INHIBITION OF CHRONIC REJECTION OF AORTIC ALLOGRAFTS BY DIETARY GLYCINE

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Abstract

Background. Chronic rejection is influenced by a variety of risk factors, including histoincompatibility and ischemia. Glycine, a cytoprotective agent, has been shown to protect against ischemia-reperfusion injury in the liver, inactivate hepatic resident macrophages, minimize cyclosporin A-induced nephrotoxicity, and exhibit immunosuppressive properties in vitro. The aim of this study was to investigate whether dietary glycine could reduce development of chronic rejection.

Methods. Lewis recipients of Fisher-344 abdominal aortic allografts received diets that contained either 5% glycine plus 15% casein or 20% casein as control for 10 weeks. Vascular lesions of aortic isografts and allografts were evaluated quantitatively with image analysis and cell counting.

Results. No significant vascular changes were observed in isografts (mean medial areas of 3.3±0.3×10^5 µm^2). However, dramatic intimal thickening (neointimal area 2.1±0.3) and medial thinning (1.5±0.3) were observed in allografts from rats fed control diet. In contrast, glycine significantly reduced the neointima by 45% (1.2±0.3) and protected the media (3.5±0.2). This led to intima to media area ratios almost twice as large in the control group as in glycine-fed rats (2.2±0.4 vs. 1.1±0.3, P <0.05).
Moreover, infiltrating leukocytes, especially macrophages, were reduced significantly in
the adventitia by glycine. In addition, glycine inhibited proliferation and migration of rat aortic smooth muscle cells in culture by 45 and 60%, respectively.

Conclusion. These results indicate that dietary glycine minimizes histopathological changes of chronic rejection by reducing the immune response and, in part, by minimizing proliferation and migration of smooth muscle cells.

Chronic rejection, or transplant allograft vasculopathy, has been identified as the most important cause of renal graft failure after the first posttransplant year (1), and is a major factor affecting long-term survival of transplanted organs (2). Vascular changes due to intimal hyperplasia, an important characteristic of this process, constitute the morphological hallmark of chronic rejection (3, 4). The proliferative vascular changes in chronic rejection are similar in all transplanted solid organs (5), and there is no effective treatment. In cardiac allografts undergoing chronic rejection, concentric vascular intimal thickening occurs together with medial thinning, interstitial fibrosis and perivascular infiltration of mononuclear cells (5, 6). Intimal hyperplasia involves proliferation of transformed secretory type smooth muscle cells and infiltration of inflammatory macrophages and activated T lymphocytes (5). Transplant vasculopathy affects the entire length of the vessels including major vessels as well as the small penetrating intramyocardial branches in the heart (7). Congestive heart failure, ventricular arrhythmia, myocardial infarction, and sudden death may occur.

Although the exact pathogenesis of chronic rejection remains to be established, several risk factors, some dependent and some independent of allogenic immune responses, have been identified (8). Long-term graft survival of HLA identical sibling renal transplants in humans and experimental syngenic transplants in inbred animals support the role of host immunological responsiveness in the process (9). The frequency and intensity of acute rejection episodes are also associated with an increase in the prevalence and severity of chronic rejection (10). However, the influence of factors independent of immune responses has attracted increasing interest (11). Potential risk factors include early injury secondary to prolonged ischemia and reperfusion, cytomegalovirus infection, disorders of lipoprotein metabolism and the impact of immunosuppressive drugs (3, 8). Cyclosporin A- (CsA) induced nephrotoxicity, hypercholesterolemia and hypertension may ultimately worsen or accelerate the chronic process of graft loss (8). Moreover, some of these factors, such as ischemia-reperfusion injury and infection, may be confounding variables contributing to the disease process (12). Therefore, to improve long-term survival, the primary strategy is to avoid known risk factors and to immunosuppress the recipient sufficiently although avoiding over immunosuppression.

