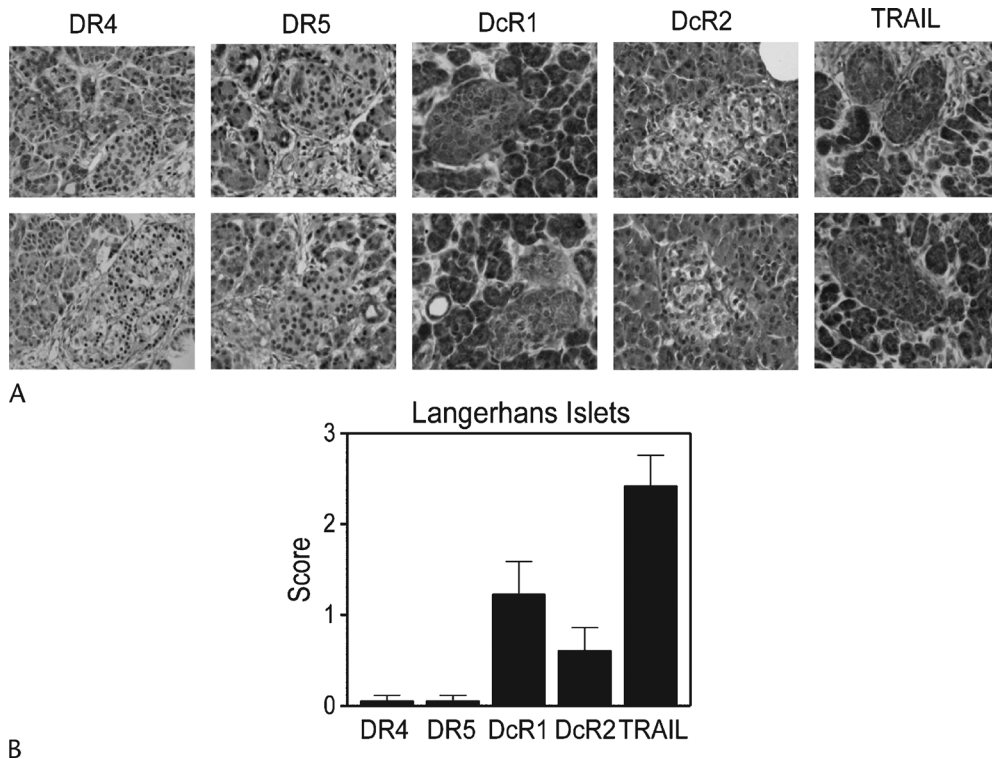


FIGURE 4. The TRAIL and TRAIL receptor expression profile in Langerhans islets. A, Representative images of immunohistochemical staining. Original magnification $\times 200$. B, Quantitative analysis of scoring. Error bars represent \pm SEM.

between apoptotic cells and DcR1 expression in acinar versus islets cells.

TRAIL and TRAIL Receptor Expression Profiles in Spleen

Because TRAIL is preferentially expressed by immune cells, we also analyzed TRAIL and TRAIL receptor expression profiles in the spleen using immunohistochemistry

for comparison with those observed in the pancreas. Death and decoy receptors, as well as TRAIL, were all expressed in the spleen (Fig. 6A). Statistical analysis of the normality of the group was tested by the Shapiro-Wilk method. Because a Gaussian distribution was not observed, the Friedman test followed by the Wilcoxon signed rank test was applied to reveal the statistical differences in the group. Equivalently high levels of staining were observed in the

TABLE 3. Differential Expression Profiles of TRAIL and TRAIL Receptors in Langerhans Islets as Illustrated by Spearman ρ Correlation Test

Spearman ρ Correlation (Islets)		DR4	DR5	DcR1	DcR2	TRAIL
DR4	Correlation coefficient	1.000	-0.033	-0.115	-0.089	-0.220
	Significance (2-tailed)		0.859	0.538	0.634	0.234
	N	31	31	31	31	31
DR5	Correlation coefficient	-0.033	1.000	0.332	-0.089	0.262
	Significance (2-tailed)	0.859		0.068	0.634	0.154
	N	31	31	31	31	31
DcR1	Correlation coefficient	-0.115	0.332	1.000	0.061	0.450*
	Significance (2-tailed)	0.538	0.068		0.743	0.011
	N	31	31	31	31	31
DcR2	Correlation coefficient	-0.089	-0.089	0.061	1.000	-0.256
	Significance (2-tailed)	0.634	0.634	0.743		0.165
	N	31	31	31	31	31
TRAIL	Correlation coefficient	-0.220	0.262	0.450*	-0.256	1.000
	Significance (2-tailed)	0.234	0.154	0.011	0.165	
	N	31	31	31	31	31

*Correlation is significant at the 0.05 level (2-tailed).

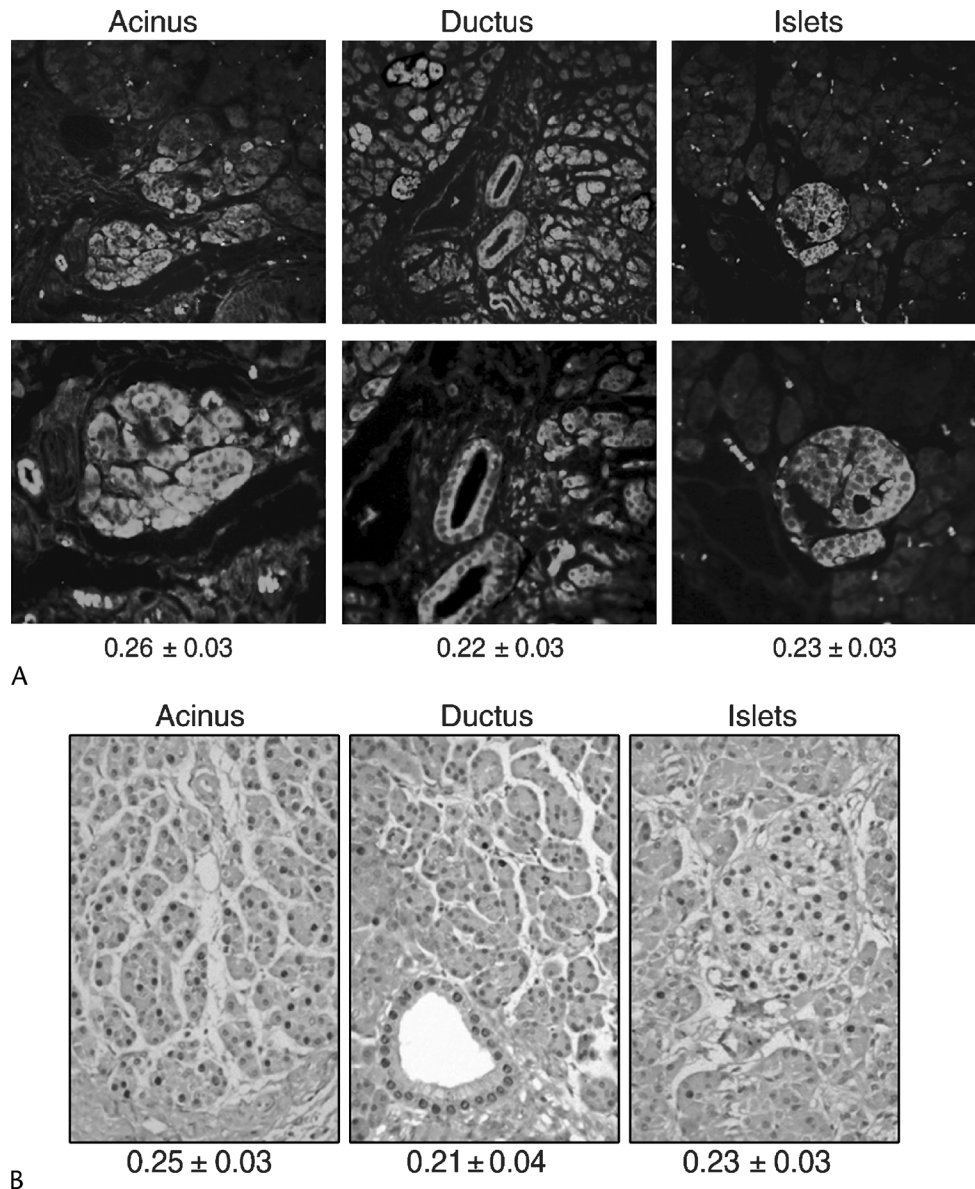


FIGURE 5. A, Annexin V-FITC binding assay on pancreas. Apoptotic cell staining and the quantifications were performed as described in "Materials and Methods." Upper row shows representative views of acinar (left), ductal (middle), and islet cell (right) at 200× magnification; lower row shows high-power magnifications (400×) of the same fields. Quantitative analysis of apoptotic scoring is given below each panel as a percentage of Annexin V-stained cells. B, TUNEL assay on pancreas. Only 1 representative image (views of acinar [left], ductal [middle], and islet cells [right]) for each condition is shown for clarity. The numbers below each panel represent quantitative analysis of TUNEL assay results in percentages (apoptotic index). Original magnification ×200. Error bars represent ±SEM in both panels.

spleen for all the TRAIL markers tested (Fig. 6B). Despite the fact that the mean DcR2 expression level was low in comparison with other markers, the difference was not statistically significant. Nonetheless, our results demonstrated that the amount of TRAIL and its receptor expression on average were much higher in the spleen than those of the pancreas (Fig. 6B).

DISCUSSION

Immunostaining approaches, either by immunohistochemistry or flow cytometry, have become valuable tools to

analyze TRAIL and TRAIL receptor expression profiles in tissues such as prostate, lung, breast, and synoviocytes.^{19–22} Despite its anticancer properties,^{23–25} TRAIL has also recently been implicated in the destruction of pancreatic β cells because the analysis of activated T cell lines derived from 29 children with new-onset T1D showed an increase in TRAIL expression in the infiltrating β cell-specific T cells (CD56⁺).²⁶ The fact that TRAIL induced much stronger cytotoxicity to the human β cell lines (CM and HP62) than did TNF and FasL further strengthened the argument of TRAIL mediating β-cell destruction.²⁷

TABLE 4. Correlation of Apoptotic Cells With TRAIL and TRAIL Receptor Profiles in Pancreas

Apoptotic Cell Staining		Acinus	Ductus	Islets
DR4	Correlation coefficient	-0.044	NA	-0.218
	Significance (2-tailed)	0.813		0.239
	N	31		31
DR5	Correlation coefficient	0.135	NA	0.177
	Significance (2-tailed)	0.468		0.342
	N	31		31
DcR1	Correlation coefficient	0.383*	0.310	0.433*
	Significance (2-tailed)	0.034	0.090	0.015
	N	31	31	31
DcR2	Correlation coefficient	0.001	0.012	-0.168
	Significance (2-tailed)	0.994	0.947	0.367
	N	31	31	31
TRAIL	Correlation coefficient	0.872†	0.693†	0.978†
	Significance (2-tailed)	0.000	0.000	0.000
	N	31	31	31

*Correlation is significant at the 0.05 level (2-tailed).

†Correlation is significant at the 0.01 level (2-tailed).

NA indicates not applicable.

Because of its presumed role in the development of T1D, our study assessed TRAIL and TRAIL receptor expression profiles using immunohistochemistry of 31 human pancreata specimens. Our investigation revealed varying degrees of TRAIL and TRAIL receptor expression in the acinar cells. Although DR4 and TRAIL expressions were the highest, there were substantial levels of TRAIL decoy receptor expression in the acinar cells. Ductal cells, in

contrast, exclusively expressed TRAIL and TRAIL decoy receptors. Similar observations were made for the Langerhans islets, despite the presence of very low, but detectable, amounts of TRAIL death receptor expression. Thus, the absence of TRAIL death receptor expression was clearly evident in ductal cells and in Langerhans islets. Despite equivalent levels of apoptosis observed among the cell types tested, high levels of TRAIL expression were correlated with the increased amount of cell death in all 3 cell types (acinar, ductal, and islet cells) analyzed. This may strengthen the arguments implicating TRAIL as an apoptosis-inducing agent in pancreas.^{26,27} Some degree of correlation was also observed between Annexin V-FITC-stained cells and DcR1 expression in acinar versus islet cells, but not in ductal cells. Although the biologic basis of this finding is not clear and cannot be explained with our current knowledge, our studies on prostate cancer cells suggest that DcR1 expression by itself is not sufficient to prevent TRAIL-induced cytotoxicity.^{24,28} Yet, the removal of DcR1 from the membrane increased the susceptibility of primary islet cells to TRAIL-induced apoptosis,²⁷ further suggesting the potential protective roles of TRAIL decoy receptors in protecting Langerhans islets from the death ligand-mediated apoptosis.

