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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 autoimmune 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 (insulitis) generally 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 insulitis 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 autoantigen 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,

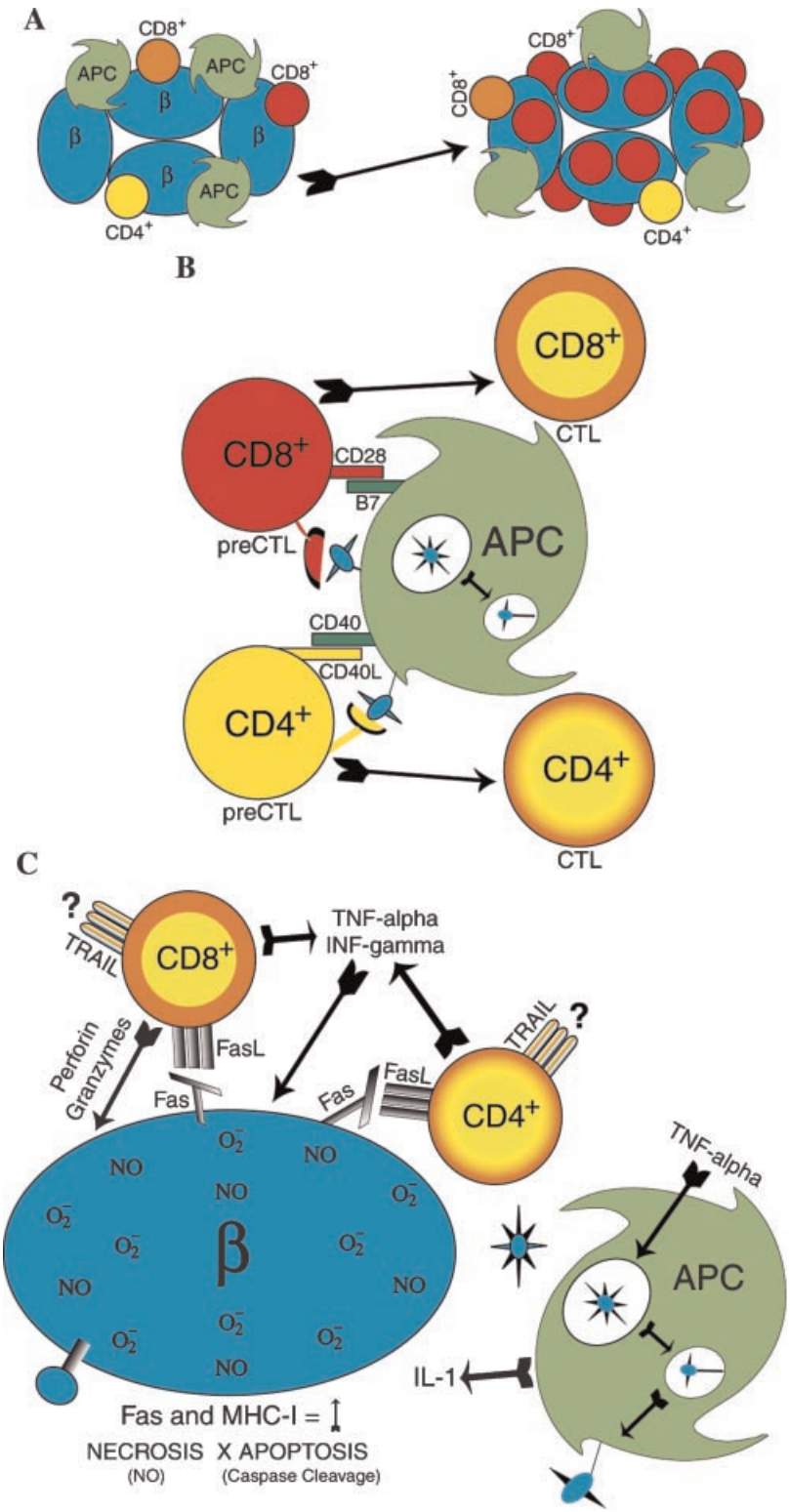

Fig. 1. An inflammatory model for the pathogenesis of *type 1 diabetes*. Prolonged insulitis leads to preferential amplification of autoreactive $CD8^+$ T cells bearing high affinity receptors for islet antigens (**Panel A**). Differentiation of $CD8^+$ and $CD4^+$ T cells into effector cytotoxic T cells (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 INFγ 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-κB 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

