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Prevention of Pancreatic Islet Xenograft Rejection by Dietary Vitamin E

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In pancreatic islet transplantation, the adhesion of activated leukocytes to endothelial cells and the loss of microvascular integrity represent the critical microcirculatory events, which promote loss of graft function due to rejection. With the view that oxygen radicals may contribute to graft rejection, we studied the effect of the antioxidant vitamin E on microvascular rejection of islet grafts. Islets were transplanted syngeneically and xenogeneically (rat) into dorsal skinfold chambers of hamsters, which received a non-vitamin-E-supplemented laboratory chow. Treated animals with xenografts were fed with a diet supplemented with vitamin E in a low (150 mg/kg) and high (8000 mg/kg) concentration. Intravital fluorescence microscopy demonstrated complete vascularization of syngeneic grafts at day 10 after transplantation, intact islet microcirculation at day 20 with a functional capillary density of 653 ± 6 cm^{-1}, and only few leukocytes adherent to the endothelial lining of the islets' microvasculature (88 ± 23 mm^{-2}). Xenogeneic islets showed initial signs of rejection at day 6, including adhesion of leukocytes to the microvascular endothelium (610 ± 110 mm^{-2}) and loss of endothelial integrity. After 20 days, functional capillary density was significantly lower (173 ± 68 cm^{-1}) when compared with syngeneic grafts, indicating failure of graft acceptance. Supplementation of the diet with low and high concentrations of vitamin E resulted in a significant ($P < 0.05$) reduction of xenograft leukocyte-endothelium interaction (146 ± 29 mm^{-2} and 109 ± 42 mm^{-2}) at day 6 after transplantation and adequate development of functional capillary density at day 20 (478 ± 36 cm^{-1} and 539 ± 86 cm^{-1}; $P < 0.05$), indicating prevention of microvascular rejection. We conclude that dietary supplementation of the lipophilic antioxidant vitamin E attenuates leukocyte-endothelial cell interactions, preserves microvascular integrity, and thus inhibits microvascular rejection in a dose-dependent fashion. Our study underscores the pivotal mediator role of reactive oxygen species in islet xenograft rejection and, furthermore, suggests that dietary vitamin E may act as an adjunct anti-rejection treatment in clinical islet transplantation. (Am J Pathol 1997, 150:1487–1495)

Based on the experience that insulin of bovine and porcine origin has been successfully administered to diabetic patients, the xenotransplantation of isolated pancreatic islets represents an attractive approach for the curative treatment of insulin-dependent diabetes mellitus. However, the loss of graft function due to immune-mediated rejection still remains the major obstacle for the long-term viability and function of porcine pancreatic endocrine tissue transplanted to man.[1]

The microcirculation is the primary target in organ graft rejection.[2–4] With the use of intravital fluorescence microscopy and a rat-to-hamster transplantation model, we have previously demonstrated the impact of microvascular leukocyte recruitment, leukocyte-endothelium interaction, and subsequent destruction of the grafts' microvascular network in islet xenograft rejection.[5,6] However,

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as islet grafts are vascularized by host endothelial cells,[7] rejection-induced leukocyte-endothelial cell interactions may not be mediated by species/strain-specific cell surface antigens but may rather involve distinct endothelial adhesion molecules, which trigger general inflammatory responses, such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1.[8,9] This view is supported by recent experiments, demonstrating that blockade of ICAM-1- and VCAM-1-mediated leukocyte-endothelium interaction by appropriate monoclonal antibodies successfully prevents islet allograft and xenograft rejection.[10,11] However, both the trigger and the effector mechanisms that underly these microvascular and cellular events and that lead to immune/inflammation-mediated graft destruction are not completely understood.

Reactive oxygen species contribute to leukocyte recruitment and adhesion in experimental models of inflammation.[12,13] Moreover, in both experimental and clinical studies, oxygen radicals have been suggested to be involved in cardiac and renal allograft rejection.[14–16] The idea that oxygen radicals might also contribute to the rejection of pancreatic islets is supported by previous experiments in which islet allograft survival was improved by desferrioxamine, an iron-chelating agent that exerts powerful antioxidant effects by interfering with hydroxyl radical formation.[17,18] Another, less toxic, antioxidant is the lipid-soluble vitamin E (α-tocopherol), which is incorporated in biomembranes, prevents the propagation of lipid peroxidation, and thus effectively reduces oxidative cell damage. As vitamin E has also been shown to attenuate leukocyte chemotaxis/adhesion and leukocyte-mediated endothelial injury *in vitro* and *in vivo* in models of oxygen-radical-induced tissue damage,[19–22] we studied, with the use of intravital fluorescence microscopy, whether dietary supplementation of vitamin E attenuates microvascular rejection in rat pancreatic islet xenotransplantation.

