American Journal of Pathology, Vol. 146, No. 5, May 1995

Short Communication

Up-Regulation of Endothelin-1 mRNA and Peptide Expression in Rat Cardiac Allografts with Rejection and Arteriosclerosis

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Acute and chronic rejection are frequent and significant complications of cardiac transplantation, and graft arteriosclerosis is the leading cause of death beyond the first year after transplant. Levels of endothelin-1 (ET-1) are elevated in plasma of patients with cardiac allografts and those with symptomatic vascular atherosclerosis, but little is known about the role of ET-1 in these processes. This study examined intragraft ET-1 expression in rat cardiac models of acute rejection and chronic rejection associated with graft arteriosclerosis. Corrected ET-1 gene transcript levels were measured with a [^{32}P]dCTP reverse transcription polymerase chain reaction assay normalized with glyceraldehyde-3-phosphate dehydrogenase, and the gene product was evaluated by immunohistology with a monospecific anti-ET-1 antibody at different time points after transplant. ET-1 mRNA levels were significantly increased in acutely rejected (Wistar-Furth rat cardiac allografts transplanted into Lewis rat recipients) and chronically rejected (Lewis allografts transplanted into F344 recipients) vascularized cardiac allografts as compared with isograft controls. In acutely rejected allografts, peak expression occurred on day 5 after transplant. In chronically rejected allografts, the increase in ET-1 mRNA was sustained on days 7, 28, and 75. In both acutely and chronically rejected allografts, ET-1 mRNA up-regulation was not seen in host spleens or paired host hearts. Immunohistological analysis confirmed that the bulk of ET-1 peptide expression was localized to mononuclear cells that diffusely infiltrated the graft interstitium (acute rejection and early chronic rejection) and accumulated within the neointima of chronically rejecting hearts with arteriosclerosis. These observations, taken together with* in vitro *data showing that ET-1 production is stimulated by certain cytokines, indicate that the allogeneic stimulus within rejecting vascularized cardiac allografts, presumably cytokine mediated, leads to significant intragraft up-regulation of ET-1 mRNA and peptide expression. The local up-regulation of this vasoactive and mitogenic peptide within acutely and chronically rejected cardiac allografts suggests that ET-1 may be involved in the development of graft arteriosclerosis. (Am J Pathol 1995, 146:1065–1072)

Endothelin-1 (ET-1), a member of a family of 21-amino-acid peptides, was first isolated from the supernatant of cultured endothelial cells.[1] Plasma ET-1 levels have been shown to be elevated at various time

Supported by research grants from Bristol-Myers Squibb. B. W. is a recipient of an Erwin Schrödinger Stipendium of the Austrian Science Foundation.

Accepted for publication February 1, 1995.

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points after solid organ transplantation[2–7] and in patients with severe symptomatic atherosclerosis.[8,9] As ET-1 acts predominantly in a paracrine/autocrine fashion,[10,11] circulating peptide levels may not reflect the microenvironmental regulation and local pathophysiological effects of the peptide within a particular tissue or organ. It has been demonstrated that ET-1 mRNA and protein expression are increased in atherosclerotic lesions compared with normal vessels.[8,12] Besides its potent vasoconstrictive effects,[1] ET-1 also exerts mitogenic effects on endothelial and smooth muscle cells *in vitro*.[13–17] Recent studies in the rat carotid artery balloon injury model showed that neointimal formation is promoted by ET-1 and can be inhibited by an ET receptor antagonist.[18] In addition, there are data to indicate that certain cytokines stimulate production of ET-1 *in vitro*.[19–21] Little is known about the local expression of ET-1 in vascularized organ allografts. The present study was designed to test whether the allogeneic stimulus influences the regulation of ET-1 production and to assess local ET-1 mRNA and peptide expression within vascularized rat cardiac allografts during both acute rejection and during various phases of chronic rejection and arteriosclerosis.