Glycine, a cytoprotective agent and immunomodulatory amino acid, has been shown to protect against hypoxia-reoxygenation injury in the liver (13), inactivate hepatic resident macrophages (14), and exhibit immunosuppressive properties in vitro (15). Moreover, diets supplemented with glycine, a simple long-term dietary therapy, minimize cyclosporin A-induced nephrotoxicity, inhibit hepatocyte proliferation caused...
by peroxisome proliferators, and improve survival in endotoxin shock in rats (14, 16, 17). These findings led to the hypothesis that dietary glycine would minimize chronic allograft rejection. Therefore, our study was designed to test this hypothesis using a rat aortic transplant model. Indeed, a glycine-containing diet inhibited allograft vasculopathy by reducing intimal thickening and medial thinning in vivo and suppressed smooth muscle cell proliferation in vitro. Preliminary accounts of this work have appeared elsewhere (18).

**MATERIALS AND METHODS**

**Animals and diets.**

Two inbred strains of female rats weighing 200 to 240 g with minor histocompatibility differences were chosen for these studies: Fisher-344 and Lewis. Allogenic transplantations were performed from Fisher-344 donors to Lewis recipients (n=10 per group), and syngeneic transplantations (i.e., isografts, n=4 per group) were from Lewis to Lewis rats. The animals were housed in an AALAC-approved facility for one week before surgery with free access to standard laboratory chow (Agway PROLAB RMH 3000, Syracuse, NY) and tap water. Two days before transplantation, recipient rats were randomly assigned to two experimental groups and fed a semisynthetic powdered diet (AIN-93G (19), Teklad test diets, Madison, WI) containing 5% glycine and 15% casein (glycine group) or 20% casein (control group). After transplantation, each recipient rat continued to receive its assigned diet throughout the entire experimental period of 10 weeks. All animals received humane care in compliance with guidelines approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

**Aortic transplantation.**

Rats were anesthetized by inhalation of methoxyflurane (Mallinckrodt Veterinary, Inc., Mundelein, IL). The abdominal aorta of each recipient rat was dissected free below the left renal arteries to the bifurcation of the abdominal aorta, as described previously (20). A 14- to 18-mm long segment of the aorta was removed from the donor rat and stored at 4°C in lactated Ringer’s solution (Baxter, Deerfield, IL) during preparation of the recipient. Each graft was immediately transplanted orthotopically by an end-to-end anastomosis with 8–0 nylon sutures (Ethicon, Somerville, NJ). The aortic clamps were then removed and patency of the new vascular connection was confirmed. Average cold ischemia time was about 35 min, and no pharmacological immunosuppression was used. Ten weeks after transplantation, recipient animals were anesthetized and the aortic grafts were excised. Each graft was divided into four parts. Two parts were fixed in 4% buffered formaldehyde and embedded in paraffin, and the other two parts were frozen and stored at -80°C until processed for immunohistochemistry.

**Measurement of glycine concentration in blood.**

Blood samples were collected at necropsy and serum was stored at -80°C until
measurement. Concentrations of glycine in serum were determined as described by Ohmori (21). Briefly, glycine was extracted, benzoylated, and the resulting hippuric acid was extracted and dried. Subsequently, the concentration of a colored conjugate of hippuric acid was determined spectrophotometrically at 458 nm.

Image analysis of rat aortic grafts.

A minimum of two cross-sections (5 µm thickness) of each isograft or allograft was taken from each animal, embedded in paraffin, and stained with elastic-van Gieson and hematoxylin. The cross-sectional areas of intima and media of the aortic allografts were measured using a BioQuant TCW95 image acquisition and analysis system (Nashville, TN) incorporating an Olympus BH2 microscope (Olympus America Inc., Lake Success, NY). The medial area was defined as the area between the external and internal elastic lamina. The neointimal area was defined as the area between the internal lamina and arterial lumen (22).

Histology.

Paraffin-embedded isografts and allografts were cut into 5-µm thick cross-sections and stained with hematoxylin and eosin. Evaluation of grafts was performed semiquantitatively by scoring the severity of vascular changes (intimal thickening, medial thinning and adventitia inflammation) from 0 to 3 (0=no changes, 1=mild, 2=moderate, and 3=severe). Sections were scored by an outside investigator without knowledge of group assignment. The number of nuclei in a given anatomical area using straight, cross-sectional lines and a 10-mm grid in the eye-piece was counted in four random areas of the adventitia layer of each graft (magnification ×200), and means were calculated. It has been shown that nuclei in the allograft adventitia are predominantly inflammatory infiltrates (23, 24).