The expression profiles of TRAIL and TRAIL receptors on fetal pancreas were recently analyzed using laser scanning confocal microscopy.²⁹ In this study, TRAIL-positive cells were primarily positioned on the periphery of the pancreatic islets. Although DcR1 and DcR2 expressions were noticeable on a few cells, no DR4 or DR5 expression was detected in the pancreatic islets. Our study however revealed uniform but substantial levels of TRAIL and TRAIL decoy receptor expressions in the Langerhans islets. Differences between these

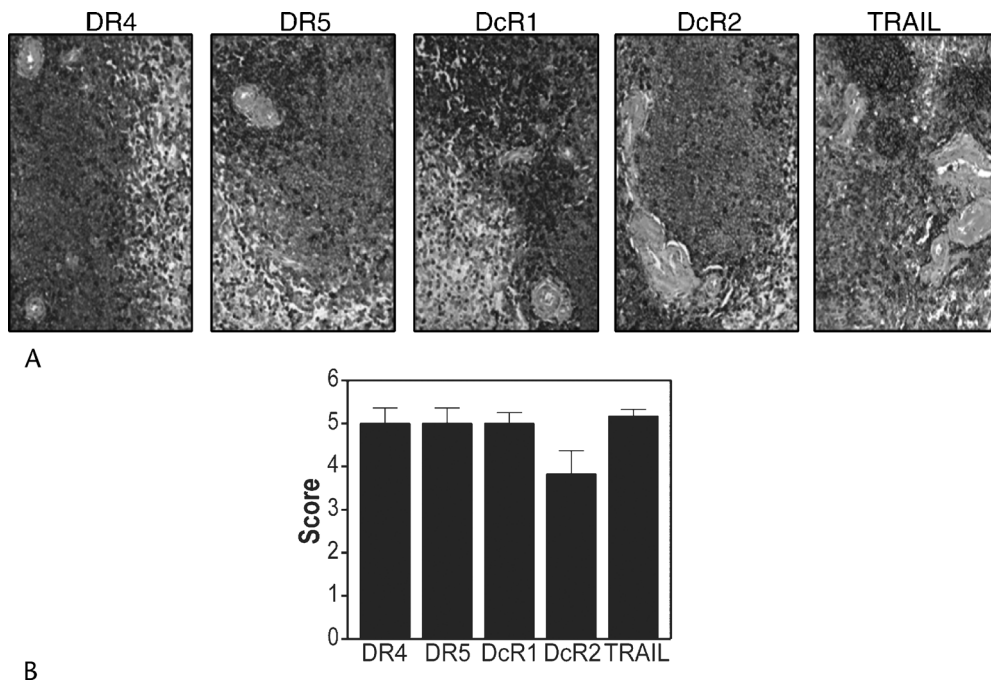


FIGURE 6. Expression profile of TRAIL and TRAIL receptors in spleen (n = 6). A, Examples of immunohistochemical staining representing a single patient are provided. Original magnification $\times 200$. B, Quantitative immunohistochemical scoring results. Error bars represent \pm SEM.

2 studies can be attributed to the use of fetal versus adult pancreata, in addition to the differences in antibodies used. Nevertheless, the presence of substantial levels of TRAIL decoy receptor expression in the absence (or trivial levels) of TRAIL death receptor expression may suggest that Langerhans islets are naturally resistant to the cytotoxic effects of apoptotic ligands. Not surprisingly, certain differences in the TRAIL sensitivity of cancerous versus normal islets were also reported.³⁰ Although β cell lines were sensitive to TRAIL treatment, normal primary islet cells isolated from most donors displayed resistance to TRAIL-mediated cytotoxicity.²⁷ Moreover, because TRAIL-transduced dendritic cells protected mice from acute graft-versus-host disease and leukemia relapse through the suppression of antigen-specific T-cell activity,³¹ it is reasonable to assume that high levels of TRAIL expression may provide immune protection to Langerhans islets. In summary, our study revealed differential expression profiles of TRAIL and TRAIL receptors in the pancreas with potential implications in T1D. More importantly, this study demonstrated that high levels of TRAIL expression correlated with increased amount of cell death in the pancreas.

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PROSPECTS

Molecular Mechanisms of Death Ligand-Mediated Immune Modulation: A Gene Therapy Model to Prolong Islet Survival in Type 1 Diabetes

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Abstract *Type 1 diabetes* results from the T cell-mediated destruction of pancreatic beta cells. Islet transplantation has recently become a potential therapeutic approach for patients with type 1 diabetes. However, islet-graft failure appears to be a challenging issue to overcome. Thus, complementary gene therapy strategies are needed to improve the islet-graft survival following transplantation. Immune modulation through gene therapy represents a novel way of attacking cytotoxic T cells targeting pancreatic islets. Various death ligands of the TNF family such as FasL, TNF, and TNF-Related Apoptosis-Inducing Ligand (TRAIL) have been studied for this purpose. The over-expression of TNF or FasL in pancreatic islets exacerbates the onset of type 1 diabetes generating lymphocyte infiltrates responsible for the inflammation. Conversely, the lack of TRAIL expression results in higher degree of islet inflammation in the pancreas. In addition, blocking of TRAIL function using soluble TRAIL receptors facilitates the onset of diabetes. These results suggested that contrary to what was observed with TNF or FasL, adenovirus mediated TRAIL gene delivery into pancreatic islets is expected to be therapeutically beneficial in the setting of experimental models of *type 1 diabetes*. In conclusion; this study mainly reveals the fundamental principles of death ligand-mediated immune evasion in diabetes mellitus.

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Key words: type 1 diabetes; gene therapy; adenovirus; TRAIL; islet transplantation

Diabetes is a disease that drastically reduces life-expectancy and the quality of life. It is predicted that 250 million people will be affected

with diabetes in the world by 2010 [McCarty and Zimmet, 1994], making it the third most common disease and the fourth leading cause of death in North America [Boyle et al., 2001]. Insulin injection is the main treatment modality for patients with type 1 diabetes. While this approach protects patients from nephropathy, neuropathy and retinopathy, it does not prevent the recurrence of hypoglycemic events, seizures, and coma. In addition, the loss of physiologic insulin secretion cannot be compensated through the insulin administration into the patients. While pancreas transplantation can prolong and improve the quality of life, the procedure is controversial because of the less favorable outcome due to major surgery and the need for long-term immunosuppression

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[Gruessner et al., 1997]. As an alternative, transplantation of pancreatic islets is one means of avoiding major surgery and the complications associated with insulin injections [Bromberg and LeRoith, 2006]. Even though islet transplantation has been a promising approach for the treatment of patients with *type 1 diabetes*, the success of the approach was challenged due to the high frequency of non-functioning grafts and secondary graft failure leading to the necessity of the majority of recipients to resume the administration of insulin at 5 years [Shapiro et al., 2005]. Despite all the methods devised to protect the beta cells from the immune mediated destruction, the agents could only delay, not prevent, the eventual failure of the transplanted beta cells [Skyler et al., 2002].

The ultimate goal of islet transplantation is to completely correct the diabetic syndrome without the need for chronic immunosuppressive drug therapy. In order to maintain long term graft function, both alloimmune and auto-immune barriers must be overcome. Thus, tolerance induction is one of the objectives in islet transplantation. Initial studies investigating the protection of islets from the immune system involved the transplantation of islets into immune privileged sites, such as the testis, brain or thymus. It quickly became obvious that these sites did not protect the grafts through sequestration, but relied on the activation of apoptotic pathways, such as Fas ligand (FasL)-induced apoptosis [Takeda et al., 1998]. This observation revealed the feasibility of an immune modulation strategy consisting of death ligand expression in pancreatic islets by means of gene therapy for the purpose of destroying (or avoiding) beta cell reactive cytotoxic T cells. Thus, gene therapy arose as an alternative treatment modality for the treatment of *type 1 diabetes* patients [Harlan, 2004]. FasL, TNF [Dajani et al., 2007] and TNF Related Apoptosis Inducing Ligand (TRAIL) are well known as apoptosis inducing members of the TNF family, which are all implicated in the pathogenesis of *type 1 diabetes*. Below is the summary of the current literature on what we know about these death ligands, their prospective roles in *type 1 diabetes* as well as their potential therapeutic applications in the context of gene therapy. A particular emphasis will be given to TRAIL, since it has some discrete immune-modulatory properties compared to TNF or FasL.

TNF-ALPHA AND FASL PLAY ESSENTIAL ROLES DURING THE COURSE OF TYPE 1 DIABETES

Despite the fact that apoptosis mediates beta-cell death both in rodents and humans, the effector molecules responsible of the development of *type 1 diabetes* are still disputed [Santamaria, 2001]. A model depicting the molecular pathogenesis of *type 1 diabetes* is given on Figure 1. An islet inflammation (insulinitis) precedes the development of type 1 diabetes. This process requires the involvement of local professional antigen presenting cells (APC), such as dendritic cells, macrophages and B cells, in addition to CD4⁺ T cells and CD8⁺ T cells (Fig. 1A). A prolonged period of insulinitis may lead to the preferential amplification of autoreactive CD8⁺ T cells bearing high affinity T cell receptors (TCR). The differentiation of high affinity CD8⁺ pre-CTLs into CTLs is accomplished via TCR recognition of target peptide-MHC I complexes on local APC in CD4⁺ T helper (Th) independent manner (Fig. 1B). Co-stimulatory pathways involving CD28-B7 are believed to be essential for this process. T cell effector pathways involving Fas/Fas-ligand (Fas-FasL) interaction or the perforin/granzyme system are primarily responsible for the beta cell destruction. According to this model, perforin production from CD8⁺ T cells initiates the immune response, and then Fas/FasL interaction causes CD4⁺ T cell-induced beta cell death [Augstein et al., 1998; Eizirik and Mandrup-Poulsen, 2001]. In addition, the interaction between the APC and T cells initiates an inflammatory response resulting in the production of high concentrations of proinflammatory cytokines locally in the islets. These cytokines then facilitate the induction of apoptotic signaling cascades in the pancreatic beta cells [Miwa et al., 1998; Heimberg et al., 2001]. Both CD8⁺ and CD4⁺ T cells can secrete TNF and INF- γ upon antigen recognition. TNF enhances auto-antigen presentation and IL-1 secretion by local APC. By binding to specific receptors on beta cells, these proinflammatory cytokines induce either apoptosis through caspase cleavage (TNF) or necrosis through NO production (INF- γ and IL-1). These three cytokines can also upregulate Fas and MHC I expression on beta cells in order to facilitate cell recognition and cell death [Yamada et al., 1996]. All these results suggest that TNF and Fas signaling play major roles during the development of *type 1 diabetes*. Yet,