Materials and Methods

Cardiac Transplantation and Experimental Design

Adult male Lewis (LEW), Wistar-Furth (WF), and Fisher (F-344) rats (8 to 10 weeks old) were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and Charles River Laboratories (Kingston, NY). Abdominal heterotopic rat cardiac transplantation was performed as previously described.[22,23] In the acute rejection model, WF cardiac allografts were transplanted into LEW recipients and harvested on days 3, 5, and 7 after engraftment (rejection usually occurs between days 6 and 8, as defined by the cessation of palpable heartbeats). In the chronic rejection model, LEW allografts were transplanted into F-344 recipients and harvested on days 7, 28, and 75 after engraftment. These time points represent various stages of transplant arteriosclerosis.[22–24] Isografts harvested at day 5 (WF→WF, n = 3) and day 75 (LEW→LEW, n = 3) served as controls for acute and chronic allograft rejection, respectively. Midventricular sections of the grafts and paired host hearts and, in some cases, sections of host spleens were used for mRNA extraction and histological evaluation, as previously described.[25–27] The animals received no immunosuppressive drugs to study ET-1 expression in unmodified acute and chronic allograft rejection.

ET-1 Gene Transcript Analysis

ET-1 oligonucleotides spanning exon/intron borders within the coding region were chosen by using the MAC VECTOR program (International Biotechnologies, New Haven, CT).[28] The 5′ primer (AT GGA TTA TTT TCC CGT GAT C) and 3′ primer (C TGT AGT CAA TGT GCT CGG) were synthesized in the Protein/Nucleic Acid Laboratory, Department of Medicine, Brigham and Women's Hospital. Polymerase chain reaction (PCR) studies were carried out on a GeneAmp system 9600 (total volume, 25 μl) as previously described.[25–27] Reaction conditions included 1.25 μl of cDNA, 1 μmol/L (each) 5′ and 3′ primer, 10 mmol/L Tris-HCl, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.001% (w/v) gelatin, 800 μmmol/L dNTPs, and 0.625 U of AmpliTaq DNA polymerase. The 641-bp amplified product was cloned directly into the PCRII vector (Invitrogen, San Diego, CA). Sequence analysis (Sequenase kit 2.0; US Biochemical, Cleveland, OH) by the dideoxynucleotide chain termination method confirmed that the amplified PCR fragment had 100% identity with the previously submitted rat ET-1 sequence (m64711).[28]

ET-1 Gene Transcript Assay

In an effort to conserve RNA, we measured gene transcript levels by a published reverse transcription PCR assay.[25–27] To identify the optimal PCR conditions for accurate measurement of gene transcript levels, we established the logarithmic assay range with respect to cycle number and starting template concentration against different dilutions of cDNA. Subsequent measurements of ET-1 transcript levels were then completed at 28 cycles with 1.25 μl of cDNA in a 25-μl reaction (denaturation at 94 C for 15 seconds, annealing at 50 C for 20 seconds, and extension at 72 C for 60 seconds (with a final extension of 7 minutes at the end of all cycles). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH), which represents ubiquitously expressed mRNA, was used as an internal reference to reflect total cellular RNA, as previously described.[25–27] G3PDH amplification was performed for 22 cycles at 94 C, 58 C, and 72 C.

For semiquantitative PCR analysis, 150,000 cpm of [^{32}P]dCTP (800 Ci/mmol/L; 1 Ci = 37 GBq; DuPont/NEN, Boston, MA) was included per reaction. Negative control experiments were performed

by substituting water for cDNA or omitting reverse transcriptase in cDNA synthesis. Agarose gels (1%) containing the amplified products were dried and exposed to storage phosphor plates for 20 to 24 hours. Incorporated ^{32}P in the amplified band was measured on a PhosphorImager system (Imagequant software, Molecular Dynamics, Sunnyvale, CA). Corrected values were derived by dividing the measured ^{32}P value for ET-1 by the mean G3PDH value (quadruplicate samples). Relative transcript levels were then determined from cDNA sets that included negative control samples (for which reverse transcriptase had been omitted during cDNA synthesis or water used instead of cDNA) performed in the same evaluation. The results represent the mean corrected levels obtained by pooling the levels obtained from two or three animals per group that had been analyzed in quadruplicate. Differences in the corrected ET-1 mRNA expression were examined in three different studies: 1), time course in the acute rejection model; 2), comparison of allografts, paired spleens, and isografts for the acute (day 5) and the chronic (day 75) model; and 3), time course in the chronic rejection model. Results from each experimental set were subjected to multivariate analysis of variance without replication. Individual comparisons were made, and the level of significance was corrected by the Bonferroni method.