Materials and Methods

Islet Isolation

Pancreatic islets were isolated from adult Sprague Dawley rats for xenogeneic transplantation and from Syrian golden hamsters for isogeneic transplantation using a collagenase digestion technique that has previously been described in detail.[23] In brief, donor animals were anesthetized with 50 mg/kg sodium pentobarbital intraperitoneally. Islets were stained *in situ* by perfusion of the pancreas with Neutral Red (3 mg dissolved in 20 ml of Hanks' balanced salt solution). The gland was then excised cautiously, cut into 30–40 pieces, and digested with collagenase from Clostridium histolyticum (4 mg/ml Hanks' balanced salt solution) at 37°C for 10 to 15 minutes. The islets were separated from the digest by multiple sedimentations. The islet yield per isolation procedure was 600 to 800 for hamsters and 400 to 600 for rats. Handpicking of the isolated islets using a dissecting microscope guaranteed single, exocrine-free grafts.

Islet Transplantation

After isolation, 8 to 12 islets (~200 μm diameter) were transplanted into the dorsal skinfold chamber of Syrian golden hamsters, which contains one layer of skin and striated muscle and serves as a microvascular bed for the free islet grafts. The dorsal skinfold chamber model in the hamster represents a versatile model for repeated intravital microscopic studies of the microvasculature of pancreatic islet grafts.[24,25] For the preparation of the chamber, hamsters aged 6 to 8 weeks were anesthetized with 50 mg/kg sodium pentobarbital intraperitoneally. Two symmetrical titanium frames were implanted into the back of the animals so as to sandwich the extended double layer of skin. One layer of skin and retractor muscle was completely removed in a circular area of 15 mm in diameter, and the remaining layers of striated skin muscle, subcutaneous tissue, and epidermis were covered with a glass cover slip incorporated into one of the frames. Vitamin E supplementation did not influence the quality of the preparations. The animals tolerated the chambers well and showed no signs of discomfort or changes in their feeding or sleeping habits. All animal experiments were performed according to protocols approved by the local ethical committee.

Intravital Microscopy

For the visualization of the islet grafts' microvasculature and the study of the microvascular manifestations of xenograft rejection, the technique of intravital fluorescence microscopy was applied. Therefore, the awake animals were immobilized in a plexiglass tube, and the chamber, containing the islet grafts, was attached to the microscope stage. Microscopy was then performed using epi-illumination with a 100 W mercury lamp, attached to a Ploema-Pak illuminator with an I_2 blue and a N_2 green filter block (Leica, Wetzlar, Germany). The microscopic observations (10× long distance and 25× water immersion objec-

tives) were recorded on videotape and analyzed off-line using a computer-assisted image analysis system. Contrast of plasma was enhanced with 5% fluorescein isothiocyanate (FITC)-dextran intravenously (MW 150,000; Sigma, Deisenhofen, Germany) and leukocytes were stained *in vivo* with 0.2% rhodamine G6 intravenously (MW 489; Serva, Heidelberg, Germany).

The microvasculature of the islet grafts was quantitatively analyzed at days 6, 10, 14, and 20 after transplantation. The analyses included 1) the percentage of grafts presenting with initial signs of angiogenesis, 2) the size of the islet's microvascular network, 3) the functional capillary density, 4) microvascular leukocyte accumulation, and 5) permanent leukocyte adhesion to the microvascular endothelium. Initial signs of angiogenesis in an islet graft were defined as the formation of new microvessels, originating from capillaries and postcapillary venules of the host tissue.[24] The size of the microvascular network (mm^2) included the area of newly formed microvessels within the islet grafts.[26] Functional capillary density (cm^{-1}) was defined as the length of red-blood-cell-perfused capillaries per islet area.[23] Microvascular leukocyte accumulation (mm^{-3}) and permanent leukocyte adhesion to the microvascular endothelium (mm^{-2}) were analyzed in postcapillary venules (diameter, 15 to 50 μm) that drained the capillary network of the grafts and were defined as intravascular leukocyte count and the number of adherent cells (>20 seconds) per mm^2 of endothelial surface, respectively.[27]

