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Mouse-ear-heart transplant model : evaluation using TNFR 1 knockout

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MOUSE-EAR-HEART TRANSPLANT MODEL: EVALUATION USING TNFR 1 KNOCKOUT

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ABSTRACT

MOUSE-EAR-HEART TRANSPLANT MODEL: EVALUATION USING TNFR 1 KNOCKOUT

By Daniel W. Woolley

The role of TNFα and its receptor TNFR 1 are implicated in the cardiac transplant rejection process but their roles have yet to be identified in murine allograft cardiac transplant rejection models. This thesis uses the mouse-ear-heart transplant model to evaluate the role of TNFR 1 in the cardiac transplant rejection processes. The experiment utilizes allograft (Balb/c to C57Bl/6; H-2^d to H-2^b) and isograft (C57Bl/6 to C57Bl/6; H-2^b to H-2^b) mouse transplanted cardiac tissues along with C57Bl/6 TNFR 1 knockout subjects to evaluate the receptor's role in cardiac rejection. Increased viability was identified in TNFR 1 deficient subjects compared to controls. Mean transplant survival time for allograft knockouts was 15.2 ± 2.7 and 10.5 ± 2.1 ($P = < 0.0001$) days for allograft controls. These results demonstrate the possible involvement of TNFR 1 in cardiac transplant rejection.

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Introduction

Cytokines are communication proteins produced by immunologically competent cells during the host response to infection, invasion, injury, or inflammation (1). The predominate cause of illness (e.g., fever, shock, and weight loss) are often not caused directly by invading pathogen, but are caused by a cascade of cytokine effects. Indeed, disease morbidity and mortality are frequently caused by an over-expression of cytokines by the body's immune system (1). Fever, shock, and weight loss are typically seen in transplant patients undergoing transplant organ rejection. Although it remains unclear as to the extent each cytokine undertakes in the host response to an invading organism, it is accepted that cytokines, in general, are major components in the overall mechanism leading to an immune response. One such cytokine, Tumor Necrosis Factor alpha (TNFα), has been linked to transplant rejection (2). TNFα has also shown significant involvement in the inflammatory process (2). TNFα is mediated through two receptors, Tumor Necrosis Factor Receptor One (TNFR 1) and Two (TNFR 2) (2, 3). TNFR 2 has not been implicated as having a potential role in the rejection process or inflammation.

Mechanism of Action

In responding cells, TNFα alters gene expression of transcription factors tyrosine kinase, serine kinase, NFκB, phospholipase A2, and GTP-binding proteins (1, 4). *In vitro* studies have demonstrated that TNFα causes activation and differentiation of monocytes and macrophages and this activation initiates two pathways producing substances toxic to both host and invading cells (3). The superoxide pathway produces

O_2 and H_2O_2 (5). The L-arginine dependent pathway causes activation of nitric oxide synthase (iNOS) and produces reactive nitrogen intermediates (5, 6).

Until recently, the precise mechanism and signal transduction pathway of TNFα binding to TNFR 1 was not completely understood. The activation of these pathways has detrimental consequences to the cell and surrounding tissue. Chen and Goeddel have found transcription factors are eventually responsible for the biological processes of immune and inflammatory responses initiated by TNFR 1 and the pathway responsible for these observations (7). Once TNFα is bound to TNFR 1, there is a release of the inhibitory protein silencer of death domains (SODD) from TNFR 1's intracellular domain (ICD). The resulting aggregated TNFR 1 ICD is recognized by the adaptor protein TNF receptor-associated death domain (TRADD), which recruits additional adaptor proteins receptor-interacting protein (RIP), TNF-R-associated factor 2 (TRAF2) and Fas-associated death domain (FADD) (7). This complex is responsible for the activation of several pathways. RIP plays an essential role in the activation of transcription factor, nuclear factor κB (NF- κB), but enzymatic activity of RIP is not required for its TNF-induced activation (7). NF- κB is a ubiquitous transcription factor that, when activated, translocates to the nucleus, binds to DNA, and promotes transcription of many target genes (8). NF- κB activation is required for cytokine induction of the human iNOS gene and activation has been demonstrated in inflammatory conditions (9).