Immunohistology

Cardiac allografts, isograft controls, and paired host hearts and spleens were snap-frozen and stored at −70 C until sectioning on a cryostat. Immunoperoxidase labeling was performed as described previously[27,29–31] with a specific rabbit antibody to ET, kindly provided by Biomedica (Vienna, Austria). This antibody identifies ET-1 in human renal endothelial cells.[32] Negative controls included omission of the primary antibody and use of an irrelevant primary antibody (von Willebrand factor). In addition, smooth muscle cells and macrophages were localized by using monoclonal antibodies to desmin and ED-1 antigen, respectively.[29–31]

Results

ET-1 mRNA Expression during Acute Cardiac Allograft Rejection

ET-1 gene transcript levels were significantly higher in acutely rejected cardiac allografts than in the paired host hearts on day 5, but not on day 3 or day 7 (n = 3/group; $P < 0.002$) after transplant (Figure 1). Transcripts from the paired host hearts were low and comparable at all time points (days 3, 5, and 7). ET-1 mRNA levels of day 7 allografts were significantly lower than those on day 5 ($P < 0.02$). ET-1 transcript levels in day 5 allografts were significantly higher ($P < 0.05$) than those of day 5 isograft controls (Figure 2), suggesting that the rejection process, rather than the surgical procedure, was responsible for the ET-1 mRNA increase. ET-1 mRNA expression in matched recipient spleens during the rejection process was significantly less than in the allograft ($P < 0.02$) and comparable with host heart levels (Figure 2), indicating that the microenvironment of the rejecting allograft is crucial for the induction of ET-1 synthesis.

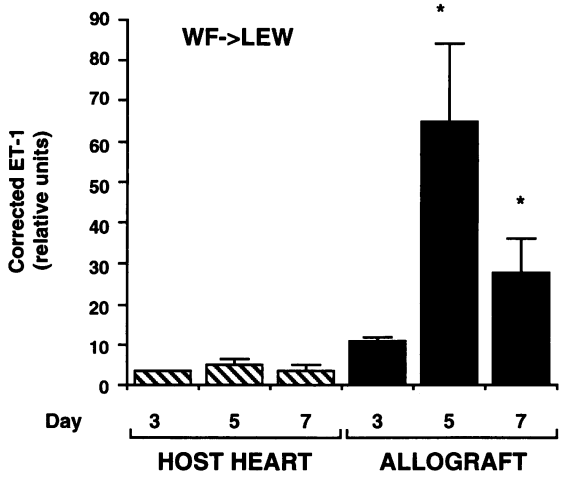

Figure 1. *Time course of ET-1 mRNA expression in acutely rejected cardiac allografts and in paired host hearts. Columns represent the mean ± SEM of corrected levels shown in relative units (reverse transcription PCR values for ET-1 normalized against those for the control gene G3PDH). Experiments were performed in quadruplicates (n = 3 per group per time point).*

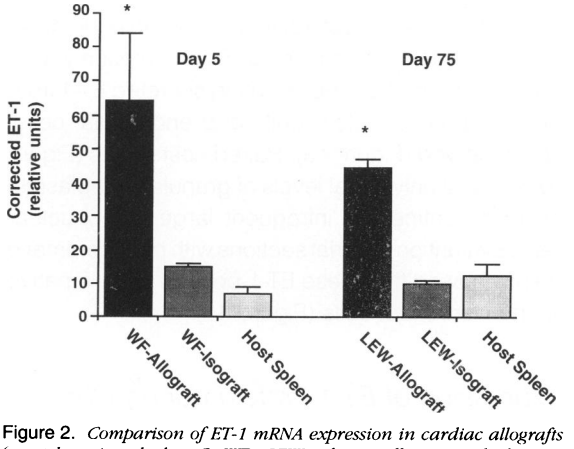

Figure 2. *Comparison of ET-1 mRNA expression in cardiac allografts (acutely rejected day 5 WF→LEW, chronically rejected day 75 LEW→F344), paired host spleens, and control isografts in the acute and chronic allograft rejection model (n = 2 to 3 per group per time point).*