Immunohistochemical staining.

Five-µm thick cross-sections of paraffin-embedded grafts were stained for detection of monocyte/macrophages (ED1+ cells). In addition, 5-µm thick cross-sections of frozen sections were stained for pan T-lymphocytes (CD5+ cells) and proliferation cell nuclear antigen (PCNA) using the Dako Envision System. Primary antibodies against ED1 and CD5 were purchased from Biosource International (Camarillo, CA). Immunohistochemistry was quantitated by counting the number of positive-stained cells using the above-mentioned eye-piece with a magnification of 400×. Four areas per section were randomly selected and counted. Data were pooled to determine means.

Smooth muscle cell proliferation assay.

Smooth muscle cells were isolated from rat aortas, cultured for 12-16 generations and maintained in Dulbecco's modified Eagle's medium (GIBCO Laboratories Life Technologies, Grand Island, NY) supplemented with 10% bovine calf serum and penicillin/streptomycin in a humidified, 5% CO₂ atmosphere at 37°C, as described
previously (25). Cells were trypsinized, seeded onto 60-mm tissue culture dishes (Corning Glass Works, Corning, NY) at a density of 10 (5) cells per dish and cultured in minimum essential medium (MEM) supplemented with 10% serum and nonessential amino acids (100 µmol of alanine, asparagine, aspartic acid, glutamic acid, proline, and serine) in the presence or absence of glycine (0.1-10 mmol/liter). On days 2, 4, 6, and 8, cells were trypsinized and counted.

Smooth muscle cell adhesion assay.

Two six-well culture plates (60 mm) were precoated with 10 µg/ml of collagen (type 1, Collaborative Biomedical Products, Bedford, MA). Cells were resuspended in modified MEM (see above) with or without 10 mM glycine to give a final concentration of 2×10^5 cells/ml. One ml of cells was added to each well and incubated in a humidified, 5% CO₂ atmosphere at 37°C. After 2.5 hr, nonadherent cells were removed, and the number of cells adhering to the bottom of the wells was determined microscopically (100×) by counting five randomly selected fields of constant area per well. All experiments were performed in triplicate.

Smooth muscle cell migration assay.

Rat smooth muscle cells were preincubated in MEM supplemented with 10% serum and nonessential amino acids as described above in the presence or absence of glycine (10 mmol/liter) for 48 hr. The migration of smooth muscle cells was quantitated using a Transwell culture chamber (Costar, Cambridge, MA) where the upper and lower culture chambers are separated by a polycarbonate filter with 8-µm pores. Each membrane was coated with 30 µg/ml of rat tail collagen (Collaborative Biochemical Research) in 0.2 mol/liter acetic acid for 24 hr before each chemotactic assay. For each assay, 25 µl of PDGF (5 ng/ml, Life Technologies, Inc.) in MEM plus 10% serum was loaded in quadruplicate wells in the bottom chamber. Cultured smooth muscle cells with or without glycine (10 mmol/liter) were trypsinized and suspended at a concentration of 1×10^5 cells/ml. A volume of 50 µl of the smooth muscle cell suspension was seeded in the upper chamber. The culture media with glycine (10 mmol/liter) was placed into the lower chamber of the well and cells preincubated with glycine were placed in the upper chamber. Control was glycine-free media and cells preincubated without glycine treated in the same manner. After 24 hr of incubation at 37°C, the filters were removed and the smooth muscle cells on the upper side of the filter were removed. The smooth muscle cells that had migrated to the lower side of the filter were fixed in methanol and stained with Diff-Quik staining solution (Dade International Inc., Miami, FL). The filters were excised from their plastic supports and mounted on glass slides with Permount (Sigma Chemical Co.). The number of migrating cells was determined microscopically by counting five high-power (400×) fields of constant area per well. The migration assays were performed with two wells per group each time and repeated a total of three times.

Statistics.

All results were expressed as means±SEM. Statistical differences between means
were determined using analysis of variance, analysis of variance on ranks or $t$ test where appropriate. $P < 0.05$ was selected before the study to determine statistical differences between groups.