Immunohistochemistry

At day 20 after transplantation, the tissue containing the islet grafts was excised and fixed in 10% formaldehyde for immunohistochemical staining of intracellular insulin.[25] After 24 hours, the preparations were dehydrated through a graded series of alcohol, embedded in paraffin, and sectioned at 3 μm thickness. For the detection of intracellular insulin, the dewaxed sections were incubated with a polyclonal guinea pig anti-porcine insulin antibody (1:200; Dako Corp., Hamburg, Germany). Cross-reactivity of the anti-porcine insulin antibody with hamster and antigen specifity to insulin was confirmed by Western blotting. A peroxidase-conjugated rabbit anti-guinea-pig IgG (1:50; Dako) was used as secondary antibody. Specimens were then reacted serially with a biotinylated secondary mouse anti-rabbit IgG and an avidin-biotin-horseradish peroxidase complex (both diluted according to the manufacturer's instructions; Vector Laboratories, Burlingame, CA). Finally, all specimens were stained with 3-amino-9-ethylcarbazole (Sigma), dimethylsulfoxide, H_2O_2 (Merck, Darmstadt, Germany), and were counterstained with hemalaun (Merck). Controls were performed without primary and secondary antibodies to exclude nonspecific staining.

Dietary Vitamin E Supplementation

Hamsters were bred receiving a non-vitamin-E-supplemented standard laboratory chow (Sniff, Soest, Germany). Four weeks before xenogeneic islet transplantation, the diet was supplemented with vitamin E (DL-tocopheryl acetate, Omega Pharma, Berlin, Germany) in a low (150 mg/kg chow dry weight; n = 9 animals and 81 islet xenografts) and high (8000 mg/kg chow dry weight; n = 6 animals and 56 islet xenografts) concentration. Nine hamsters (n = 82) were continued on the standard laboratory chow with no vitamin E supplement and served as controls. To exclude a direct influence of vitamin-E-depleted nutrition on the process of vascularization of islet grafts, syngeneic hamster islets (n = 63) were grafted to six animals receiving the identical standard chow as the untreated animals with xenogeneic transplants. Water and diets were given *ad libitum*. None of the supplemented animals demonstrated adverse effects to the dietary regimens as assessed by clinical and behavioral observations. At the end of the 20-day observation period, blood samples were withdrawn and plasma vitamin E levels were analyzed by high pressure liquid chromatography.[28] The dietary regimens with low-dose and high-dose vitamin E supplementation resulted in a 3.4-fold (22.6 ± 4.8 μg/mg cholesterol) and 10.6-fold (77.0 ± 20.0 μg/mg cholesterol) increase of plasma vitamin E levels when compared with that of control animals receiving standard chow (6.6 ± 1.8 μg/mg cholesterol).

Statistics

All data are presented as mean ± SEM. For analysis of differences between the groups, one-way analysis of variance was performed, followed by Student's *t*-test, including Bonferroni probabilities (CSS, StatSoft, Tulsa, OK). Sample number for comparison between the groups referred to the number of animals studied and included the mean values of the individual islet grafts per animal. Statistical significance was set at $P < 0.05$.