ET-1 mRNA Expression during Chronic Cardiac Allograft Rejection

ET-1 transcript levels were significantly higher in cardiac allografts compared with paired host hearts on days 7, 28, and 75 ($P < 0.02$, 0.003, and 0.001 respectively, Figure 3). These time points were chosen to examine the following three stages of arteriosclerosis: day 7, when the adhesion of scattered mononuclear cells to the lumen is seen; day 28, when mild degrees of concentric intimal thickening are first apparent; and day 75, when neointimal thickening and smooth muscle cell proliferation are typical features.[23,24,27] The increase in ET-1 mRNA expression occurred early (day 7) and was sustained over time (days 28 and 75). ET-1 transcript levels in the paired host hearts were low throughout the observation period (Figure 3) and comparable with those seen in the acute rejection model (Figure 1). ET-1 transcripts levels in day 75 allografts were significantly higher ($P < 0.0003$) than those of day 75 isograft controls (Figure 2). In addition, the recipient spleens from the day 75 transplants expressed low levels of ET-1 mRNA compared with the transplanted allografts ($P < 0.0007$). Thus, in the chronic cardiac rejection model there was also up-regulation of ET-1 localized within the allograft.

Expression of ET-1 Protein during Acute Cardiac Allograft Rejection

Intragraft labeling for ET-1 was detected in association with infiltrating mononuclear cells from day 3 after transplant. Similar to the mRNA data, peak staining for ET-1 protein was observed on day 5 after transplant (Figure 4a), when dense ET-1 expression by infiltrating mononuclear cells was noted. In addition, less intense focal staining of adjacent capillary endothelial cells and weak and diffuse labeling of most myocardial cells (most likely representing secreted ET-1 from infiltrating mononuclear cells and endothelial cells) was observed (Figure 4a). Paired host hearts (Figure 4b) showed only basal levels of granular cytoplasmic labeling confined to infrequent large mononuclear cells. Evaluation of serial sections with metachromatic stains showed that these ET-1-positive cells in native hearts were mast cells (Figure 4c).

Expression of ET-1 Protein during Chronic Cardiac Rejection

In the chronic cardiac allograft rejection model, samples collected on day 7 showed dense staining of

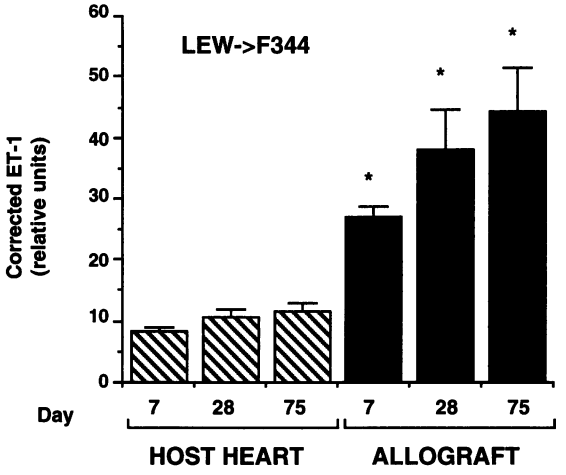

Figure 3. *Time course of ET-1 mRNA expression in chronically rejected cardiac allografts and in paired host hearts. Columns represent the mean ± SEM of corrected levels shown in relative units (reverse transcription PCR values for ET-1 normalized against those for the control gene G3PDH). Experiments were performed in quadruplicate ($n = 2$ per group per time point).*

>75% of infiltrating mononuclear cells and weaker staining of adjacent endothelial cells (Figure 4d). Day 28 and 75 allografts showed similar patterns of intragraft labeling of ET-1 (Figure 4, e–g). In these grafts ET-1 staining was predominantly seen in the neointima. Analysis of serial sections with desmin (Figure 4e) as a marker of vascular smooth muscle cells and ED-1 (Figure 4f) as a marker of macrophages[31] showed that the ET-1-positive neointimal cells (Figure 4g) were primarily a subset of ED-1-positive macrophages.

Discussion

This study investigated the expression of ET-1 mRNA and peptide in cardiac allografts undergoing acute rejection and during development of chronic rejection, a process for which graft arteriosclerosis is a characteristic feature.[23,24,27] The heterotopic cardiac transplant model allowed us to compare ET-1 expression in the allograft and in the recipient's native heart (exposed to the same circulation), as the latter is not removed during transplantation. We demonstrate that rejecting vascularized cardiac allografts lead to local up-regulation of ET-1 mRNA and protein expression within the transplanted organ. This up-regulation was not seen in isograft controls. Two distinct patterns of mRNA ET-1 regulation were seen in cardiac allografts. In the acute rejection model, peak ET-1 expression occurred on day 5 after engraftment followed by a significant decrease on day 7. This reduction on day 7 may reflect the decreasing viability of cells capable