RESULTS

Glycine concentration in blood.

Animals received either glycine-containing or control diets during the entire 10-week experimental period, and blood samples were taken at necropsy. The blood concentration of glycine from glycine-fed recipients was $0.56 \pm 0.06$ mmol/liter, which was 3-fold higher than values of samples from rats fed control diet ($0.18 \pm 0.03$ mmol/liter, $P < 0.05$).

The effect of dietary glycine on histological manifestations of transplant vasculopathy.

Intimal proliferation, medial thinning, and perivascular inflammation are characteristics of allograft vasculopathy in this model (20, 23), and these pathological features of chronic rejection were found in our study. Figure 1 displays representative photomicrographs of aortic grafts stained with elastic-van Gieson. Isografts from rats fed either control or glycine diets were completely free of arteriosclerotic lesions, i.e., no intimal proliferation, medial necrosis, or adventitial inflammation was observed (Fig. 1A). In contrast, allografts from rats given control diet showed typical intimal thickening, medial thinning, and perivascular inflammation (Fig. 1B) and exhibited a pathology score of 3.7 (Table 1). The media showed loss of nuclei and breaks of elastic laminae, and in the neointima, nuclei accumulated in the subendothelial space. Intimal proliferation and medial necrosis were reduced dramatically by dietary glycine (Fig. 1C), with a nearly 50% lower pathology score (Table 1).
A

Isograft

B

Allograft + Control diet

C

Allograft + Glycine diet

I

M
Figure 1. Representative photomicrographs of aortic grafts 10 weeks after transplantation. Lewis recipients of Lewis (syngenic transplantation) and Fisher-344 (allogenic transplantation) abdominal aortic grafts received either control or glycine-containing (5%) diets for 10 weeks. An isograft (A), an allograft from a rat fed control diet (B), and an allograft from a rat given glycine diet (C) are shown. Intima is marked “I,” media are labeled “M” and adventitia “A.” Animal treatments and surgical procedures are detailed in Materials and Methods. The grafts were stained with elastic-van Gieson. Original magnification, 100×.

Table 1. Effects of dietary glycine on cells in aortic grafts

<table>
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<tr>
<th>Markers</th>
<th>Isografts&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Allografts + control diet</th>
<th>Allografts + glycine diet</th>
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<td>Pathology score</td>
<td>0 ± 0</td>
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<td>2.0 ± 0.3&lt;sup&gt;bc&lt;/sup&gt;</td>
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<td>ED1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.5 ± 0.3</td>
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<td>27 ± 4&lt;sup&gt;bc&lt;/sup&gt;</td>
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<td>CD5&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>135 ± 20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>138 ± 17&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>PCNA&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>25 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12 ± 4&lt;sup&gt;bc&lt;/sup&gt;</td>
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Pathology was scored semiquantitatively as described in Materials and Methods.<sup>a</sup> There were no differences between control and glycine groups in isografts; therefore, values of these two groups were combined.<sup>b</sup> P<0.05 compared to isograft.<sup>c</sup> P<0.05 compared to allografts from rats fed control diet, by one-way analysis of variance using Tukey’s post hoc test.<sup>d</sup> Monoclonal antibodies ED1 and CD5 recognize monocyte/macrophages and pan T lymphocytes, respectively.<sup>e</sup> PCNA is an abbreviation of proliferation cell nuclear antigen.

Table 1. Effects of dietary glycine on cells in aortic grafts. Pathology was scored semiquantitatively as described in Materials and Methods. There were no differences between control and glycine groups in isografts; therefore, values of these two groups were combined. P<0.05 compared to isograft. P<0.05 compared to allografts from rats fed control diet, by one-way analysis of variance using Tukey’s post hoc test. Monoclonal antibodies ED1 and CD5 recognize monocyte/macrophages and pan T lymphocytes, respectively. PCNA is an abbreviation of proliferation cell nuclear antigen.