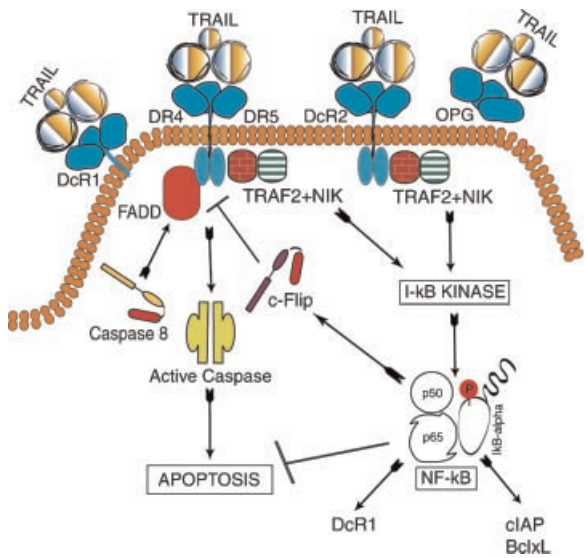

Fig. 2. TRAIL receptor signaling pathway. Activation of TRAIL receptor 1 (DR4) or 2 (DR5) by trimeric TRAIL ligands leads to the recruitment of Fas-associated death domain protein (FADD) to the membrane. Then, FADD recruits procaspase 8 to form death inducing signaling complex (DISC). DISC-induced signaling 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-κB signaling can also be activated by TRAIL and TRAIL-R4 (DcR2) interaction in an IκB Kinase (IKK) dependent fashion. TRAIL-R3 (DcR1) has a truncated cytoplasmic domain, allowing it to serve as a decoy receptor for TRAIL. Interestingly proinflamotory cytokine (TNF and IL-1) dependent activation of IKK and thereby NF-κB, has been claimed to induce apoptosis in pancreatic islets. If so, the reason why cells do not undergo apoptosis when DcR2 activates NF-κB 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-κB signaling via TRAF-2 and NIK complicates this issue further. Four different ways of inhibiting TRAIL-mediated apoptosis by way of NF-κB 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-κB signaling pathways [Degli-Esposti et al., 1997; Sanlioglu et al., 2005, 2006]. Thus, TRAIL over-expression in islets is expected to activate NF-κB signaling as well. However, the consequence of TRAIL-induced NF-κB activation in islets is not known. NF-κB is well known for its anti-apoptotic properties as demonstrated in cancer cells. Intriguingly, proinflammatory cytokine activation of NF-κB 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-κB signaling is beneficial or detrimental for the islet-graft survival in islet transplantation [Kim et al., 2007]. Nevertheless, the consequence of TRAIL-mediated NF-κB activation might differ from proinflammatory cytokine-induced NF-κB signaling.

Previous studies demonstrated that NF-κB inducing agents up-regulated cFLIP synthesis blocking caspase activation [Kreuz et al., 2001]. In addition, NF-κB 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-κB signaling [Mitsiades et al., 2002]. Based on these results, there are at least four different ways to block TRAIL-induced apoptosis through NF-κB (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-κB 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 [Satoh 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

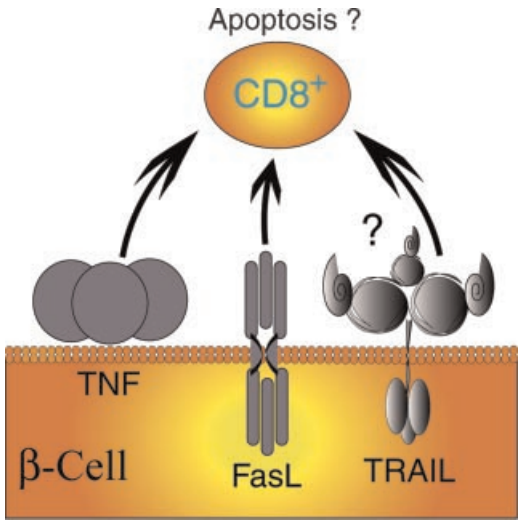

Fig. 3. Death ligand expression in pancreatic islets and its potential outcome. These therapeutic approaches are designed to prolong the graft survival in patients with *type 1 diabetes*. Pancreatic islet grafts protected from the immune-mediated cytotoxic T cell attack are expected to function longer 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 insulitis [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 regiments. For example, an adenovirus vector carrying hepatocyte growth factor reduced the minimal islet