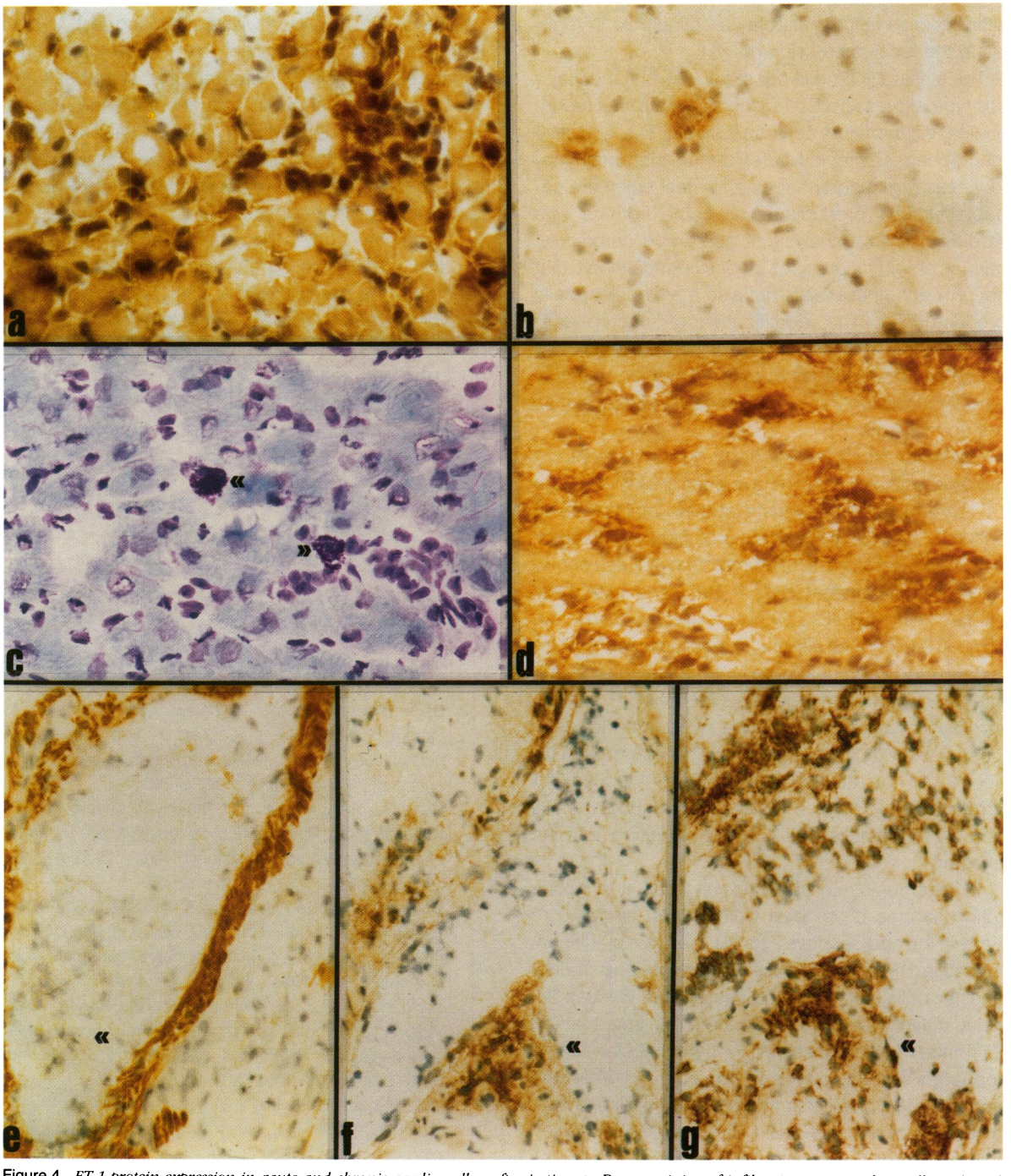

Figure 4. *ET-1 protein expression in acute and chronic cardiac allograft rejection.* a: *Dense staining of infiltrating mononuclear cells and weak and diffuse labeling of adjacent endothelial and myocardial cells were seen at day 5 after transplant (acute rejection).* b: *In the corresponding paired host heart, ET-1 labeling was restricted to large and infrequent mononuclear cells.* c: *Serial section of tissue from* b *stained with Diff-Quik shows that the large cells have coarse granules that extend over their nuclei (arrowheads) and are typical of mast cells* d: *Dense ET-1 labeling of mononuclear cells and adjacent endothelial cells and weaker, diffuse labeling of myocytes at day 7 (chronic rejection model).* e *to* f: *Serial staining of a large artery and associated neointimal cells (arrowheads) present at day 28 after transplant (chronic rejection model).* e: *Vascular smooth muscle cells stain for desmin, whereas most neointimal cells lack desmin expression.* f: *Many of the neointimal cells (arrowheads) and some mononuclear cells within the adventitial area on left show dense ET-1 expression.* g: *ED-1$^+$ macrophages present within neointima, media, and adventitia. Cryostat sections; hematoxylin counterstain, except* c; *original magnifications, ×630 (*a *to* d*) and ×400 (*e *to* g*).*

of RNA or protein synthesis, as the ongoing immunological process leads to necrosis of the allografted tissue.[22,33] In chronic rejection, the increase in ET-1 expression observed on day 7 persisted through day 75. The findings that ET-1 mRNA and peptide expression is restricted to the cardiac allografts and does not

occur in host spleens or in isografts suggest that the allogeneic stimulus must play a critical role in regulating the local expression of ET-1.

The striking finding in our study was that the major cell type expressing ET-1 in the allografts was mononuclear inflammatory cells, specifically, ED-1-positive macrophages. Previously, evidence that inflammatory cells (eg, macrophages and mast cells) are capable of ET-1 production has come from *in vitro* studies.[34–36] Our analysis of acute and chronic rat cardiac allografts provides clear evidence *in vivo* that not only endothelial cells but also mononuclear cells produce ET-1. The identification of macrophage-derived ET-1 extends our earlier studies examining activated macrophages in chronic cardiac rejection by demonstrating that mitogens as well as cytokines are up-regulated.[26,27]