Effects of TNFα

The TNFα effect varies with respect to tissue type and is determined by receptor

subtype. *In vivo* consequences of systemically increased TNFα levels are fever, lethal shock, tissue injury, anorexia, catabolic state, and increased expression of inflammatory cytokines (IL-1, IL-6, IFN-gamma) (10). TNFα has a positive feedback effect resulting in self-amplification and increased severity of symptoms (2).

TNFα strongly activates and attracts polymorphonuclear leucocytes (PMN). The activation is made evident by enhanced phagocytosis, induction of degranulation, and stimulation of superoxide production (11). TNFα also induces the expression of endothelial leucocyte adhesion molecule-1 (ELAM-1) on endothelial surfaces, which is a powerful inducer of PMN migration (12). In addition, TNFα also induces rapid adhesion of neutrophils and eosinophils to monolayers of endothelial cells, thus damaging endothelial tissue (13). Finally, TNFα induces the release of growth factors granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) (14). These growth factors not only act on bone marrow but also affect existing PMN and monocytes. This is substantiated by the observation that circulating levels of TNFα correlate with a marked increase in levels of high circulating leukocytes *in vivo* (14). The induction of superoxide production, regulation of key cellular adhesion molecules, induction growth factors might significantly contribute to the body's rejection processes and the promotion of graft-versus-host disease (GVHD) severity.

TNFα in Transplantation

Elevated levels of TNFα in both tissue and serum were observed during rejection of transplanted organs (15). Administration of anti-TNFα antibodies improves

vascularized cardiac allograft survival in rats (15). There is no direct evidence, however, linking allograft animal models and human GVHD since both are not fully understood. It would be a reasonable assumption that the same mechanism of tissue damage may be operating in both GVHD and allograft rejections. Models using isograft and allograft transplants, as is the case here, can be used to study the involvement of cytokines and their receptors. An isograft is tissue transplanted between genetically identical donor and recipient. This results in the best-matched transplantation with the lowest chance of rejection. Alternatively, an allograft is transplanted tissue between dissimilar members of the same species. The results of such studies can then be correlated to rejection processes occurring in GVHD.

It has been previously reported that murine and human TNFR 1 appear to play identical roles in the inflammatory process of allograft rejection (16). In this paper, an investigation of TNFR 1 and its involvement in organ rejection using a murine animal model was studied. By incorporating a TNFR 1 knockout into the model, the extent of the receptors involvement in the rejection process was investigated.

The mouse ear-heart transplant model has been used to determine graft viability when viability is defined as the length (days) a beating transplanted neonatal heart continuously produces a consistent rhythmical contraction (17, 18, 19). The procedure, as presented here, involves transplanting neonatal allograft (Balb/c to C57Bl/6; H-2^d to H-2^b) and isograft (C57Bl/6 to C57Bl/6; H-2^b to H-2^b) cardiac whole hearts into the pinnae of adult mouse ears. Balb/c to C57Bl/6 transplants are known to be dissimilar by

their histocompatibility complex noted as H-2^d or H-2^b these cells posses on their surface. Immune cells can identify these cells as foreign and mount an immune offensive against the foreign tissue. C57Bl/6 to C57Bl/6 cardiac transplants are genetically identical in their histocompatibility complex and are less likely to induce an immune response. This is an established and accepted model for performing cardiovascular transplant rejection research (17, 18, 19). In an attempt to understand the role TNFR1 in the rejection of cardiac rejection in transplant recipients, the procedure will include TNFR 1 knockout mice of C57Bl/6 background. Specifically, TNFR 1 knockout mice are hypothesized to have an increased viability as opposed to the allograft wild type in the mouse ear-heart transplant model.