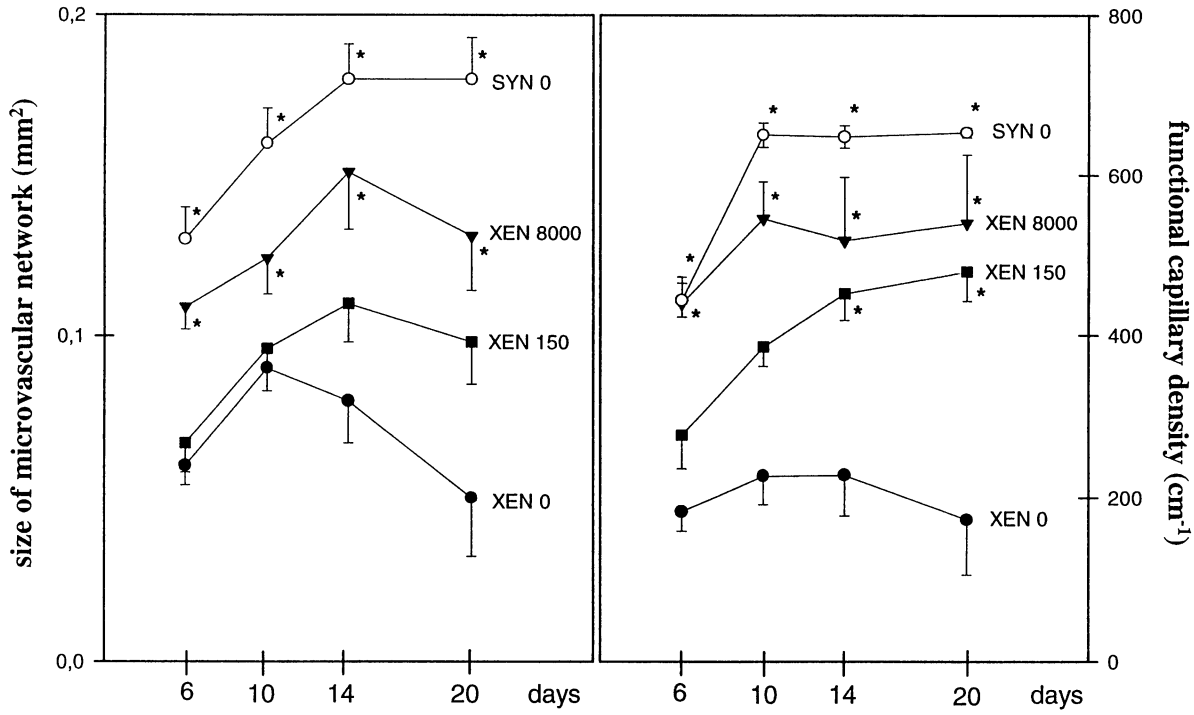


Figure 1. *Influence of vitamin E on microvascular manifestation of pancreatic islet xenograft rejection. Size of the microvascular network* (left) *and functional capillary density* (right) *of the microvascular network of pancreatic islet grafts on days 6, 10, 14, and 20 after syngeneic (hamster-to-hamster) and xenogeneic (rat-to-hamster) transplantation. Recipients of xenografts were treated with dietary vitamin E supplementation at concentrations of 150 mg/kg chow dry weight (XEN 150; n = 9) and 8000 mg/kg chow dry weight (XEN 8000; n = 6). Control animals with syngeneic (SYN 0; n = 6) and xenogeneic (XEN 0; n = 9) islet grafts received a standard laboratory chow with no vitamin E supplement. Size of the microvascular network and functional capillary density were quantitatively analyzed by a computer-assisted image analysis system. Mean ± SEM;* $*P < 0.05$ versus *XEN 0.*

Results

In recipients that were fed a diet with no vitamin E supplement, 97% of islet isografts (hamster-to-hamster) elicited initial signs of angiogenesis after 2 to 3 days, ie, formation of capillary sprouts and new microvessels, originating from capillaries and postcapillary venules of the recipient tissue. Quantitative analysis of both the size of the islets' microvascular network and its functional capillary density revealed a significant increase ($P < 0.05$) during the first 10 to 14 days with no additional changes until day 20, indicating complete revascularization during the initial 2-week period after transplantation (Figure 1). Within the newly formed microvessels, neither accumulation of leukocytes nor significant interactions between leukocytes and the microvascular endothelium were observed (Figure 2). Immunohistochemistry showed homogeneous staining for intracellular insulin.

Xenotransplantation of rat islets into the skinfold chamber of hamsters fed the same diet initially stimulated an isograft-like angiogenic response, and 82% of the islets showed vascularization by day 6 after transplantation. However, the islets' microvascular network and the functional capillary density were significantly smaller than in hamster-to-hamster isografts ($P < 0.05$; Figure 1). During further observation until day 20, there was no increase of these two microvascular parameters, indicating failure of both appropriate vascularization and adequate nutritive perfusion of the grafts (Figure 1). In parallel, the initial 14-day period after transplantation was characterized by an inflammatory response within these xenografts with marked accumulation of leukocytes and their adhesion to the microvascular endothelium of postcapillary venules (Figures 2 and 3). Leukocyte recruitment was detected predominantly in postcapillary venules and not in arterioles or nutritive capillaries. Leukocyte-endothelial cell interaction was associated with increased extravasation of the high molecular weight fluorescent plasma marker FITC-dextran 150,000, indicating the loss of endothelial integrity (Figure 3). By day 20 after transplantation, leukocyte-endothelial cell interaction returned to values comparable with those of isogeneic controls (Figure 2); however, islets had failed to develop adequate vascularization, as demonstrated by the significantly lower values of the size of the microvas-