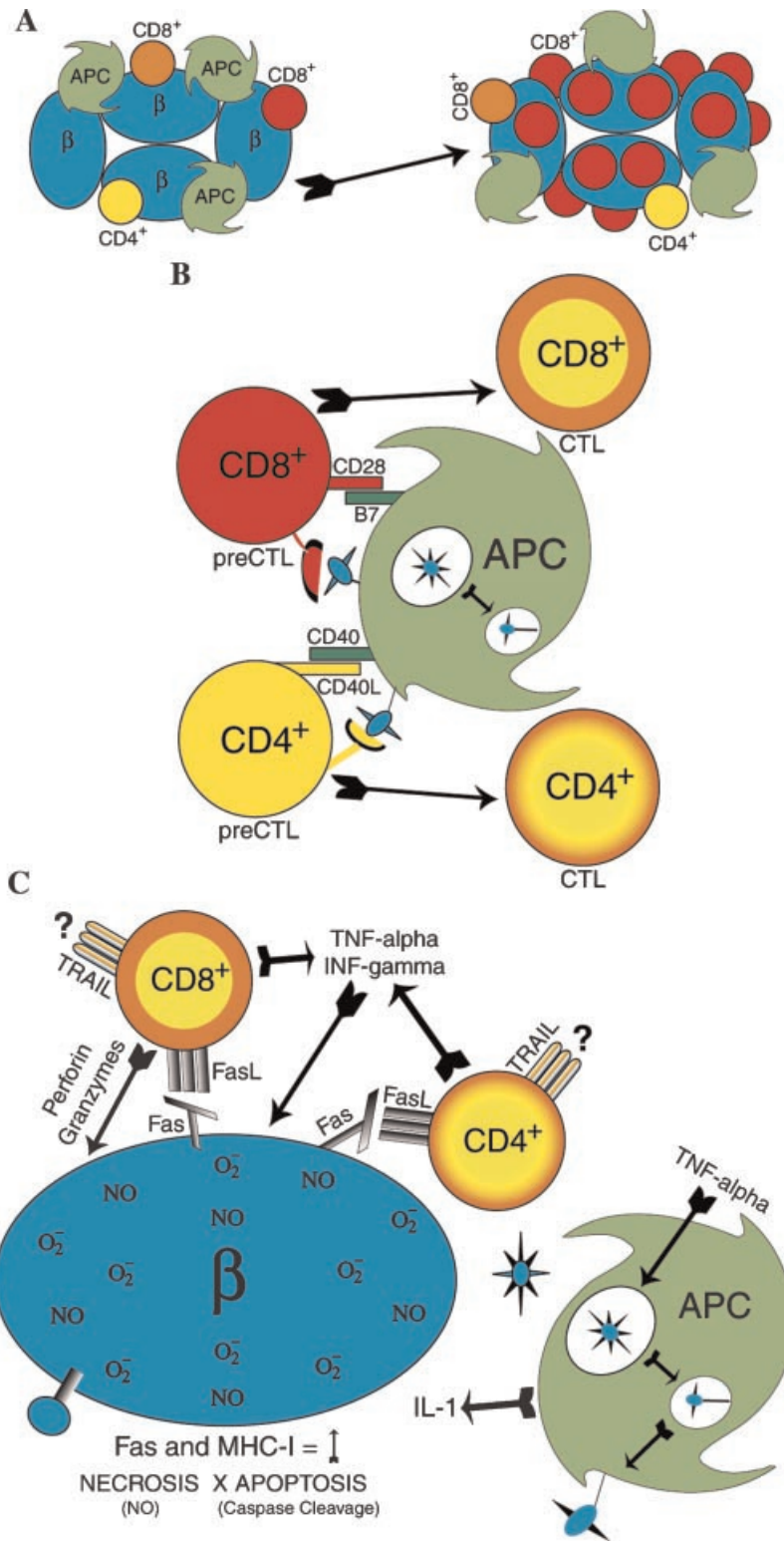


Fig. 1. An inflammatory model for the pathogenesis of *type 1 diabetes*. Prolonged insulinitis leads to preferential amplification of autoreactive CD8⁺ T cells bearing high affinity receptors for islet antigens (**Panel A**). Differentiation of CD8⁺ and CD4⁺ T cells into effector cytotoxic T cells (CTLs) (**Panel B**). Effector phase of *type 1 diabetes* (**Panel C**). Unknown environmental factors cause MHC class I restricted presentation of the beta cell antigen on the cell surface. CD8⁺ T cells recognizing this antigen generates MHC class I restricted beta cell damage through the secretion of INF γ or

TNF/TRAIL or the perforin/granzyme system. Liberated beta-cell components, such as insulin are taken up by the dendritic cells in islets and transported to the regional pancreatic lymph nodes, where the antigens are processed and presented to CD4⁺ T cells. After the clonal expansion, CD4⁺ T cells will move to the islets to perform CD4⁺ T cell-mediated killing using FasL/Fas system. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

another member of TNF superfamily, TRAIL, has recently been linked to have a profound impact on autoimmune diabetes.

MOLECULAR EVIDENCES CONNECTING TRAIL SIGNALING TO AUTOIMMUNE DIABETES

Two animal models of autoimmune diabetes were utilized to understand the potential roles of TRAIL in *type 1 diabetes* [Lamhamedi-Cherradi et al., 2003]. In the first model, a soluble TRAIL receptor was injected into NOD mice to counteract TRAIL function. Blocking TRAIL in this manner considerably increased the onset of diabetes and augmented the degree of autoimmune inflammation in pancreatic islets. In the second model, multiple low-doses of streptozotocin (STZ) were given to normal and TRAIL-deficient C57BL/6 mice. Contrary to TNF or FasL, TRAIL-deficient animals manifested a higher degree of islet inflammation leading to an earlier onset of diabetes [Lamhamedi-Cherradi et al., 2003]. This finding suggests that TRAIL expression might be required for the down-regulation of autoimmune inflammatory response in *type 1 diabetes*.

A recent study suggested that TRAIL receptors are expressed both in the human beta cell lines and in the normal primary islet cells [Ou et al., 2002]. Most of the human beta cells expressed all four TRAIL receptors and/or TRAIL. Interestingly, both of the beta cell lines (CM and HP62) were sensitive to TRAIL, whereas normal primary islet cells isolated from the most donors were resistant to the TRAIL-induced cytotoxicity [Ou et al., 2005]. Moreover, the fact that TRAIL induced much stronger cytotoxicity to the human beta cell lines than did the other cytokines brought up the possibility of TRAIL involvement in the development of *type 1 diabetes*. Freshly isolated T cells do not express TRAIL unless they are treated with the type I interferon or CD3 ligation [Kayagaki et al., 1999]. This was further confirmed by the studies showing an increased expression of TRAIL in the infiltrating cells of the pancreatic islets in patients with *type 1 diabetes* [Cheung et al., 2005]. Intriguingly, TNF and IFN- γ treatment up-regulated TRAIL gene expression in pancreatic islets of NOD mice but still TRAIL failed to induce apoptosis of freshly isolated pancreatic islets [Mi et al., 2003].

FUNCTIONAL CONSEQUENCE OF TRAIL SIGNALING IN PANCREATIC ISLETS

TRAIL is a type II membrane protein that can bind to five different receptors: TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR1), TRAIL-R4 (DcR2), and osteoprotegerin (OPG) [Wiley et al., 1995]. Current TRAIL receptor signaling and NF- κ B activation pathway, as well as their cross-talk, are displayed on Figure 2 [MacFarlane, 2003; Sanlioglu et al., 2003]. DR4 and DR5 function as authentic death receptors that signal for apoptosis, while DcR1 and DcR2 are unable to induce such signaling because they lack the intracellular

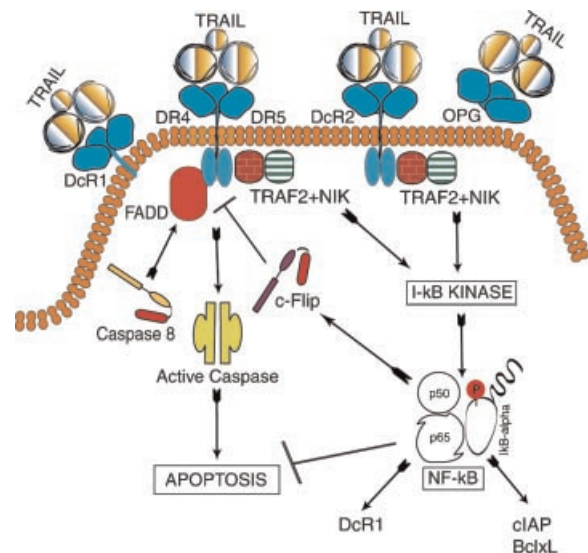


Fig. 2. TRAIL receptor signaling pathway. Activation of TRAIL receptor 1 (DR4) or 2 (DR5) by trimeric TRAIL ligands leads to the recruitment of Fas-associated death domain protein (FADD) to the membrane. Then, FADD recruits procaspase 8 to form death inducing signaling complex (DISC). DISC-induced signaling activates caspase pathway pushing cells into apoptosis. cFlip, a procaspase 8 homologue, competes with procaspase 8 for binding to FADD, thereby inhibiting apoptosis. Antiapoptotic NF- κ B signaling can also be activated by TRAIL and TRAIL-R4 (DcR2) interaction in an I κ B Kinase (IKK) dependent fashion. TRAIL-R3 (DcR1) has a truncated cytoplasmic domain, allowing it to serve as a decoy receptor for TRAIL. Interestingly proinflammatory cytokine (TNF and IL-1) dependent activation of IKK and thereby NF- κ B, has been claimed to induce apoptosis in pancreatic islets. If so, the reason why cells do not undergo apoptosis when DcR2 activates NF- κ B signaling presents itself as an intricate dilemma to resolve. Knowing that the death receptor activation (DR4 or DR5) also may lead to the activation of NF- κ B signaling via TRAF-2 and NIK complicates this issue further. Four different ways of inhibiting TRAIL-mediated apoptosis by way of NF- κ B is outlined in the figure. Osteoprotegerin (OPG) is also another receptor interacting with TRAIL. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

death domain [Griffith and Lynch, 1998; Karacay et al., 2004; Aydin et al., 2007]. The engagement of TRAIL with its receptors DR4, DR5, and DcR2 (but not DcR1) activates anti-apoptotic NF- κ B signaling pathways [Degli-Esposti et al., 1997; Sanlioglu et al., 2005, 2006]. Thus, TRAIL over-expression in islets is expected to activate NF- κ B signaling as well. However, the consequence of TRAIL-induced NF- κ B activation in islets is not known. NF- κ B is well known for its anti-apoptotic properties as demonstrated in cancer cells. Intriguingly, proinflammatory cytokine activation of NF- κ B has been linked to beta cell death [Larsen et al., 2005; Ortis et al., 2006], but its inactivation correlated with islet graft function [Eldor et al., 2006]. Consequently, the controversial issue that remains is whether the activation of NF- κ B signaling is beneficial or detrimental for the islet-graft survival in islet transplantation [Kim et al., 2007]. Nevertheless, the consequence of TRAIL-mediated NF- κ B activation might differ from proinflammatory cytokine-induced NF- κ B signaling.

Previous studies demonstrated that NF- κ B inducing agents up-regulated cFLIP synthesis blocking caspase activation [Kreuz et al., 2001]. In addition, NF- κ B activation increases TRAIL-R3 synthesis, a decoy receptor for TRAIL [Bernard et al., 2001], and the expression of apoptosis inhibitor Bcl-x_L [Hatano and Brenner, 2001; Ravi et al., 2001] resulting in the inhibition of TRAIL-mediated apoptosis. Apoptosis inhibitors such as cIAP are also induced by NF- κ B signaling [Mitsiades et al., 2002]. Based on these results, there are at least four different ways to block TRAIL-induced apoptosis through NF- κ B (Fig. 2). Because TRAIL can neutralize its own apoptosis inducing effects, it is not clear how cells decide whether to go under apoptosis or not following TRAIL treatment. Nevertheless, activation of antiapoptotic NF- κ B signaling by TRAIL itself might constitute one of the possible ways of avoiding TRAIL cytotoxicity in pancreatic islets.