Research Design and Methods

Animals

The procedure described here was approved by the Institutional Animal Care and Use Committee (IACUC) of San Jose State University and Tularik, Inc. C57Bl/6 and Balb/c (6-10 weeks) mice were purchased from Harlan-Sprague-Dawley (Indianapolis, IN) and housed in the Tularik, Inc. mouse breeding facility. TNFR 1 knockout mice were obtained from Tularik breeding facility via Dr. Tak Mak, Amgen Institute, Ontario Cancer Institute. TNFR 1 homozygous knockout mice were tail snipped and had DNA tested for presence of TNFR 1 using Genomic DNA Tissue Kit® from Bio-Rad Laboratories. TNFR 1 knockout mice are homozygous for the absence of the cell surface receptor TNFR 1. One male and two female adult mice were housed until sufficient

experimental neonatal donors and recipient adult mice were obtained. Recipient mice were housed in cages consisting of 4-5 mice. All animals were housed in Thoren, 24x24 inch plastic cages. Mice were under controlled 12-hour light/dark cycles, fed a standard laboratory diet (Purina Picolab 5053), and given water *ad libitum.* Due to the unpredictability of liter size and the length of time required to successfully fill outlined protocols, the number of animals per group was modified according to availability.

Surgical Procedure

Recipient mice (6-10 weeks) were anesthetised using ketamine HCl at 80mg/kg body weight with xylazine 16mg/kg body weight (intraperitoneal) and placed on a mat. Animals were observed every five minutes using response to toe pinch as an indicator of the level of anesthesia. The dorsal base of the mouse ear was shaved with an electric clipper and washed with 70% ethanol. All surgical instruments were sterilized in a hot bead sterilizer for 30 seconds. Using spring scissors, a small incision was made through the epidermis and dermis caudal to the centrally located blood vessels. The incision was then spread open down to the cartilage, parallel to the head. The ear was gripped at the base and forceps were advanced through the incision between the skin and cartilage. Downward pressure was maintained toward the cartilage to form a tunnel approximately 10-12 mm in length running parallel to the major arteries.

Neonatal TNFR 1 wild type unsexed mice, 24-48 hours old, were cervically dislocated using sterile scissors. The ventral surface was then disinfected with 70% ethanol and wiped with sterile gauze. The thorax was opened using scissors. Neonatal

hearts were then grasped at the apex with sterile forceps and cut superior to the atrial chambers. The heart was placed in a sterile dish containing phosphate buffered saline and allowed to contract. A single whole heart was then placed into the open space made in the recipient mouse ear using a sterile forceept. If there was any air in the pocket, it was gently expressed out of the tunnel leading from the pocket to the incision point. No suturing or bonding was required in this experiment. Wounds usually posessed a small scab that completely healed by day 6-7 after surgery. Mild scar tissue was noticable at base of ear where initial incision had been made.

Graft Viability

Visual observations or an electrocardiogram (Biopac Systems, Inc. MP100® Electrocardiogram Recording System) were used to determine if grafts were viable. Electrocardiogram were taken only when visual confirmation was unattainable. When electrocardiograms were needed, recipient mice were anesthetised using ketamine HCl at 80mg/kg body weight with xylazine 16mg/kg body weight (intraperitoneal). A pair of teflon-coated 28-gauge needle electrodes were then placed through the ear in opposition 1-4 mm from the graft. Care was taken to avoid any blood vessels while connecting electrodes. If no beating was observed, the graft was gently taped to stimulate rythmical beating. Consecutive beating of at least one minute was the criteria for viability.

The observation of graft viability was made on days 3, 5, 7, 9-20, 30, 33, and 35. Rejection was defined as cessation of electrical activity on two consecutive days. Upon

determination of cessation of beating, the animal was sacrificed and grafts immediately placed in 10% formalin at room temperature for eventual haematoxylin and eosin (H & E) staining. Sectioning and staining of tissues was performed at Histo-Tec Laboratories; Hayward, CA. Briefly, tissue was embedded in parafin wax and sectioned at a thickness of 3-5 microns. The film was then H & E stained according to the protocol described by Kiernan, 1996 (20).

Statistics

Data shown are mean ± standard deviation of the number of days post-transplant that the graft remained viable. An analysis of variance was performed ($\alpha = 0.05$, 95% confidence) to determine significance. Final comparison between the groups was done by performing multiple t-tests. Minitab® (Minitab, Inc. State College, PA) statistical package software was used for all statistical outputs.

Results

Group size varied due to success rates of the surgical procedure and neonatal availability. A transplanted heart that beat continuously for two consecutive days was considered a surgical success. A total of 59 surgeries were performed and 26 resulted in a viable graft (44.1%) when solely using visual observations as an indicator of success. Electrocardiography demonstrated an enhanced surgical success rate by increasing the number of clearly identifiable viable grafts from 26 to 41 subjects out of 59 surgeries (69.5%). Figures 1-3 are actual recordings taken from the Biopac MP100® system recording electrical activity on recipient mouse ears. Clearly recognizable ECG activity

was recorded from surgeries that were successful versus those that were failures.