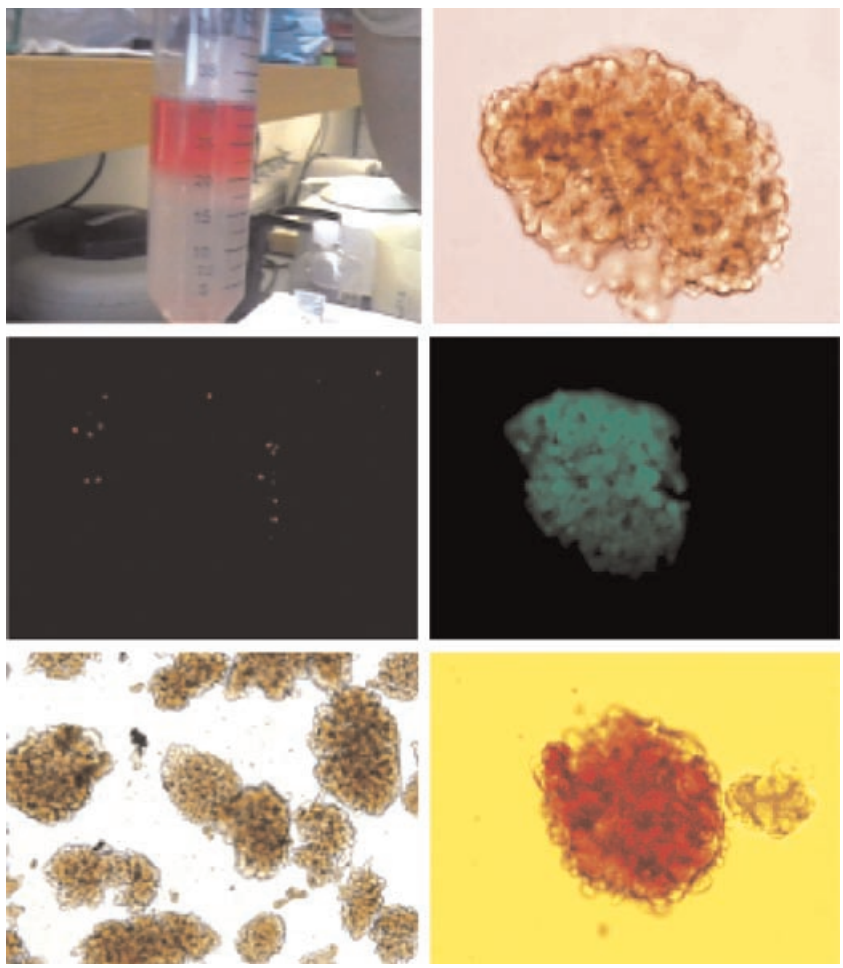

Fig. 4. Experimental pancreatic islet isolation and purification scheme for the transplantation purposes. Rat islets were isolated with in situ ductal diffusion of Liberase R1. Islets were separated with density gradient in Histopaque-1077. A view of an islet layer acquired during the pancreatic islet 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.]

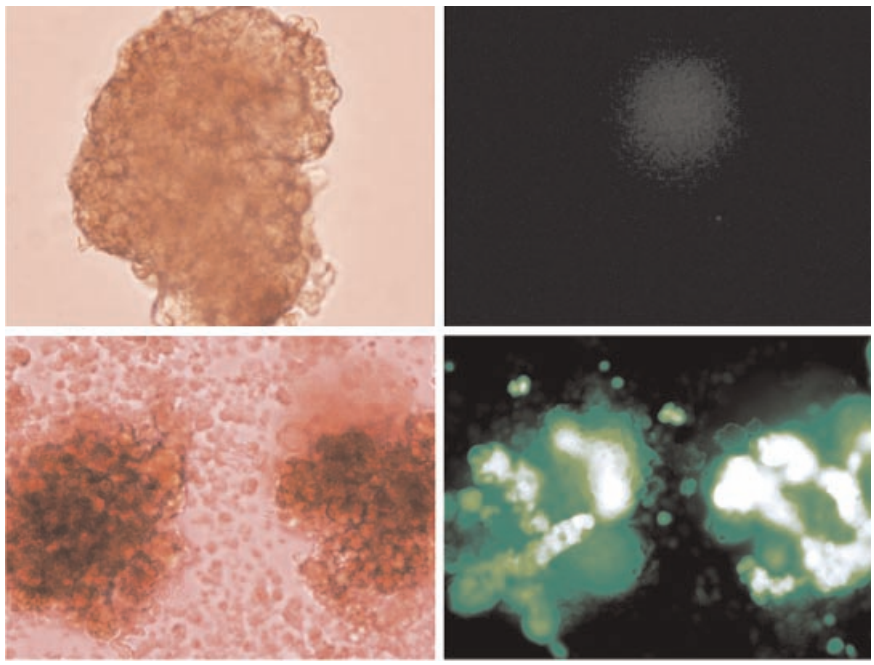

Fig. 5. First generation adenovirus transduction of rat pancreatic islets. Adenovirus vectors carrying the Enhanced Green Fluorescein Gene (AdEGFP) were infected into freshly isolated rat pancreatic islets. Fluorescein micrographs (200×) were taken 48 h after the infection using fully motorized Olympus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