The variations in the ratio of TRAIL death to decoy receptors might constitute a reason for TRAIL resistance in pancreatic islets. Immunostaining approaches became valuable tools to analyze TRAIL expression on tissues [Aydin et al., 2007; Sanlioglu et al., 2007b,c]. For example, the localization of TRAIL and its receptors on fetal pancreas were analyzed using

confocal fluorescence immunohistochemistry [Chen et al., 2003]. TRAIL-expressing cells were mainly located on the periphery of the pancreatic islets. While DcR1 and DcR2 expressions were detectable on a few cells, no expression was detected using DR4 and DR5 antibodies. The fact that the pancreatic cells expressed TRAIL and the TRAIL decoy receptors suggested the cells were resistant to apoptosis. Our recent study showing high levels of TRAIL and the decoy receptors expression in human islets also supported this notion [Sanlioglu et al., 2008]. Pancreatic ductal cell carcinoma cells expressing both the TRAIL and its receptors, however, are sensitive to TRAIL-induced apoptosis [Sato et al., 2001]. Thus, there appears to be certain differences in the TRAIL sensitivity of cancerous islet cells versus normal islets. Moreover, since cytokine-induced OPG expression protected pancreatic beta cells from destruction; this particular TRAIL interacting receptor has recently been identified as autocrine or paracrine survival factor for beta cells [Schrader et al., 2007].

IS OVER-EXPRESSION OF DEATH LIGANDS OF TNF SUPER FAMILY A VIABLE STRATEGY TO AVOID BETA CELL SPECIFIC CYTOTOXIC T CELL ATTACK?

Because *type 1 diabetes* results from the T cell-mediated destruction of the insulin-producing pancreatic beta cells [Kurrer et al., 1997], the depletion of the autoreactive T cells via apoptosis represents a viable strategy for the prevention of autoimmune diabetes (Fig. 3). Activation of the Fas-induced pathway while interfering with the co-stimulation (second signal) enhances the apoptosis of peripheral lymphocytes in vitro [Akalin et al., 1997]. Despite obtaining promising results using an adenovirus carrying both the human CTLA-4 and FasL genes (AdCTLA4-FasL) to treat diabetes [Jin et al., 2004], recent reports have challenged the use of FasL in the generation of immune tolerance. For example, CMV-hFasL transgenic mice were generated in order to investigate the role of the Fas-FasL pathway in the pathogenesis of STZ-induced *type 1 diabetes* [Lin et al., 2003]. Interestingly, the transgenic mice were more sensitive to diabetes than the control WT mice, because the over-expressed FasL stimulated IL-1 production and facilitated neutrophil infiltration [Miwa et al., 1998]. This

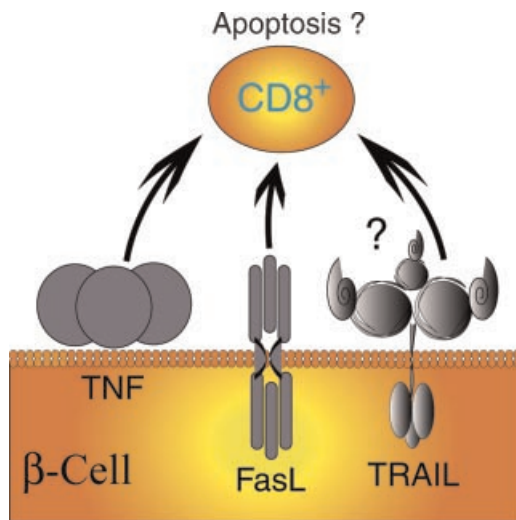


Fig. 3. Death ligand expression in pancreatic islets and its potential outcome. These therapeutic approaches are designed to prolong the graft survival in patients with *type 1 diabetes*. Pancreatic islet grafts protected from the immune-mediated cytotoxic T cell attack are expected to function longer after the transplantation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

observation is consistent with reports that transgenic expression of FasL on beta cells resulted in the earlier onset of *type 1 diabetes* [Allison et al., 1997; Chervonsky et al., 1997; Petrovsky et al., 2002]. Similarly, cardiac grafts expressing transgenic FasL were quickly rejected by neutrophils when transplanted into syngeneic or allogeneic hosts [Takeuchi et al., 1999]. FasL is synthesized as a type II transmembrane protein, but it can be cleaved by matrix metalloproteases after cell surface expression [Tanaka et al., 1998]. For this reason, it was hypothesized that the soluble FasL contributed to the graft rejection either by preventing apoptosis of the graft-reactive T cells [Suda et al., 1997], and/or acting as a chemotactic factor for neutrophils [Seino et al., 1998].

Parallel results were obtained using TNF. Specifically, transgenic production of TNF (RIP-TNF) in pancreatic islets induced insulinitis [Higuchi et al., 1992; Picarella et al., 1993; Rajagopalan et al., 2003]. Furthermore, local TNF synthesis promoted *type 1 diabetes* in NOD mice by enhancing antigen presentation [Green et al., 1998]. In accordance with this, transgenic expression of soluble TNF receptor prevented autoimmune diabetes in NOD mice [Hunger et al., 1997]. Since infiltrating cells of the pancreatic islets displayed elevated levels of TRAIL expression in patients with *type 1*

diabetes [Cheung et al., 2005], these cells might well use TRAIL death ligand in the destruction of beta cells. Conversely, it is expected that exogenous TRAIL over-expression might protect pancreatic islets from CTL invasion, as depicted on Figure 3. Below is such an assessment of potential application of TRAIL for the purpose of defying autoreactive T cells targeting pancreatic islets.

POTENTIAL OUTCOME OF TRAIL INTERACTION WITH ISLET TARGETING T CELLS

Compared to the other members of the TNF family such as FasL and TNF, TRAIL has distinct apoptosis inducing properties on cells—specifically, TRAIL is a potent inducer of tumor cell apoptosis but is nontoxic to normal cells and tissues [Griffith et al., 2002; Steele et al., 2006; Terzioglu et al., 2007; Sanlioglu et al., 2007a]. Furthermore, unlike TNF, which can initiate and exacerbate autoimmune diseases, TRAIL is reported to down-regulate immune responses. For this reason, the role of TRAIL in the lymphocyte survival was also analyzed using splenocytes isolated from BALB/c mice [Song et al., 2000]. While FasL induced apoptosis of the activated T cells, TRAIL inhibited their proliferation without inducing apoptosis. TRAIL also prevented the cell cycle transition from G1 to S phase of the lymphocytes by inhibiting DNA synthesis. For this reason, it was suggested that, unlike TNF or FasL, TRAIL inhibits the activation and the expansion of lymphocytes *in vivo*, but does not delete them from the system. Intriguingly, contrary to resting T cells, IL-2 stimulated T cells are sensitive to TRAIL-mediated apoptosis, suggesting that TRAIL might be involved in the peripheral deletion of T cells [Ashkenazi and Dixit, 1999]. All these results suggest that exogenous TRAIL expression in pancreatic islets may have beneficial results in the setting of *type 1 diabetes* by virtue of its potential to retaliate against the assault by CTL.

THE SIGNIFICANCE AND THE NEED FOR THE COMPLEMENTARY GENE THERAPY MODALITIES IN ISLET TRANSPLANTATION

Prior to *in situ* transduction of pancreatic islets with viral vectors, the pancreas must be dissected from the patient and the islets need to be properly separated from the surrounding tissue [Van Linthout and Madeddu, 2005]. An

experimental pancreatic islet isolation scenario for the purpose of transplantation is depicted on Figure 4. Here, the islets go through a quality check (the number, live-death ratio and the purity etc.) following isolation before the transplantation. Later, the islets are ready to be transduced by gene therapy vectors, such as adenovirus. Adenoviral vectors are the most commonly used viral vectors in gene therapy clinical trials [Sanlioglu et al., 2003]. The importance of gene altered islets for transplantation has recently been reviewed [D'Anneo et al., 2006; Samson and Chan, 2006]. One such example of adenoviral transduction of rat pancreatic islets is depicted in Figure 5.

Despite high transduction levels and wide tissue tropism, adenovirus can only provide transient gene expression due to its inability to integrate into the host genome. Conversely, this integration defect can be advantageous, considering the increased malignancy risks associated with retroviral vectors [Woods et al., 2006]. Despite the antigenic properties of adenovirus [Doerschug et al., 2002], which is a major concern limiting transgene expression, the induction of the cellular immune response can be minimized using appropriate immunosuppressant regimens. For example, an adenovirus vector carrying hepatocyte growth factor reduced the minimal islet

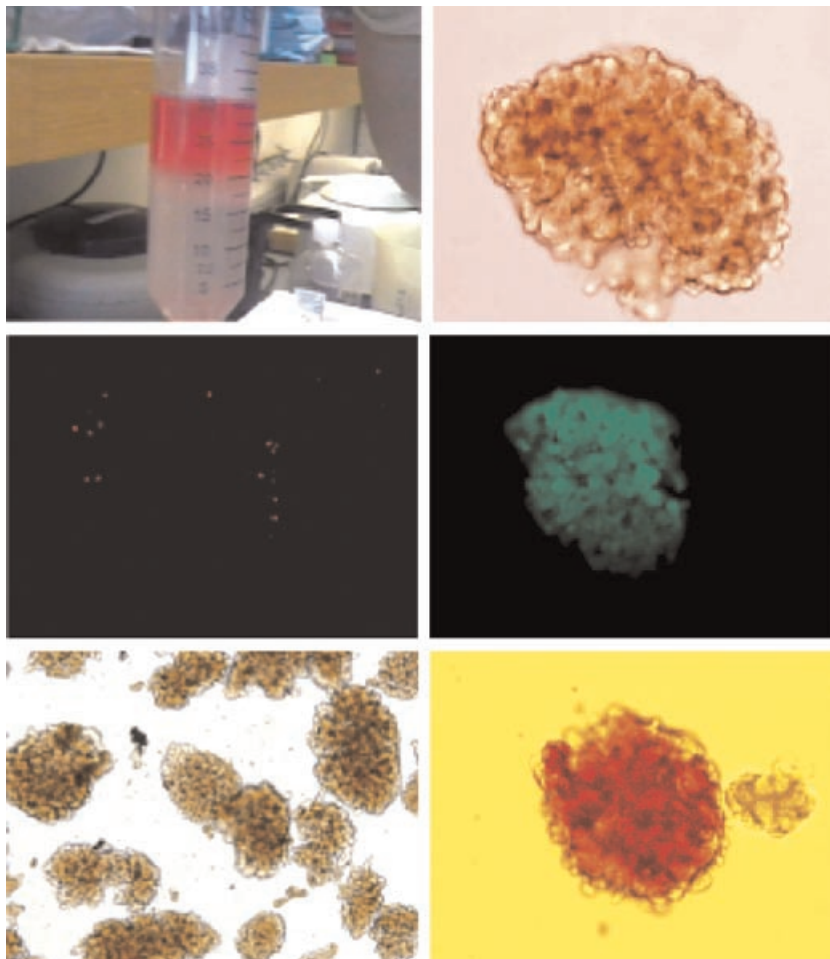


Fig. 4. Experimental pancreatic islet isolation and purification scheme for the transplantation purposes. Rat islets were isolated with in situ ductal diffusion of Liberase R1. Islets were separated with density gradient in Histopaque-1077. A view of an islet layer acquired during the pancreatic islet isolation procedure is given on the **top left panel**. A phase contrast microscopic view of a normal rat pancreatic islet is shown on the **top right panel**. Propidium Iodide (PI—**middle left panel**) and Fluorescein

Diacetate (FDA—**middle right panel**) stainings were performed for the cell viability and later analyzed under the fluorescent microscope. Bottom panels represent unstained isolated rat pancreatic islets (**left panel**) and Dithiazone (DTZ) staining indicating the cell purity (**right panel**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