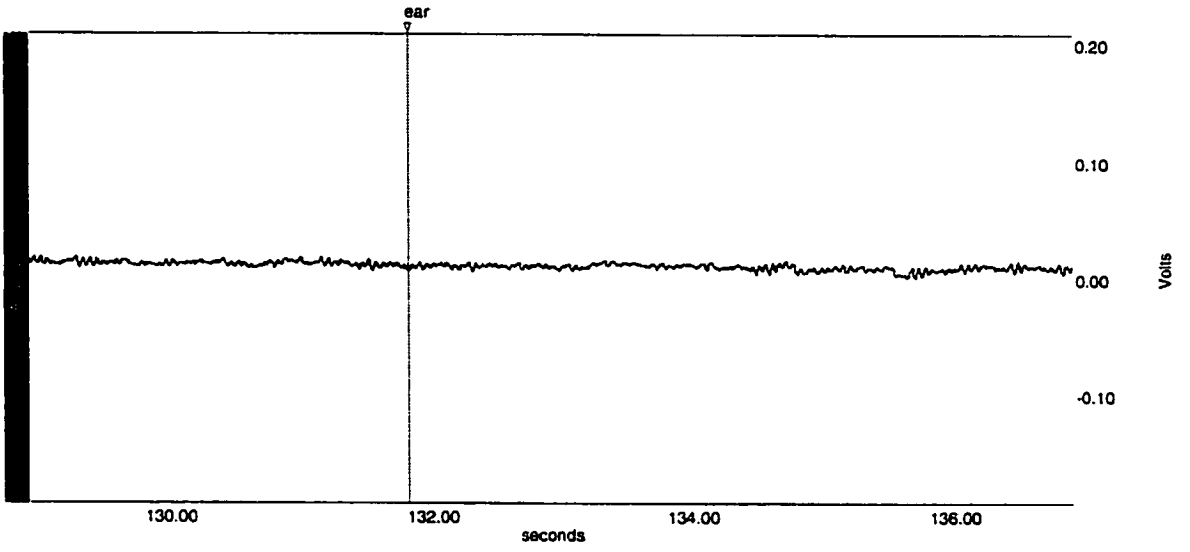

Figure 1: Recording taken on ear that did not contain a transplanted heart.

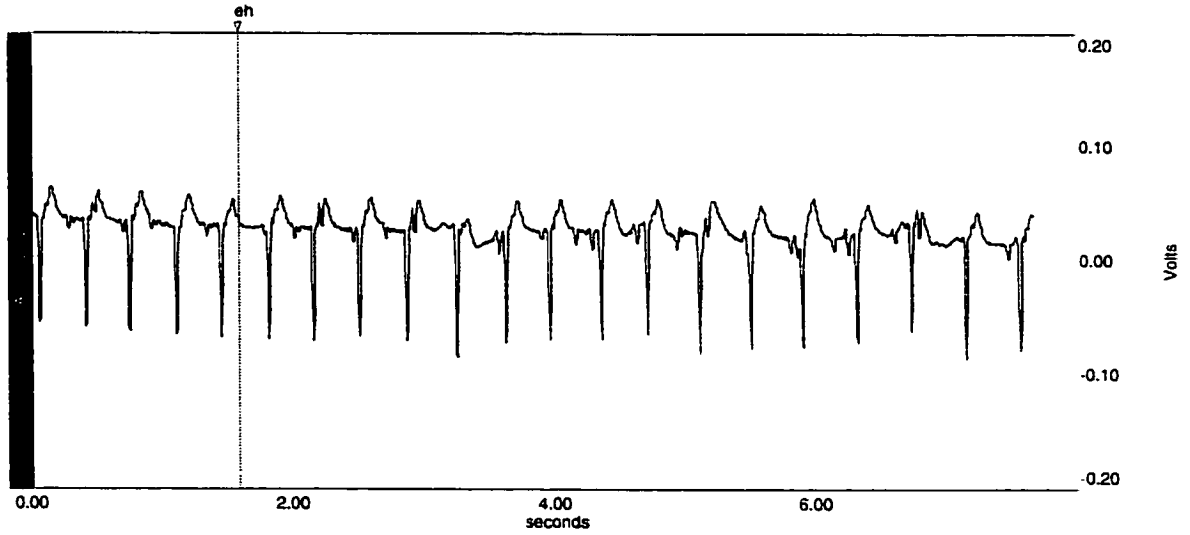

Figure 2a: Recording of allograft Balb/c to C57Bl/6 wild type TNFR 1 (+/+) on day ten. This was considered a successful transplant and a viable graft.