Image analysis was used to determine the area of each cell layer of the aortic grafts. Intimal areas (Fig. 2 A) of isografts from both control and glycine-fed animals were nearly undetectable as expected (0.01±0.01x105 µm2). However, the average intimal area of allografts from rats fed control diet for 10 weeks was increased to 2.1±0.3. Dietary glycine reduced the intimal area by 45% (1.2±0.3, P<0.05). In isografts, medial areas from rats given control diet were not different from those fed glycine-containing diet (2.9±0.1, Fig. 2B). The medial areas of allografts from animals given control diet were significantly smaller than the isografts (2.2±0.2, P<0.05). However, dietary glycine preserved the media (3.0±0.1), yielding values that were comparable to...
the syngenic grafts and significantly higher than allografts from rats fed control diet. This led to intimal to medial ratios that were about two times larger in allografts from rats fed control diet than those fed glycine (0.9±0.1 vs. 0.5±0.1, $P<0.05$) (Fig. 2C). Because the isograft intima consisted of a single layer of flat endothelial cells, the intima to media ratio was close to zero in syngenic transplants. These data demonstrate that glycine-containing diets can suppress the development of chronic rejection of aortic allografts.
The effect of dietary glycine on perivascular infiltration of aortic grafts.

Figure 3 shows the magnitude of cellular infiltration in the adventitial layer. There were only a few scattered nuclei of fibroblasts in the perivascular areas of isografts fed control or glycine diets. As expected, a strong inflammatory response in allografts from rats fed control diet was observed, with a significant increase in nuclei in the adventitia. Dietary glycine blunted this increase in the number of perivascular leukocytes in the aortic allograft by about 40%.
Figure 3. Effect of dietary glycine on perivascular inflammation of aortic grafts. Surgical procedures and animal treatments were described in Materials and Methods. Quantitation of infiltrating leukocytes, expressed by number of nuclei, was performed in hematoxylin and eosin stained grafts. Values represent mean±SEM. a, \( P < 0.05 \) compared with isografts; b, \( P < 0.05 \) compared to the allografts from rats fed control diet by analysis of variance with Tukey’s post hoc test.

Specific immunostaining with antibodies against monocyte/macrophages (ED1+) and T lymphocytes (CD5+) was negative in isografts (Table 1). Allografts from rats fed control diet demonstrated significantly more perivascular infiltration of monocytes and macrophages than isografts. Dietary glycine, however, significantly reduced the perivascular accumulation of monocytes and macrophages (Table 1). CD5 staining showed significantly more T cell infiltration in allografts from rats given control diet than isografts, but this increase in T cells was not influenced by glycine.

Effects of dietary glycine on proliferation cell nuclear antigen in neointima.

The ratio of PCNA+ stained nuclei to total nuclei in neointimal areas of allografts with or without dietary glycine supplements was calculated and is shown in Table 1. There were almost no PCNA+ nuclei in isografts. In contrast, about one-fourth of nuclei in allografts from rats fed control diet were PCNA+, values which were about 2-fold higher that those in allografts from glycine-treated rats.

Effects of glycine on rat smooth muscle cell proliferation in vitro.