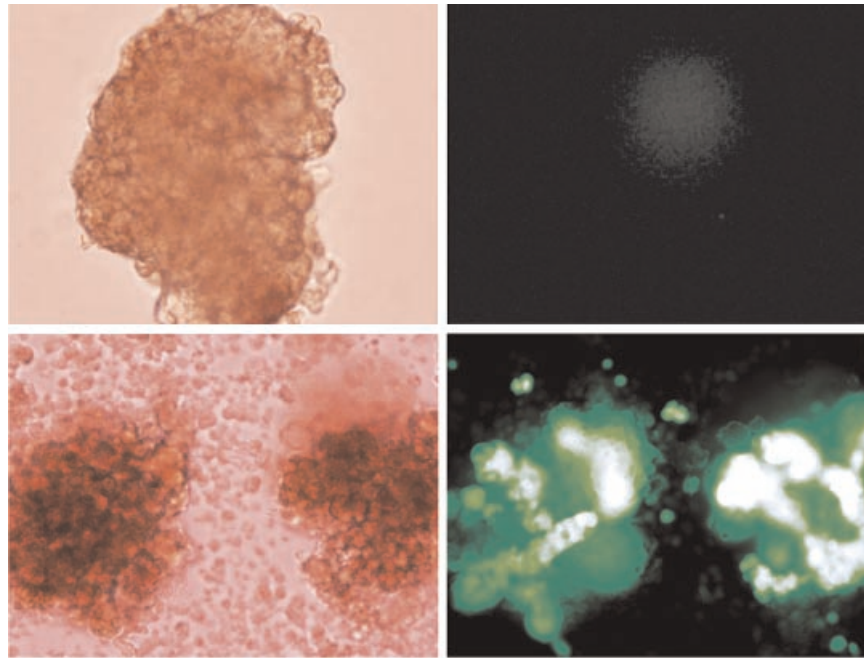


Fig. 5. First generation adenovirus transduction of rat pancreatic islets. Adenovirus vectors carrying the Enhanced Green Fluorescein Gene (AdEGFP) were infected into freshly isolated rat pancreatic islets. Fluorescein micrographs (200 \times) were taken 48 h after the infection using fully motorized Olympus IX81 inverted fluorescein microscope located at the

Human Gene Therapy Unit of Akdeniz University Faculty of Medicine. **Top panels** depict uninfected rat pancreatic islets. **Bottom panels** indicate AdEGFP transduced rat pancreatic islets. **Left panels** are the bright field images. **Right panels** are the fluorescein images. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

transplant mass required in a glucocorticoid free rat model of allogeneic portal vein islet delivery [Lopez-Talavera et al., 2004]. In addition, systemic delivery of adenovirus vectors with clamped liver circulation effectively transduced pancreatic islets in vivo [Ayuso et al., 2004]. There are various strategies currently being investigated as experimental gene therapy models for *type 1 diabetes* patients which are designed to subvert autoimmunity [Fernandes et al., 2004]. For example, recombinant adeno associated virus-IL10 (rAAV-IL10) injections reduced lymphocyte infiltration into the transplanted tissue and prolonged graft survival in NOD mice [Zhang et al., 2003]. Adenovirus vectors expressing TGF- β also protected pancreatic islets from autoimmune destruction [Suarez-Pinzon et al., 2002]. Lastly, intra-pancreatic CCL4 expression effectively suppressed inflammatory response targeting beta cells [Meagher et al., 2007]. Collectively, these studies suggest that improving islet graft survival is achievable in the experimental gene therapy animal models. Since normal adult pancreatic cells are resistant to TRAIL, this information alleviates the concerns about

TRAIL cytotoxicity upon exogenous TRAIL gene transfer into pancreatic islets and an adenovirus-mediated TRAIL gene transfer strategy (Ad5hTRAIL) [Griffith et al., 2000] should be very useful to over-express TRAIL in the pancreatic islets. Additional studies are needed, however, to understand the molecular mechanisms underlying islet graft survival to develop more effective treatment strategies against *type 1 diabetes*.

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Research article

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

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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 (AdIKK β KA) 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 AdIKK β KA 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 IKK β KA 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 TNF 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- κ B pathway [24,34,35]. Because NF- κ B 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- κ B 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- κ B activities using Luciferase reporter gene assays in MCF7 breast cancer cells. Because TRAIL-R1, TRAIL-R2 and TRAIL-R4 induced NF- κ B activation has been shown to be primarily mediated by TRAF2-NIK-I κ B kinase alpha/beta signaling cascade [35], MCF7 breast cancer cells were coinfecting with adenovirus vectors encoding a dominant negative mutant to IKK β (AdIKK β KA) [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- κ B. 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], AdIKK β KA [17], AdEGFP [18], AdCMVLacZ [40] and AdNF κ BLuc [38] was performed as previously described [41]. Amplified vectors were stored at -80 °C in 10 mM Tris with 20 % glycerol. AdIKK β KA expresses a dominant negative mutant of IKK β , which interacts with other IKK subunits to form inactive IKK complexes. The particle titers of adenoviral stocks were in the range of 10¹³ 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 % CO₂ 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 coinfecting with adenovirus vectors encoding IKK β dominant negative mutant (AdIKK β KA) and Ad5hTRAIL in order to block IKK activity thereby NF- κ B activation. NF- κ B promoter based Luciferase assay system was utilized to conduct NF- κ B transcription activation assays using AdNF κ BLuc construct. AdCMVlacZ vector was used as a control.

NF- κ B directed transcription activation assays

AdNF κ BLuc construct was utilized in order to determine the NF- κ B activation status of MCF7 cells. AdNF κ BLuc vector [38] possesses four tandem copies of the NF- κ B 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- κ B 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 100 μ g 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 μ g/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 μ g 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). $\Delta\Delta$ Ct 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- κ B activation pathway alone did not cause any reduction in the viability of MCF7 breast carcinoma cells

Because increased NF- κ B 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- κ B would reduce the viability of breast cancer cells. In order to block the intracellular anti-apoptotic NF- κ B pathway, MCF7 cells were infected with increasing MOIs of adenoviral vectors encoding a dominant negative mutant of IKK β (AdIKK β KA), a key molecule involved in the activation of NF- κ B. Cell viability was examined 48 hours following the infection under fluorescent microscope (Figure 2). Interestingly, AdIKK β KA vector alone proved inefficient in reducing the viability of MCF7 cells, even at an MOI of 10,000 DNA particles/cell.

Adenovirus delivery of IKK β KA gene expression sensitized MCF7 breast cancer cells to TRAIL-induced apoptosis

Adenovirus-mediated delivery of IKK β (Ad.IKK β KA) [17,18] or I κ B α (Ad.I κ B α SR) [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- κ B 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 coinfecting with a constant MOI of Ad5hTRAIL construct and increasing doses of AdIKK β KA 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 coinfecting with AdIKK β KA vector. For instance, nearly 55 % cell death was observed when cells were coinfecting with 1000 MOI of Ad5hTRAIL and 5000 MOI of AdIKK β KA 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 AdIKK β KA in breast cancer cells revealed no TRAIL sensitization (data not shown). These results suggested that IKK β KA expression via adenoviral vectors

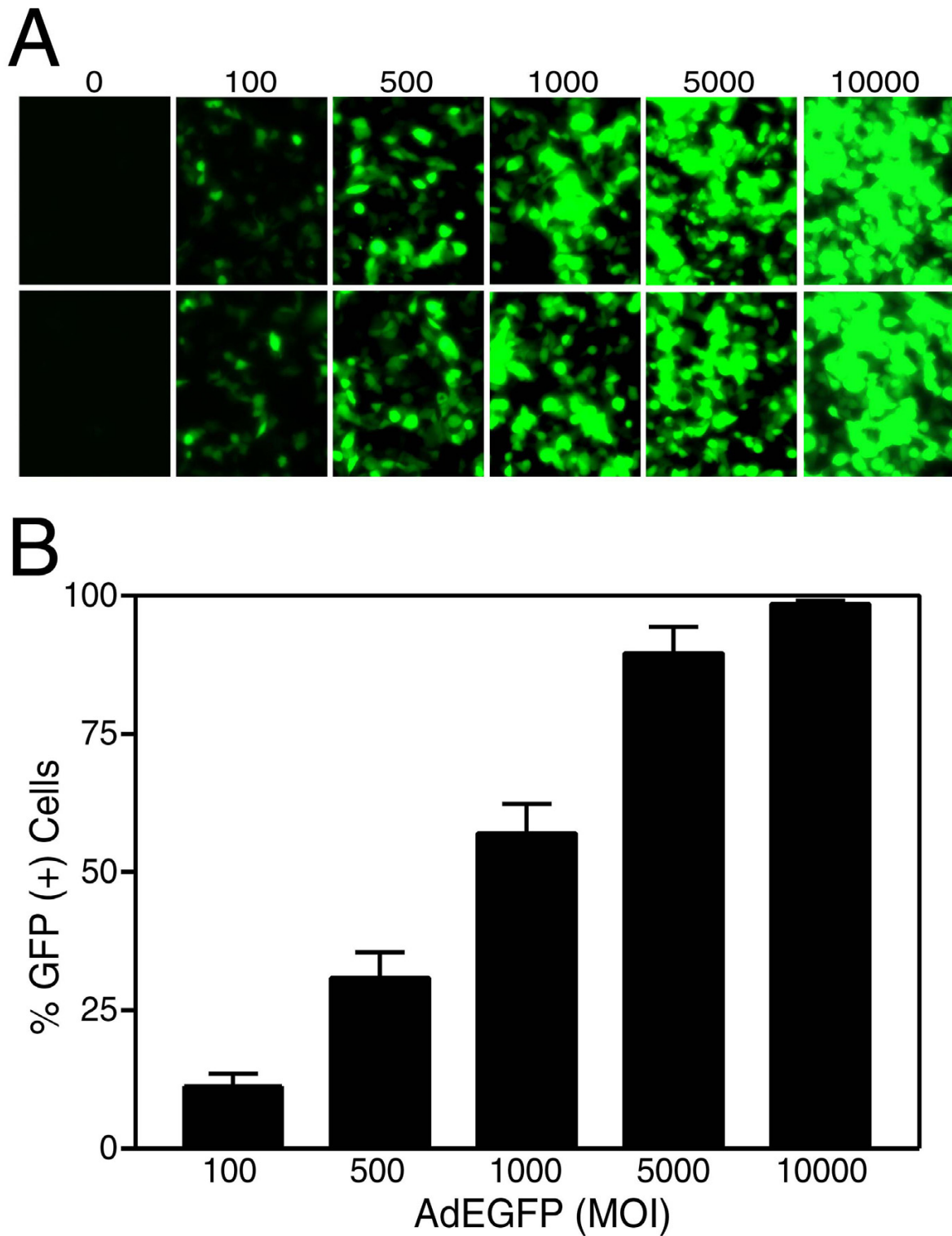


Figure 1
First generation adenoviral vectors efficiently transduced MCF7 breast cancer cells. MCF7 cells were infected with increasing MOIs of AdEGFP for 48 hours prior to analysis. The number of EGFP expressing cells was detected under fluorescent microscopy (Panel A), and analyzed by flow cytometry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

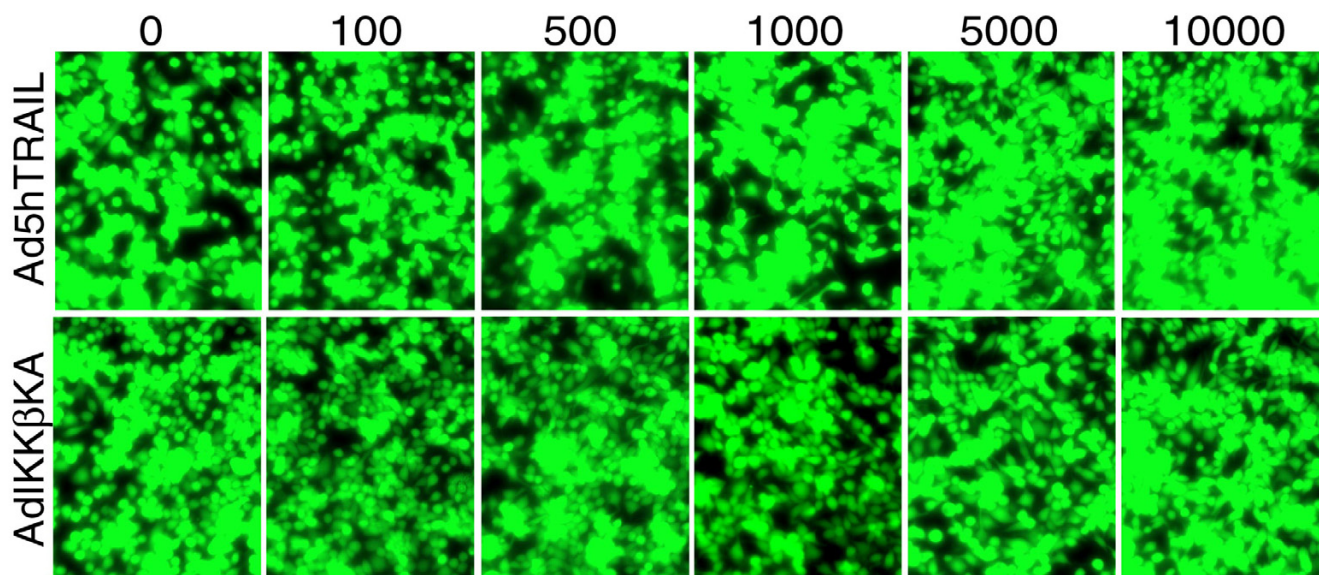


Figure 2

Ad5hTRAIL or AdIKK β KA infection alone did not decrease the viability of MCF7 breast cancer cells. MCF7 cells were infected with increasing MOIs of either Ad5hTRAIL or AdIKK β KA 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- κ B activity in MCF7 cells, whereas IKK β KA expression blocked both TRAIL-induced and basal NF- κ B activities