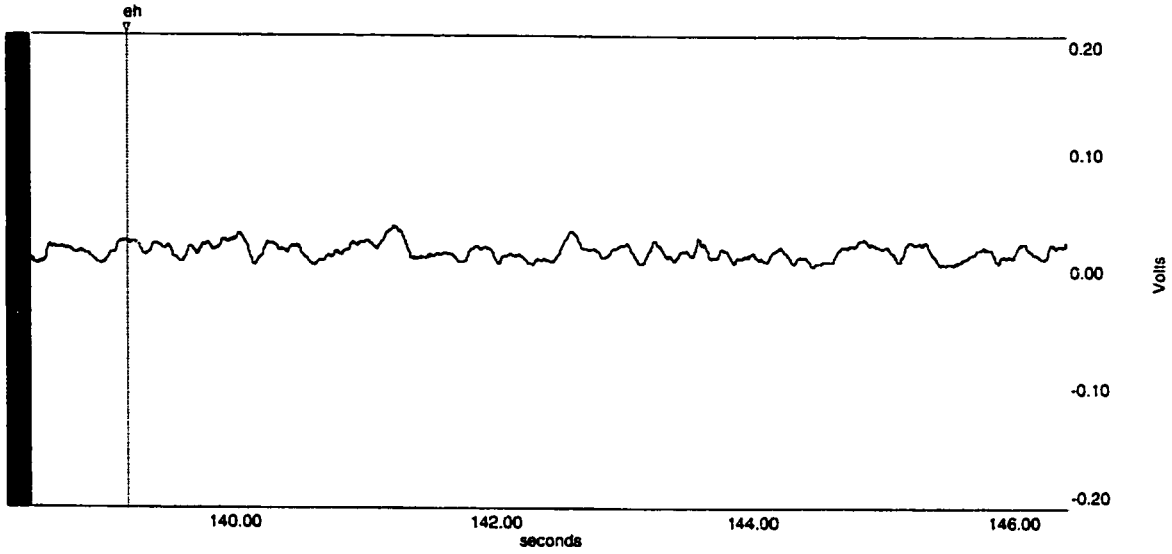

Figure 2b: Recording of same allograft wild type as in figure 2a on day twelve. The lack of rhythmical beating, as presented here, was classified as a rejected tissue.