To test the hypothesis that glycine minimizes aortic allograft vasculopathy by inhibiting smooth muscle cell proliferation, a series of in vitro experiments was performed. Indeed, when glycine (10 mmol/liter) was added to cultured smooth muscle cells, cell growth was inhibited in a time-dependent manner (Fig. 4). Significant differences between the control and glycine groups were observed as early as day 4, and on day 8, glycine suppressed growth of smooth muscle cells by ~50%. Next, an assay was performed to assess whether glycine affects the adherence of smooth muscle cells. No significant differences between the number of cells adhering to collagen-coated plates in the presence or absence of glycine (10 mmol/liter) were observed (data not shown). Moreover, culturing cells with glycine had no effect on cell morphology (Fig. 5). When the cells were incubated with different concentrations of glycine (0.1–10 mmol/liter) and cell number was determined from day 0 until day 8, a linear dose-dependent response was observed with an IC50 of about 1 mmol/liter (Fig. 6).
Figure 4. Inhibitory effects of glycine on growth of rat aorta smooth muscle cells in vitro. Rat aorta smooth muscle cells were plated at a density of 105 cells/dish in MEM supplemented with nonessential amino acids either with ([white circle], solid line) or without ([black small square], dotted line) glycine (10 mmol/liter) as described in Materials and Methods. Results are reported as mean±SEM from experiments performed in triplicate. Asterisks denote statistical differences from the corresponding time point (P <0.05, by two-way analysis of variance).
Figure 5. Effect of glycine on morphology of rat aortic smooth muscle cells in vitro. Representative photographs of smooth muscle cells in medium with or without glycine (10 mmol/liter). Rat aortic smooth muscle cells were seeded onto 60 mm/tissue culture dishes at a density of 1000 cells per dish and cultured with MEM in the presence or absence of glycine (10 mmol/liter) for 8 days. Cells were fixed and stained as detailed in Materials and Methods.
Figure 6. Inhibitory effect of glycine on in vitro growth of rat aorta smooth muscle cells is dose dependent. Rat aorta smooth muscle cells were plated at a density of 105 cells/dish in MEM supplemented with glycine (0.1–10 mmol/liter) as described in Materials and Methods. Cells were cultured for 8 days with one change of culture medium on day 4. At the end of the experiment, cells were detached by trypsinization and cell number determined. Results are reported as mean±SEM from experiments performed in triplicate. At all concentrations studied, differences from the control group were statistically significant (one-way analysis of variance with Student-Newman-Keul’s post hoc test).

Effects of glycine on rat smooth muscle cell migration in vitro.

As reported previously, cultured rat aortic smooth muscle cells migrate from the upper to the lower side of filters due to the chemotactic action of PDGF (26). Glycine (10 mmol/liter) added to culture media inhibited PDGF-induced migration of vascular smooth muscle cells by about 60% (Fig. 7), suggesting that glycine suppresses intimal thickening, at least partially, by inhibiting smooth muscle cell migration.
Figure 7. Inhibitory effects of glycine on migration of rat aorta smooth muscle cells in vitro. Rat aortic smooth muscle cell migration was determined as detailed in Materials and Methods. Cells were seeded in a Transwell culture chamber with a collagen-coated filter and grown in PDGF-containing culture medium with or without glycine (10 mmol/liter) for 24 hr. The smooth muscle cells that migrated to the lower side of the filter were fixed, stained and counted as described in Materials and Methods. The asterisk indicates $P < 0.05$ compared with the number of smooth muscle cells that migrated in control medium by $t$ test.
DISCUSSION

*Dietary glycine minimizes chronic rejection in aortic allografts.* The essential features of human chronic rejection, or allograft vasculopathy, are persistent perivascular inflammatory infiltrate associated with concentric intimal proliferation and medial necrosis (5). In our study using a model of Fisher-344 to Lewis aortic transplantation, similar lesions to human allograft vasculopathy were produced (Fig. 1). Intimal thickening, medial thinning and inflammation of the adventitia were observed in allografts, but not in isografts, confirming that antigen-dependent factors are critical in the pathogenesis of transplant vasculopathy. The mechanisms of intimal proliferation and medial thinning observed in allografts receiving control diet (compared to isografts) is unclear. Adventitia inflammation might be associated with necrosis of cellular components of the medial layer (23) or migration of smooth muscle cells from media into intima (27). Importantly, dietary glycine minimized transplant vasculopathy of aortic allografts. It reduced intimal proliferation, medial necrosis and perivascular inflammation significantly (Figs. 1–3, Table 1). Although allograft vasculopathy was not inhibited completely by dietary glycine, long-term dietary therapy is easy and exhibits no toxic side effects (28); therefore, it could be clinically useful. Moreover, dietary glycine minimizes CsA nephrotoxicity by reducing free radical production (16), which is an important risk factor in chronic rejection (24).

The mechanism of chronic rejection is poorly understood, but it most likely involves several cell types including endothelial cells and smooth muscle cells as well as leukocytes. Activation of the immune system is thought to be responsible for initiation and progression of chronic rejection and endothelial cells are the first target of the host immune system (29). Moreover, they undergo ischemia-reperfusion injury early after implantation, possibly enhancing antigenicity of the graft. This may cause up-regulation of MHC class II molecules and adhesion molecules leading to production of cytokines and growth factors, which facilitates infiltration of immune cells and migration and proliferation of smooth muscle cell (30, 31). Therefore, the protective effect of dietary glycine here might be due to effects on endothelial cells (32, 33). Moreover, humoral effects might be involved (34), and glycine inhibits B cell-mediated humoral reactions (35).