It is well known that different tumor cells display diverse levels of endogenous NF- κ B activities. Furthermore, intracellular NF- κ B 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- κ B 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- κ B driven Luciferase reporter gene (AdNF κ BLuc) and Ad5hTRAIL vector in order to study the extent of NF- κ B activation as a result of TRAIL overexpression in MCF7 breast cancer cell line. NF- κ B Luciferase assays were conducted 24 hours following the infection in order to determine cell's NF- κ B 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- κ B activation. In order to determine the magnitude of NF- κ B inhibition, a triple coinfection exper-

iment involving AdNF κ BLuc, Ad5hTRAIL and AdIKK β KA or AdCMVLacZ was performed. While IKK β KA overexpression in MCF7 cells gradually reduced both the TRAIL-induced and basal NF- κ B activities in MCF7 cells, no such NF- κ B inhibiting effect was observed in cells upon superinfection with AdCMVLacZ virus as a control (Figure 5).

Coinfection of Ad5hTRAIL and AdIKK β KA 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 AdIKK β KA 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 IKK β KA in MCF7 cells. However, considerable levels of Annexin V binding were observed in cells coinfecting with Ad5hTRAIL and AdIKK β KA 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

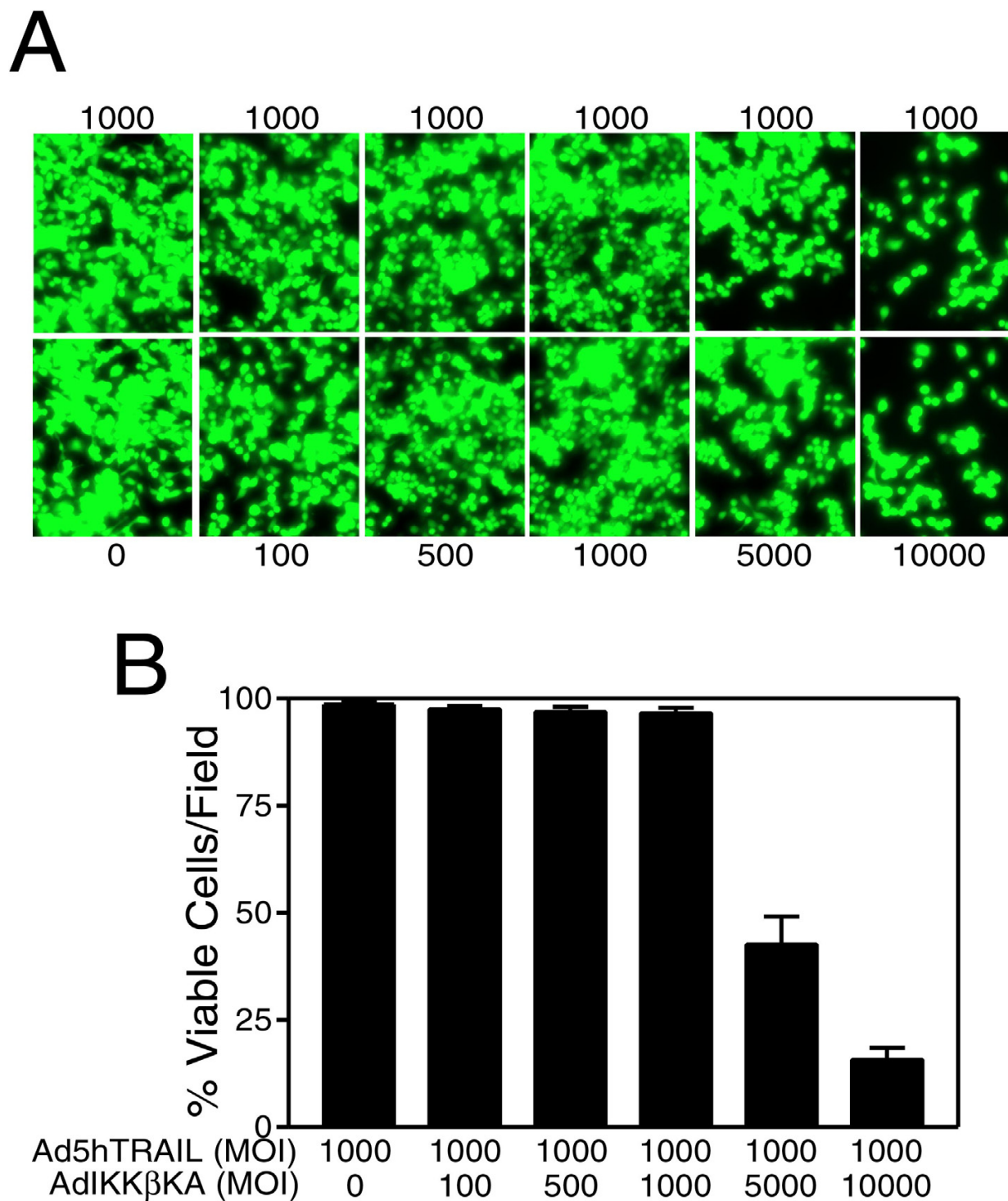
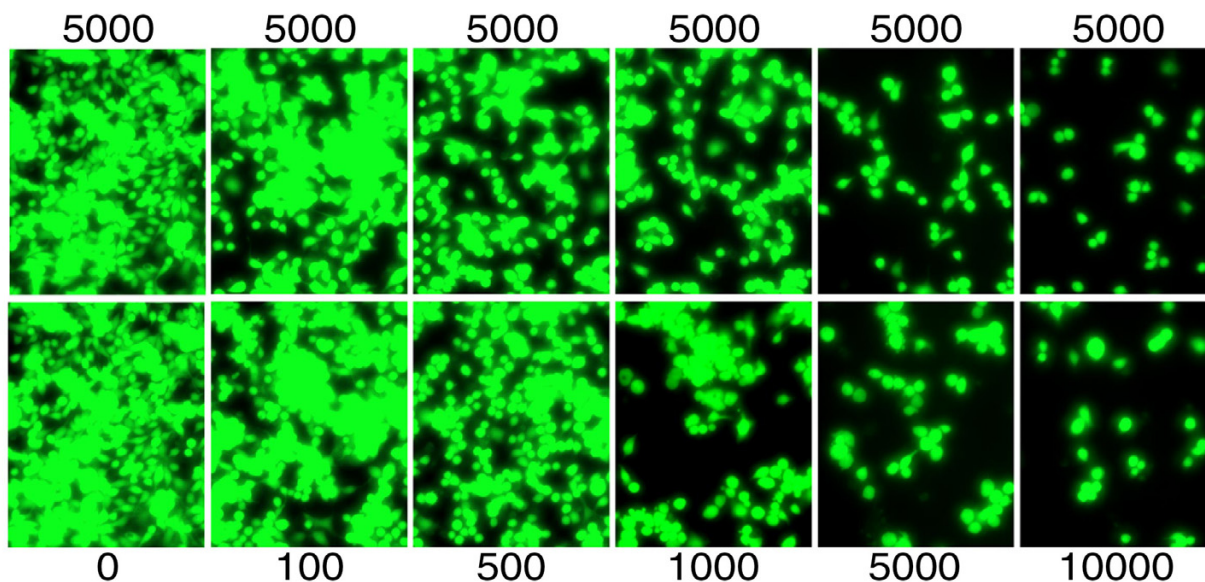


Figure 3
 IKKβKA expression via adenoviral vectors sensitized MCF7 cells to TRAIL-mediated apoptosis. MCF7 cells were infected with increasing doses of adenoviral vectors encoding dominant negative mutant of IKKβ (as shown below each panel), while simultaneous infection with Ad5hTRAIL (as shown above 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.

A



B

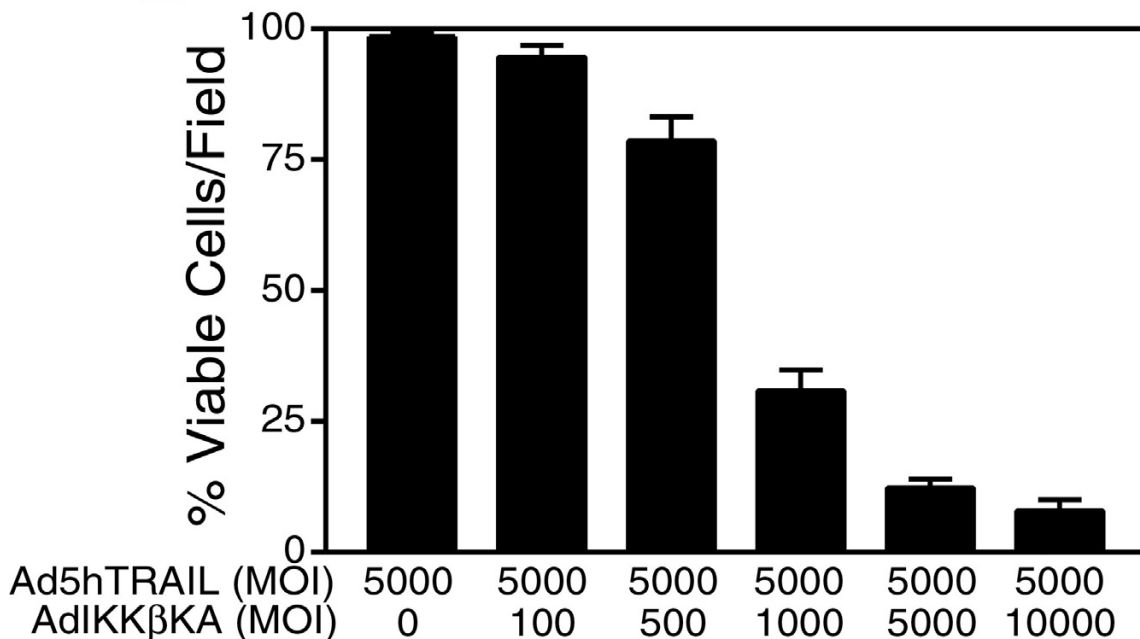


Figure 4
 AdIKKβKA infection defeated the resistance to TRAIL-induced apoptosis in MCF7 breast cancer cells. These cells were coinfecting with a constant MOI of 5000 DNA particles/cell of Ad5hTRAIL (as shown above each panel) and increasing doses of AdIKKβKA (as shown below each panel). Live/Dead Cellular 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).