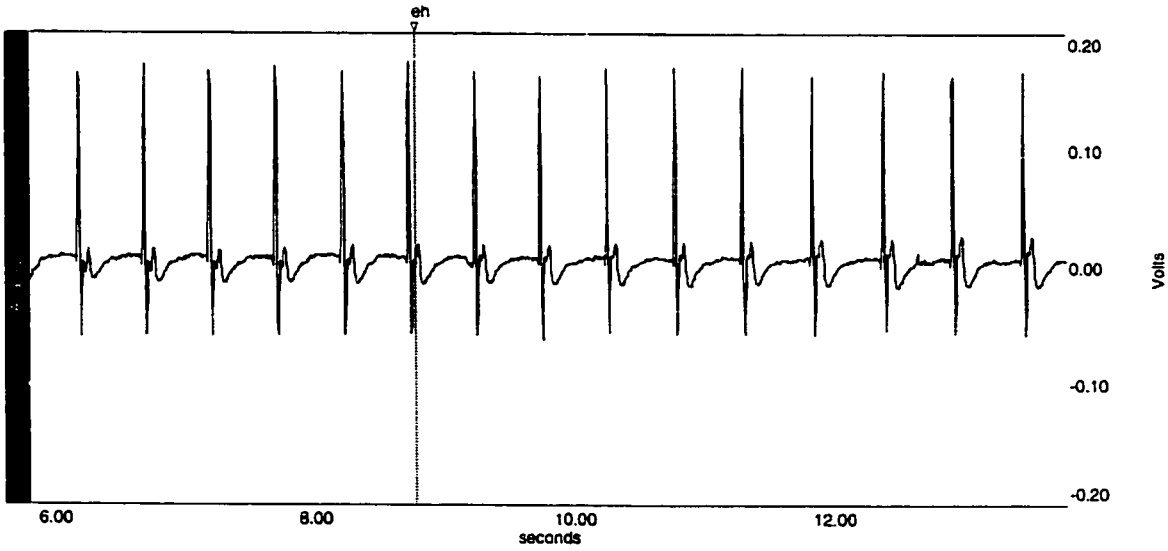

Figure 3. Isograft wild type on day thirty-five post-transplant.

Survival results are shown in Table 1 for the various groups. Statistical analysis

indicated that sex was insignificant for differentiating graft survival (P=0.2882). Table 2 shows the results for the unsexed groups after cardiac transplantation. There was no significant difference in isograft knockout versus isograft wild type in survival time. Allograft versus isograft did show a significant difference in graft survival times, supporting the hypothesis that TNFR 1 is involved in mediating graft rejection. A comparison of knockout and wild type allografts indicates a clear distinction in longevity of survival ($P < 0.0001$).

Table 1. Graft Survival Following Cardiac Transplantation, Days

Group	N	Sex	Survival[a]
Isograft TNFR1 -/-	3	Male	35.0 ± 0.0
Isograft TNFR1 -/-	3	Female	35.0 ± 0.0
Isograft TNFR1 +/+	2	Male	35.0 ± 0.0
Isograft TNFR1 +/+	4	Female	34.5 ± 1.0
Allograft TNFR1 -/-	7	Male	14.7 ± 3.2
Allograft TNFR1 -/-	8	Female	15.6 ± 2.3
Allograft TNFR1 +/+	5	Male	9.2 ± 1.5
Allograft TNFR1 +/+	9	Female	11.2 ± 2.1

[a]Post-transplant days

Table 2. Graft Survival Following Cardiac Transplantation, Days

Group	N	Survival[a]
Isograft TNFR1 -/-	6	35.0 ± 0.0
Isograft TNFR1 +/+	6	34.7 ± 0.8
Allograft TNFR1 -/-	15	15.2 ± 2.7[b]
Allograft TNFR1 +/+	14	10.5 ± 2.1[b]

[a]Post-transplant days
[b]P-value: < 0.0001

Figure 4 demonstrates the time course survival for all unsexed groups. Surgeries that did not produce a viable graft were excluded from data sets. One of the isograft

knockout mice did not survive the full thirty-five days of the experiment. This subject was thought to have damaged the ear as noted by the scratch marks. However, the epidermal layer remained intact. This subject was included in the group for data and statistical analysis.

Several subjects also scratched through the ear, but this occurred before the onset of viability. These subjects were excluded from results and were grouped with the 18 surgical failures. Of the thirteen subjects that scratched out their grafts, eleven were male. Aggressive behavior was also noted among male subjects throughout the experiment.

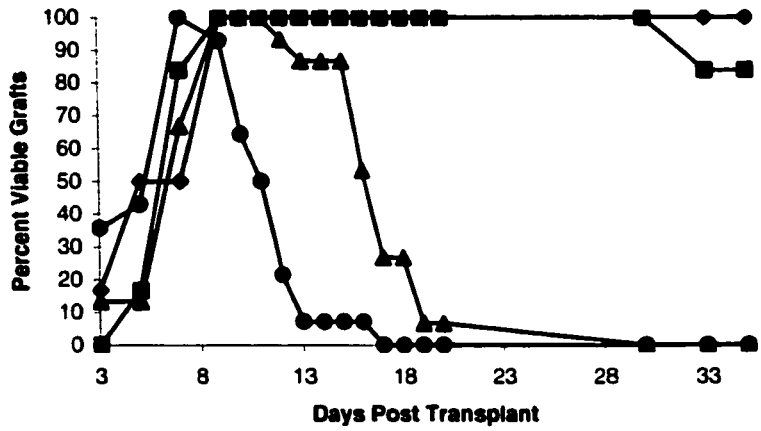

Figure 4. Time-course survival for mouse ear-heart transplant data. Data represents isograft knockout (◆), isograft wild (■), allograft knockout (▲), and allograft wild (○); n = 6, 6, 15, 14, respectively.