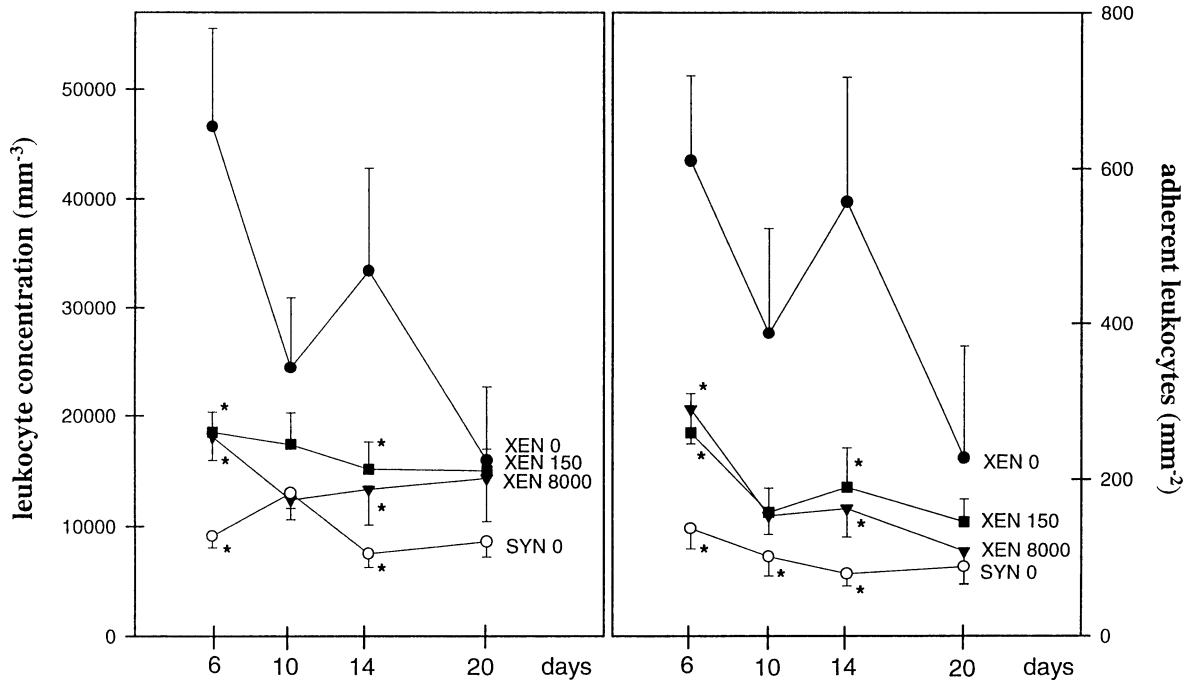


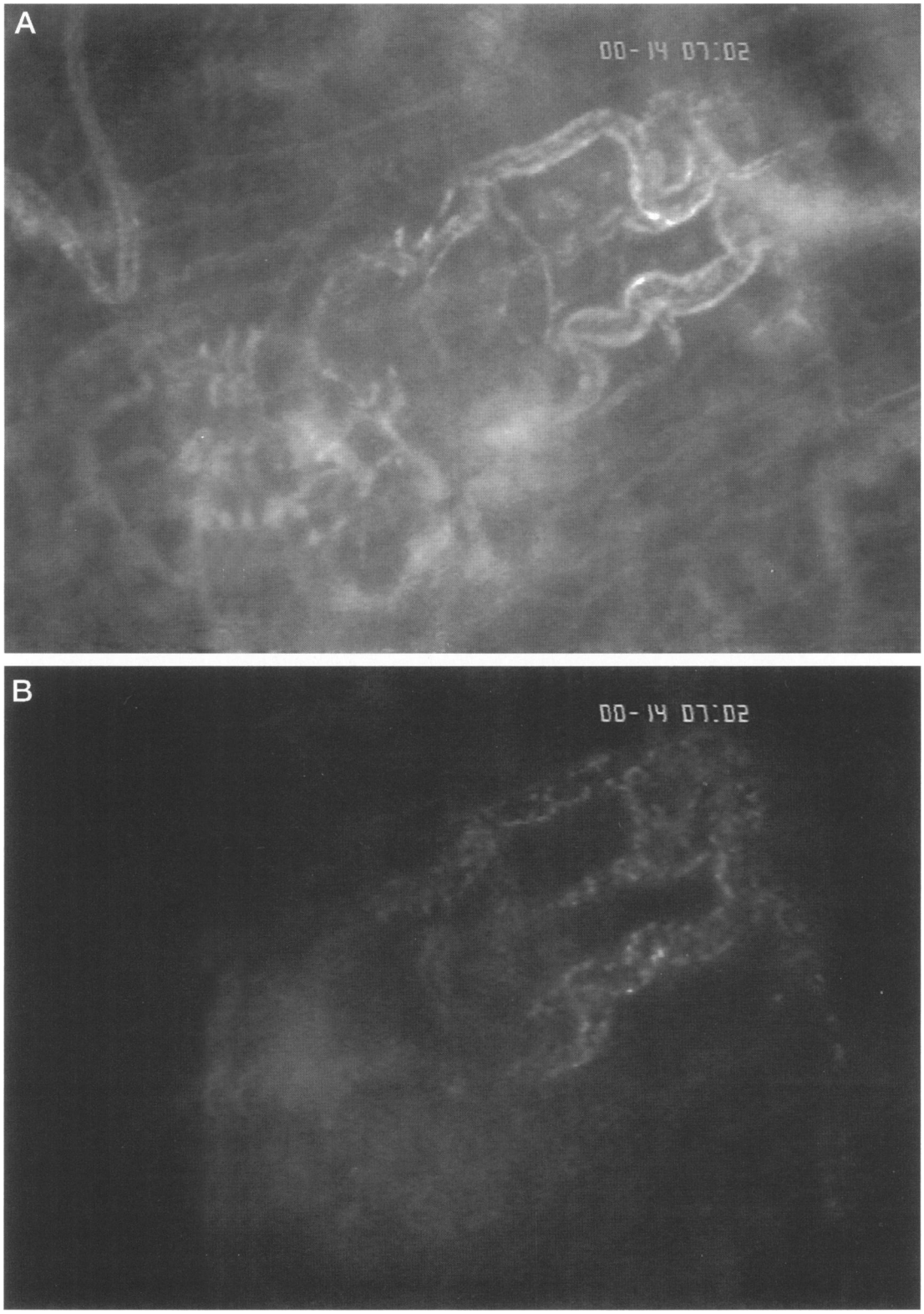
Figure 2. *Influence of vitamin E on microvascular manifestation of pancreatic islet xenograft rejection. Leukocyte accumulation* (left) *and leukocyte adhesion to the endothelium of postcapillary venules* (right) *of the microvascular network of pancreatic islet grafts on days 6, 10, 14, and 20 after syngeneic (hamster-to-hamster) and xenogeneic (rat-to-hamster) transplantation. Recipients of xenografts were treated with dietary vitamin E supplementation at concentrations of 150 mg/kg chow dry weight (XEN 150; n = 9) and 8000 mg/kg chow dry weight (XEN 8000; n = 6). Control animals with syngeneic (SYN 0; n = 6) and xenogeneic (XEN 0; n = 9) islet grafts received a standard laboratory chow with no vitamin E supplement. Leukocyte accumulation and leukocyte adhesion were quantitatively assessed by frame-to-frame analysis of video-taped images. Mean ± SEM; * $P < 0.05$* versus *XEN 0.*

cular network and functional capillary density (Figure 1). In a few of the islets, immunohistochemistry revealed some fragments of intracellular insulin, whereas in most of the untreated xenografts, intracellular insulin could not be detected.

In vitamin-E-supplemented animals, 87% (150 mg of vitamin E/kg of chow) and 92% (8000 mg of vitamin E/kg of chow) of the xenografts demonstrated initial vascularization on day 6 after transplantation. In these animals, vitamin E supplementation almost completely abrogated the inflammatory response during the entire observation period. The values of leukocyte accumulation and adhesion to the grafts' postcapillary venules were not different from those observed in syngeneic control islets (Figure 2). In parallel, vitamin E feeding prevented rejection-induced microvascular alterations, resulting in adequate development of both the size of the islets' microvascular network and its functional capillary density beyond day 10 of transplantation (Figure 1). Protection from microvascular rejection was most pronounced after supplementation of 8000 mg of vitamin E/kg of chow, which resulted in an angiogenic response and graft vascularization similar to the ones observed in isografts (Figure 4). Immunohistochemistry showed preserved islet grafts with homogeneous staining for intracellular insulin.