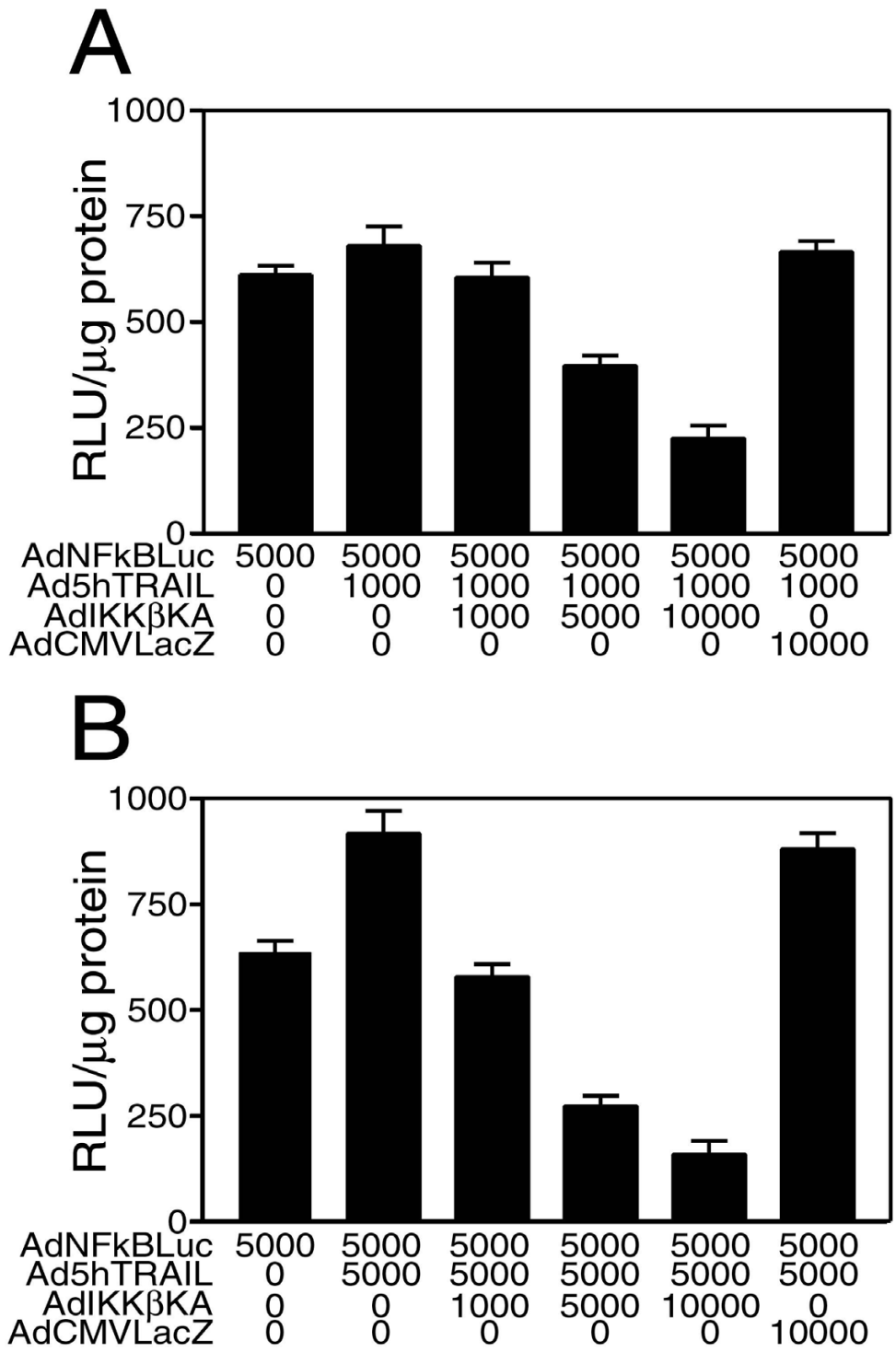


Figure 5
 Distinctive regulation of NF-κB activation in MCF7 breast cancer cells by Ad5hTRAIL and/or AdIKKβKA infections. MCF7 cells were simultaneously infected with AdNFκBLuc, Ad5hTRAIL and/or increasing doses of AdIKKβKA construct for 24 hours. AdCMVLacZ infection was also performed as a negative 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).

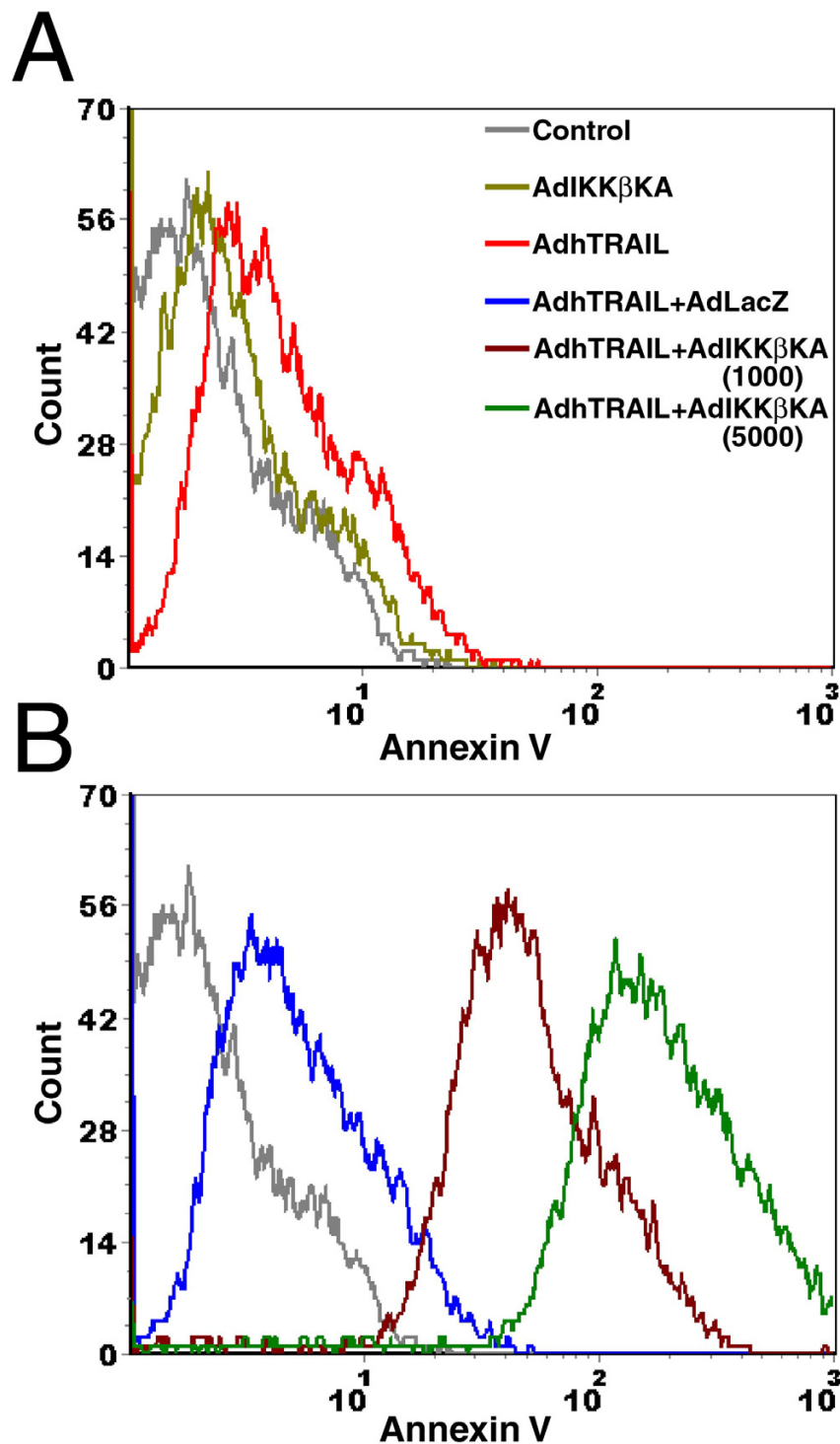


Figure 6

Ad5hTRAIL and AdIKK β KA 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 10⁴ 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- κ B 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- κ B activation has been shown to be primarily mediated by TRAF2-NIK-I κ B kinase alpha/beta signaling cascade [35], there is some doubt on whether or not NF- κ B activation can block TRAIL mediated apoptosis. For example, in one particular study it was reported that NF- κ B inhibition by way of I κ B α mutant expression sensitized MCF7 cells to TNF but not TRAIL-induced apoptosis [35]. Considering the fact that there are different ways to activate NF- κ B pathway (I κ B dependent and independent ways) [46] we decided to inhibit IKK activity rather than targeting I κ B α 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

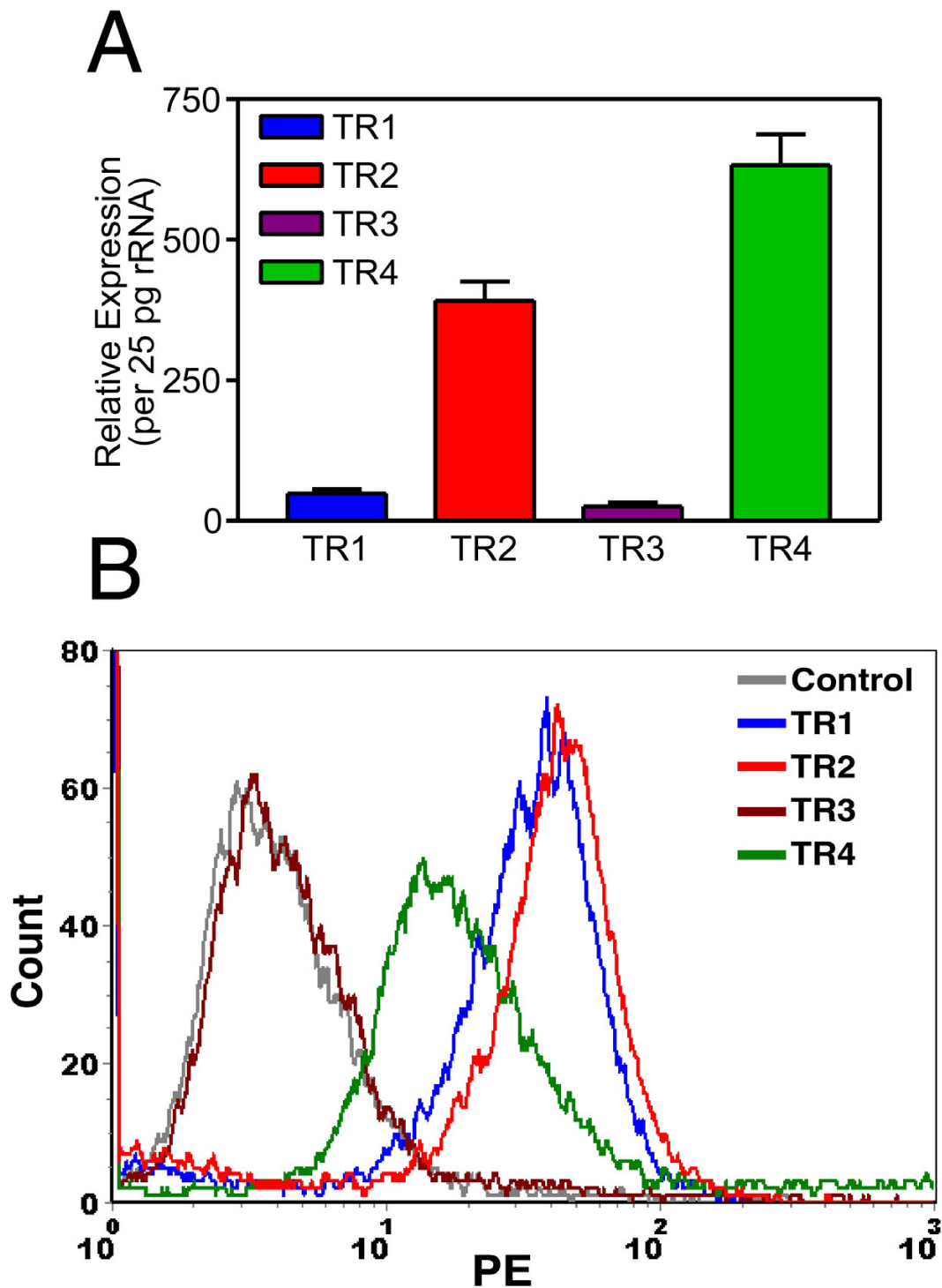


Figure 7

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.