Cytokines such as interferon-γ, tumor necrosis factor-α, and interleukin-1 have been shown to induce ET-1 production in a variety of cells maintained in culture.[19–21] During the process of allograft rejection, a main source of such inflammatory cytokines are infiltrating mononuclear cells.[37] We have previously studied select cytokine pathways up-regulated in the LEW→F344 cardiac allografts undergoing chronic rejection and demonstrated marked increases in interferon-γ and tumor necrosis factor-α[26,27] expression by mononuclear cells. We speculate that cytokines secreted by infiltrating mononuclear cells in response to the alloantigenic stimulus induce ET-1 production by other infiltrating mononuclear cells and by endothelial cells in the rejecting cardiac allograft.

In the chronic rejection model studied, arteriosclerotic changes develop in well defined stages.[23,24] Early in the course (days 7 to 14), there are significant interstitial mononuclear cell infiltrates and mononuclear cells adhere to the vessel wall. By day 28, when inflammatory cells infiltrate the intima, marked ET-1 labeling was observed in monocytes and macrophages in the neointima. On day 75, when the neointima is typically composed of both mononuclear and smooth muscle cells, ET-1 staining showed marked increases in both neointimal cell types. The presence of ET-1 immunoreactivity within the arteriosclerotic lesions of transplant vessels in the LEW→F344 chronic cardiac allograft rejection model extends the findings of ET-1-positive endothelial and smooth muscle cells in atherosclerotic vessels of humans[8] and suggests a common role for ET-1, as a vasoactive peptide and as mitogen, in the development of various forms of arteriosclerosis. *In vitro* studies showing a mitogenic effect of ET-1 on human vascular endothelial cells and on c-*fos* and c-*myc* expression in rat vascular smooth muscle cell proliferation[13–17] and *in vivo* studies suggesting that ET-1 may play an important role in development of glomerulosclerosis[38,39] support this hypothesis. In our model, we suggest that macrophage and/or endothelial cell-derived ET-1, through the process of vasoconstriction and smooth muscle cell proliferation, regulate vascular remodeling leading to development of graft arteriosclerosis.

In summary, our findings demonstrate that animal models of cardiac allograft rejection may be used to elucidate the precise role of ET-1, as a vasoactive and/or mitogenic peptide, in development of graft arteriosclerosis. The availability of specific endothelin receptor antagonists will help delineate the role of ET-1 in this process and could provide potential therapeutic strategies to prevent or ameliorate graft arteriosclerosis.

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