Discussion

We demonstrate herein that the initial angiogenic response induced by pancreatic islet xenografts is significantly reduced when compared with that induced by isografts. Moreover, microvascular leukocyte sequestration and adhesion to the endothelial lining of postcapillary venules within the grafts hallmark the microvascular manifestation of graft rejection and is associated with the loss of endothelial integrity and capillary perfusion failure. These microvascular events precede the process of graft infiltration and destruction[29,30] and support the notion that the microvasculature represents a primary target of immune/inflammation-mediated injury in xenograft rejection.[2–4] As freely transplanted pancreatic islets are revascularized from the host-derived microvascular bed, the endothelial cells are of host (hamster) origin.[7] As a consequence, the cell-cell interactions observed during graft rejection may not be triggered by the recog-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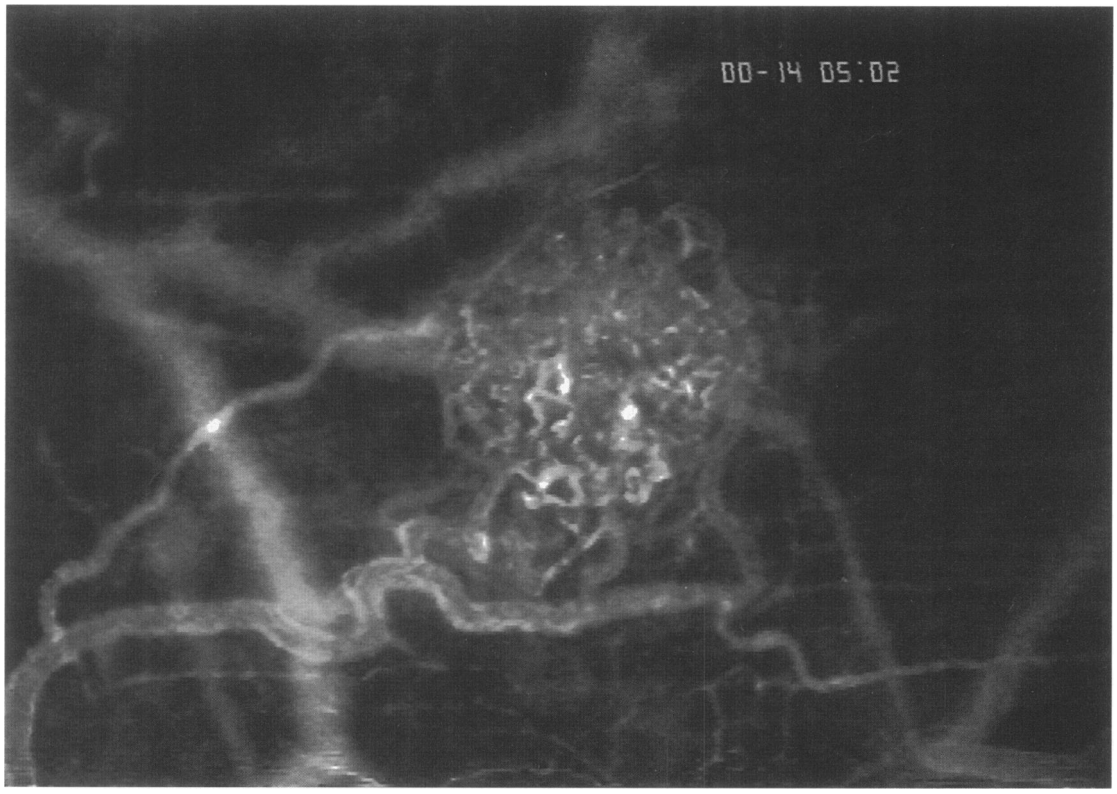

Figure 4. *Microvasculature of a xenogeneic rat islet 14 days after transplantation into the dorsal skinfold chamber of a Syrian golden hamster receiving dietary vitamin E supplementation (8000 mg/kg chow dry weight). Note the islet-specific glomerulus-like network of capillaries, indicating complete revascularization without signs of inflammation. Intravital fluorescence microscopy (10× objective); contrast enhancement with 5% FITC-dextran 150,000 i.v.; magnification, × 30.*

nition of species/strain-specific endothelial cell surface antigens but may be mediated by less specific inflammatory mediators and adhesion molecules, such as reactive oxygen metabolites and intercellular/vascular adhesion molecules.[30]