Histological examination of the cardiac grafts showed findings of moderate to severe rejection processes in all animals. Even isografts that remained beating for the entire experiment showed moderate to somewhat severe rejection. The tissue was

characterized by cellular infiltrate (data not shown). Figures 5 and 6 demonstrate isograft and allograft wild type histological (H & E) staining, respectively. The isograft shows an

Figure 5 Isograft wild type histological slide (H & E stained, 4x) on day 35. Moderate inflammation is seen around cardiac tissue and transplanted heart can clearly be distinguished from surrounding tissue.

intact heart that can clearly be distinguished from the surrounding tissue. Although the heart in this figure was beating strongly on day thirty-five, there is an obvious

Figure 6 Allograft wild type histological slide (H & E stained, 4x) on day 15. Inflammation is seen and cardiac tissue is nearly indistinguishable from surrounding tissue.

inflammatory response in progress. No indications were noted of vascularization of the transplanted cardiac tissue. Figure 6 shows an allograft wild type transplant that rejected on day 12 and was sacrificed on day 14 demonstrating a more severe rejection. The inflammatory rejection process has necrosed the tissue to the point that anatomical heart features are no longer recognizable. There was no recognizable histological difference between the groups except those found between isograft and allograft transplants.

Discussion

The electrocardiogram provided an invaluable and precise measurement of graft viability. Although cardiac rhythm and voltage amplitude were variable throughout the groups, cessation of a prolonged rhythmical beat was easily determined using the electrocardiogram. Electrical activity was the only means for exact determination of viability.

As represented in the ECG recordings in Figures 1-3, peak height and frequency of contraction differed between groups. Knockout and wild type allografts showed no distinction in these parameters, but there were noticeable differences in peak height of approximately 0.08 volts in isogenic and allogenic recordings taken on comparable days after surgery. Although the electrocardiogram was essential to determine graft rejection, the rhythm and QRS complex peak height patterns of the different groups showed no difference between wild type and knockout mice. There was modest intergroup variability that may have been the result of trauma in the surgical process or in the onset of the rejection process to the sinoatrial node, internodal atrial pathways, atrioventricular node, bundle of His, or Purkinje systems.

There was no difference in longevity of survival between the sexes. Fulmer et al. (18) also reported no difference in graft survival between the sexes for mice. However, Judd and Trentin (19), who used C57Bl/6 background mice in isograft longevity survival studies, found that female subjects rejected before male counterparts. A distinguishing difference was the length of time isographs were allowed to beat. Due to time

constraints, the isografts were not allowed to beat indefinitely. This study does not contradict nor substantiate these finding because isograft recipients were sacrificed prior to the loss of ECG activity. Judd and Trentin demonstrated that isogenic grafts using this ear-heart transplant model and of C57Bl/6 background can remain viable for 80 weeks or more. The observations presented here suggest the aggressive behavior of C57Bl/6 male mice could possibly confound the results. The aggressive behavior noted in male mice contributed to surgical success rate but remains undetermined if the aggressive behavior noted has an effect on the overall significance between TNFR 1 knockout and wild type allografts.

The difference in survival time, more than two fold, between isograft and allograft control subjects supports the validity of the model. An assumption made by the model asserts that genetically similar transplants will not be rejected by the receptor's immune system. Conversely, the recipient will reject genetically dissimilar allograft tissues relatively quickly. These two assumptions were verified by the data presented here. Though the allograft mismatch selected has previously been reported by Judd and Trentin (19), they did not mention the aggressive behavior of the strain. This behavior had negative consequences as noted in the thirteen subjects that scratched through the epidermal layer of the ear thus exposing the underlying graft tissue. These subjects were subsequently removed from study. The mouse recipient strain selected was based on the background of available TNFR 1 knockout mice. Allograft transplants of C3H to Balb/c ($H\text{-}2^b$ to $H\text{-}2^d$) background demonstrated less aggressive behavior and greater surgical

success rates (unpublished data). Surgical rates of nearly 100% were found in these experiments and no aggressive behavior was noted.