Cellular invasion in adventitia is one of the main features of chronic rejection and is regarded as a prerequisite for intimal thickening (23). When inflammatory cells infiltrate the blood vessels (36), they produce cytokines, growth factors, and chemotactic agents (37) that most likely are responsible for vascular smooth muscle cell proliferation and migration (3, 38). In addition, inflammatory leukocytes release cytokines and eicosanoids, leading to activation of graft vascular endothelium generating a vicious cycle, where increased expression of proinflammatory molecules leads to increased leukocyte extravasation, increased inflammation, and even increased expression of proinflammatory molecules (39). Subsequently, smooth muscle cells responding to these reactions and growth factors produced by endothelial cells, inflammatory cells, and smooth muscle cells themselves are induced to migrate into the media, replicate and begin remodelling the vascular wall. In the present study, dramatic perivascular inflammation was observed in aortic allografts (Fig. 3).
Interestingly, dietary glycine decreased perivascular inflammation by reducing invasion of monocytes and macrophages, but had no effect on infiltration of T lymphocytes (Table 1). The early appearance of monocytes and macrophages in the allograft intima has been reported to be related to the development of chronic rejection (38, 40). The macrophage is able to process and present antigen, leading to the production of a variety of cytokines and growth factors, e.g., TNF-[alpha] and PDGF, that are mitogenic or cytotoxic (41, 42). Moreover, in the vascular wall of atherosclerosis, macrophages have been shown to produce matrix metalloproteinases (43) which are involved in the migration of smooth muscle cells in vascular lesions (44). It is possible, therefore, that glycine acts by inactivating monocytes and macrophages, leading to reduced smooth muscle cell migration from media to intima. This possibility is strengthened by the fact that glycine inactivates macrophages in the liver by activating a glycine-gated chloride channel (45). The lack of effectiveness of glycine on infiltration of T cells in this study may partially explain the fact that chronic rejection was not blocked completely in this model.

Glycine inhibited rat smooth muscle cell proliferation and migration.

Vascular smooth muscle cell proliferation is a central event in the progression of chronic rejection (46). The increase in intimal thickness and cellularity was due, in large part, to proliferation of smooth muscle cells migrating from the media (47). The in vivo proliferative activity of smooth muscle cells in the neointima, as indicated by the number of PCNA+ cells in our study, was also reduced by glycine (Table 1). This is consistent with the hypothesis that glycine directly inhibits proliferation and migration of vascular smooth muscle cells of aortic allografts in addition to its effects on infiltrating macrophages. Indeed, glycine suppressed the growth of cultured vascular smooth muscle cells in a dose-dependent manner (Figs. 4 and 6) and inhibited the migration of cells in vitro (Fig. 7). It is known that changes in intracellular calcium are important for smooth muscle cells (48). Whether glycine influences the replication and migration of vascular smooth muscle cells by altering intracellular calcium through glycine-gated chloride channels, as is observed in resident hepatic macrophages (45), remains a possibility.

In conclusion, our results demonstrate that dietary glycine could be helpful as an adjuvant therapy to current immunosuppressive regimens to reduce development of chronic rejection. It is simple and lack of toxicity makes it possibly clinically useful.

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**IMAGE GALLERY**

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**Table 1**

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Pathology was scored semiquantitatively as described in Materials and Methods.

<sup>a</sup> There were no differences between control and glycine groups in ingraft, therefore, values of these two groups were combined.

<sup>b</sup> P<0.00 compared to ingraft.

<sup>c</sup> P<0.05 compared to allografts from rats fed control diet, by one-way analysis of variance using Tukey's post hoc test.

<sup>d</sup> Monoclonal antibodies ED1 and CD4 recognize monocyte/macrophages and pan T lymphocytes, respectively.

<sup>e</sup> PCNA is an abbreviation of proliferation cell nuclear antigen.