Dietary vitamin E supplementation effectively improved the angiogenic response and counteracted the microvascular rejection after xenotransplantation of pancreatic islets. The preservation of endothelial integrity and nutritive capillary perfusion by vitamin E indicates a protective effect on endothelial cells. This view is supported by *in vitro* experiments, demonstrating that endothelial cells are most resistant to immune-triggered, leukocyte-mediated injury when cultured in the presence of vitamin E.[19] Although the exact mechanisms by which vitamin E exerts its beneficial effects in our experiments remain unknown, the well documented antioxidant properties of vitamin E implicate reactive oxygen species as one of the key mediators in microvascular rejection of the grafts. On exposure to appropriate stimuli (eg, tumor necrosis factor-α and interleukin-1), inflammatory cells, in particular polymorphonuclear leukocytes, release a rich armamentarium of cytotoxic mediators, including superoxide anions and other reactive oxygen species.[12,31] Indeed, several studies suggest that oxygen radicals are directly involved in endothelial cell cytotoxicity by activated leukocytes,[32,33] a process that is mediated by lipid peroxidation in biomembranes.[31,34] The beneficial action of vitamin E on islet xenografts may thus be attributed to the prevention of oxygen-radical-induced lipid peroxidation by its chain-breaking properties.[20,31,34,35]

Figure 3. *Microvascular network of a xenogeneic (rat-to-hamster) pancreatic islet graft, 14 days after transplantation into the dorsal skinfold chamber of a Syrian golden hamster receiving a laboratory chow with no vitamin E supplement, visualized by intravital fluorescence microscopy (10× objective). Contrast enhancement with 5% FITC-dextran 150,000 i.v.* (A) *and* in vivo *staining of leukocytes with 0.2% rhodamine 6G i.v.* (B). (A) *Note the decreased density of the islet's microvascular network* (left) *and the local inflammatory response with leakage of the high molecular weight fluorescence marker FITC-dextran associated with edema formation, indicating loss of microvascular integrity. Extravasated FITC-dextran is observed within both the grafts pericapillary and perivenular space, as indicated by the brightness of the extravascular space compared to the perivascular space of non-inflammed grafts (see Fig. 4). Brighter appearance of the inflamed graft compared to the surrounding area with postcapillary venules results from the higher vessel density within the graft.* (B) *Leukocyte accumulation* (white dots) *is observed within postcapillary venules* (right; *identified by direction of blood flow*), *but not within the islet's network of capillaries* (left). *Magnification, × 30.*

However, oxygen radicals may also cause tissue injury indirectly either by stimulating the generation of endothelial-cell-derived chemoattractants[36] or by inducing the up-regulation of endothelial cell surface ligands for leukocyte adhesion.[37,38] Both mechanisms will ultimately contribute to the local microvascular recruitment of inflammatory cells and, consequently, to the perpetuation of graft rejection. Therefore, prevention of microvascular islet xenograft rejection by vitamin E may not only be attributed to its ability to interfere with lipid peroxidation but may also be due to its direct inhibitory effect on one or several steps in leukocyte-endothelial cell interactions. This view is supported by recent *in vitro* studies demonstrating that vitamin E reduces gene expression of individual endothelial cell adhesion molecules, such as E-selectin,[21] and thereby inhibits leukocyte-endothelial cell communication.[21,22] Furthermore, dietary vitamin E supplementation of heart transplant recipients was found to reduce platelet aggregation in response to thrombin or ADP.[39] This is of particular interest, as recent experiments have suggested a crucial role for platelets in forming bridging interconnections between activated circulating leukocytes, tethering leukocyte/platelet aggregates to the microvascular wall.[40]

In conclusion, we have demonstrated by intravital fluorescence microscopy that dietary supplementation of vitamin E effectively prevents both vascularization failure and microvascular rejection of pancreatic islet xenografts. With the fact that this dietary regimen is inexpensive, easy to apply, and safe regarding toxic side effects as well as with the view that it is potentially beneficial for glycemic control by modulating insulin action[41–43] and reducing oxidative stress,[44] which is known to be associated with both non-insulin-dependent[45] and insulin-dependent[46] diabetes mellitus, we propose that its use represents an interesting novel treatment modality aimed at improving the outcome of islet xenotransplantation.

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