Histological examination of the grafts revealed that the time of sacrifice of two days post rejection did little except to demonstrate the difference between isogenic and allogenic mismatches. It would have been more helpful to sacrifice all recipients at the same time on day 8-9 in order to distinguish knockout from wild type through histological examination. However, this would have increased group size and was not an option. The mismatch selection may have also contributed to the inability of histological determination of differences in rejection severity. The inflammatory response seen in isograft transplants was a concern. The image in Figure 5 demonstrates that even isograft control subjects had moderate inflammation occurring around the cardiac tissue. Balb/c to C57Bl/6 ($H\text{-}2^d$ to $H\text{-}2^b$) is a major mismatch where there is complete H-2 incompatibility. A consequence of this mismatch selection was that both knockout and wild type allograft groups rejected relatively quickly after demonstrating sustainable electrocardiogram activity.

During cardiovascular transplants, the rejection response is initiated by activation of CD4+ T helper cells by alloantigens. Activated T helper cells release initiator cytokines IL-1β, IL-2, and interferon γ. These cytokines induce macrophages to produce TNFα (10). It is thought that TNFα will further maintain the inflammatory response within the rejection infiltrate through up regulation of adhesion molecules, increased vascular permeability, and activation of inflammatory cells (21). In cardiac allografts,

the presence of protein TNFα correlates to graft rejection (22, 23). It was hypothesized that without TNFR 1, which as previously noted is involved in allograft rejection, the longevity of survival of cardiac transplants would increase significantly over allograft recipients with the receptor. Clearly, the results presented here demonstrate a significance in survival between allograft TNFR 1 knockout over wild type ($p < 0.0001$).

The results shown here indicate that there was increased viability in the mouse ear-heart transplant model for TNFR 1 knockouts compared to TNFR 1 wild type controls. This suggests that TNFR 1 does play a role in the rejection process for the mouse ear-heart transplant model and allograft rejection. An increase of nearly five days was observed between allograft knockout and wild type groups. While further investigation into the effects of TNFR 1 need to be identified, clearly these finding support the involvement of this receptor and its resulting pathway in the rejection process of cardiac transplanted tissues. It is unclear the exact involvement of TNFR 1 activation in cardiac transplant rejection. This is not within the boundaries of this experiment, but there does seem to be a correlation between survival time and the absence of the TNFR 1 activation.

The extent of involvement of the receptor in the rejection process is still a matter of debate. It does seem apparent that TNFα and its receptor TNFR 1 play a role in the rejection response but it no doubt works in tandem with other rejection mediating pathways. Cytokines such as IL-1β, IL-2, and interferon γ undoubtedly are involved in the induction of transplant rejection process. Further studies will be necessary to provide

any real causal relationship as this study indicates. It is mindful to consider that TNFα and its immune activities through TNFR 1 are a small piece of a complex rejection response from the host immune system. It would be optimistic to assume that one single mechanism or cytokine receptor pathway alone is involved in the graft rejection response. One must also consider the cardiac transplants shown here are of major mismatch selection type (H-2^d to H-2^b). The increased length of survival in the model warrants advocating the investigation of long term minor mismatch study design. It is difficult to know if the mouse cardiac ear-heart transplant model can be indicative to other tissue transplants, but this may also be of interest for future investigations.

The clinical implications of this experiment remain unclear. Demonstration to the effectiveness of TNFR 1 as a potential drug target is however substantiated. Existing compounds that decrease TNFα are currently in use but have toxic effects on other organs (24). Recently, it has been suggested that the glucocorticoid dexamethasone, an immunosupressant synthetic molecule, acts by blocking iNOS expression through inhibition of NF- κB (25). As noted earlier, this is a pathway for TNFα and its receptor TNFR 1. Salicylates have been observed on several nuclear transcription factors such as NF- κB, which may explain its anti-inflammatory properties (26). With highly successful compounds already on the market, the benefits could outweigh the potential toxicity risks with the development of a TNFR 1 antagonist. With the results of this experiment taken into consideration, further investigation into the role of the TNFR 1 cellular transduction pathway in the transplant rejection process is warranted.

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