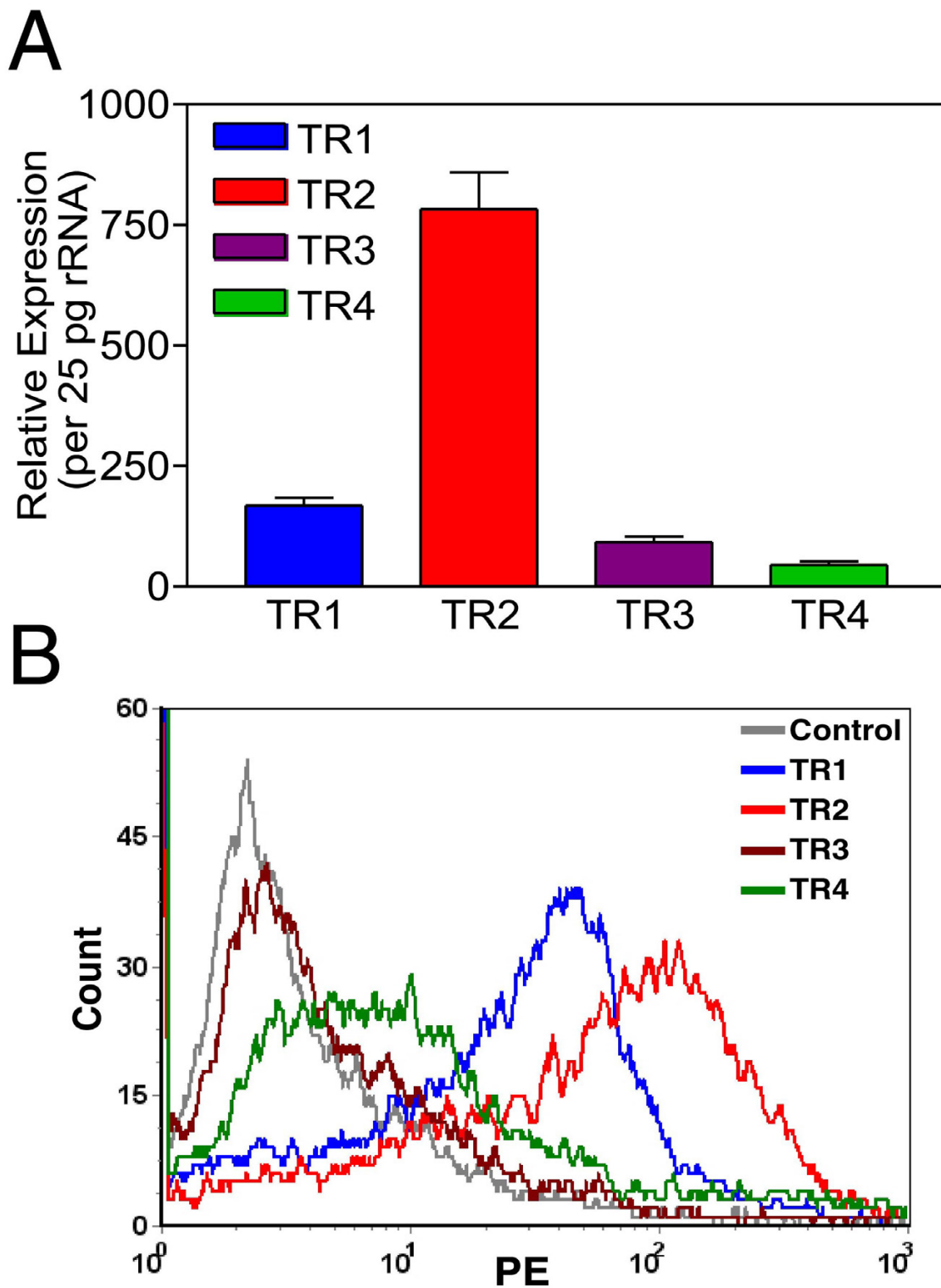


Figure 8
 MDA-MB-231 breast cancer cells displayed trivial levels of TRAIL-R4 decoy receptor expression on surface. TRAIL receptor composition of MDA-MB-231 breast cancer cells revealed by Real Time RT-PCR assay is displayed in Panel A. Panel B illustrates flow cytometry analysis showing the surface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

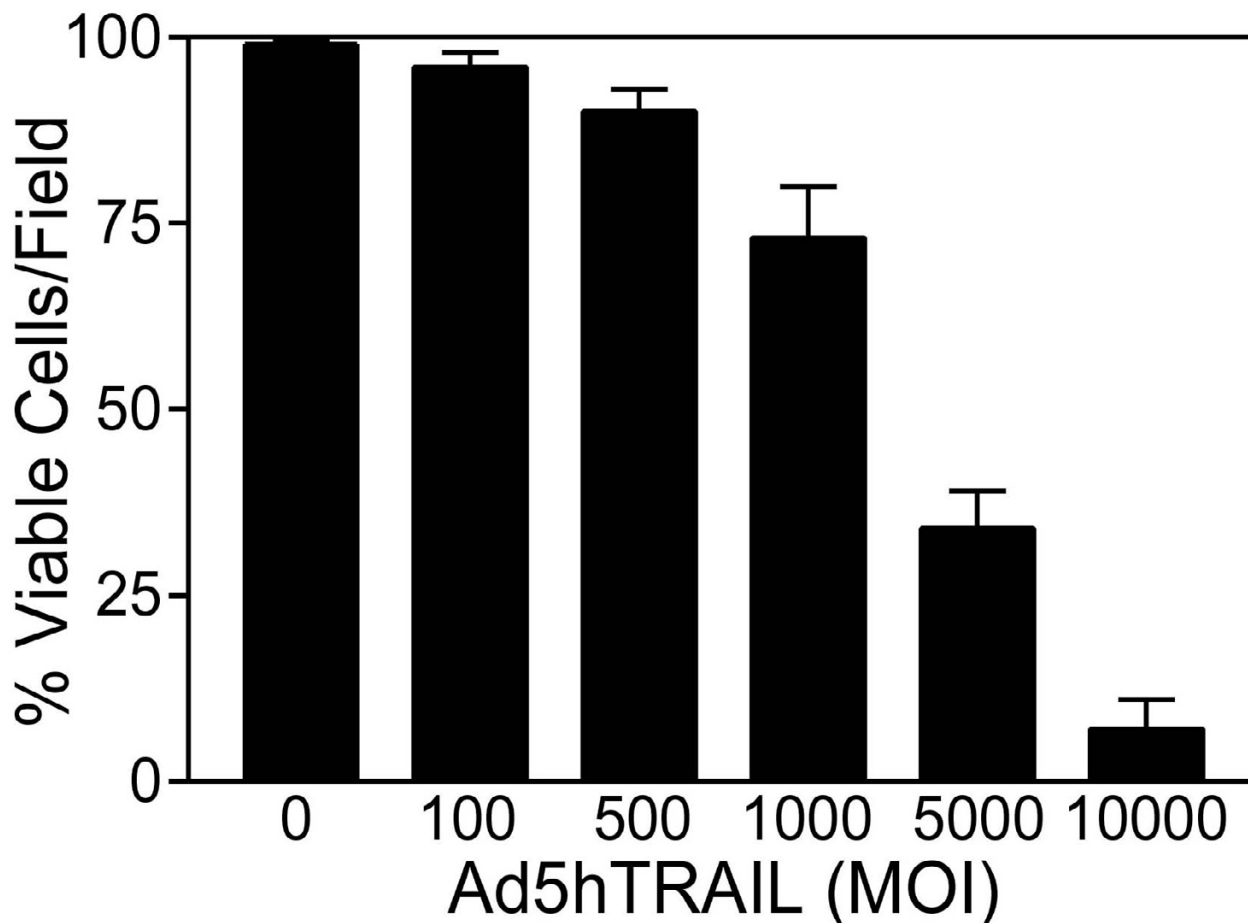


Figure 9

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 (\pm 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 AdIKK β KA 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 AdIKK β KA virus (Figure 5). Moreover, IKK β KA 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

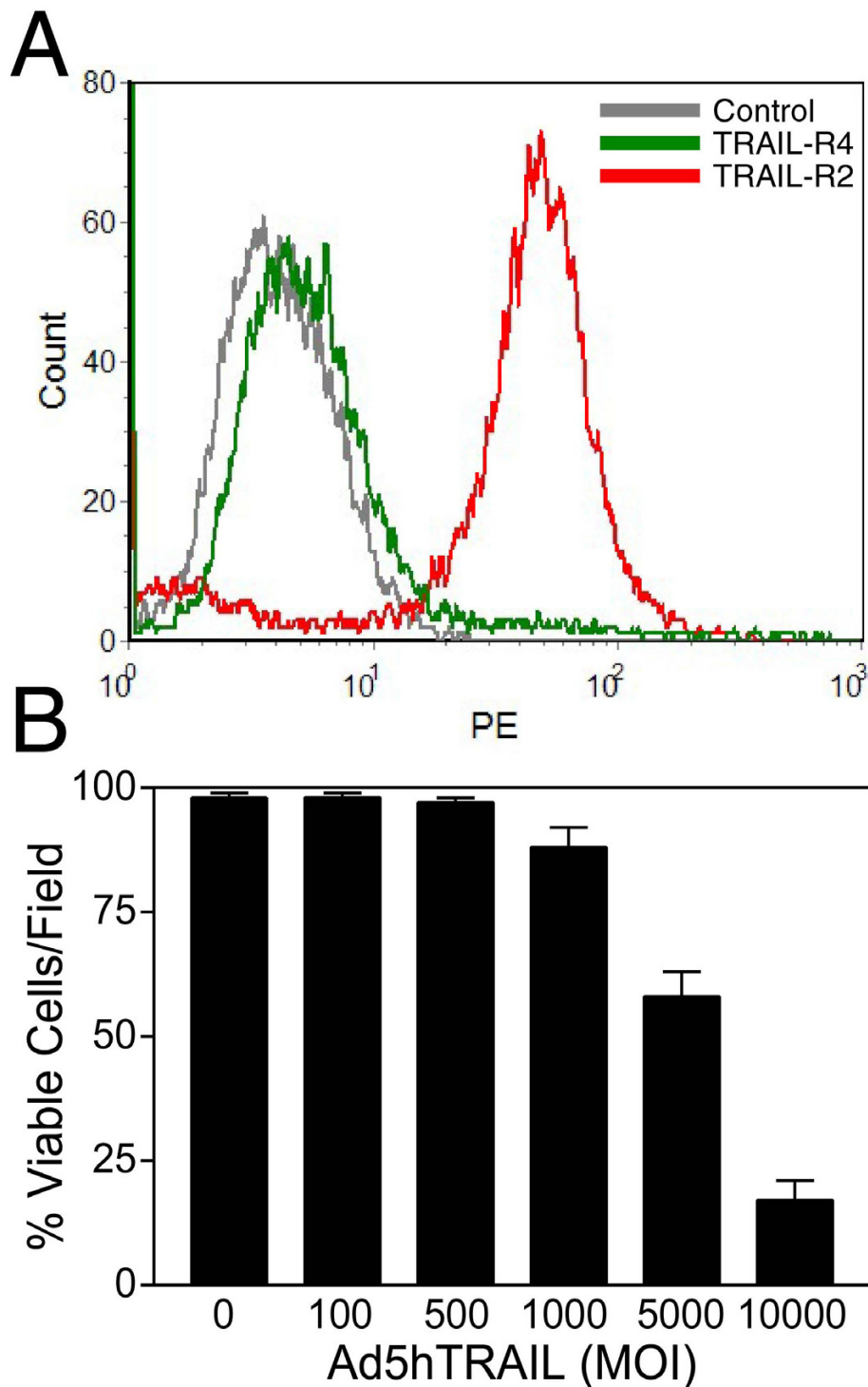


Figure 10

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 (\pm 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- κ B 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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