Intimal Thickening and Medial Necrosis in Allograft Arteriosclerosis (Chronic Rejection) Are Independently Regulated

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Rat aortic allografts from the DA (RT1\(^a\)) to the WF (RT1\(^r\)) strain but not syngeneic DA-to-DA control grafts develop arteriosclerotic changes in the vascular wall that are virtually identical to human allografts during chronic rejection. A more prominent medial cell destruction in the rat aorta, leading ultimately to complete medial necrosis, is the major difference between rat and human allografts. If the adventitia of syngeneic grafts is exposed to starch before transplantation, these grafts also develop an inflammatory reaction in the adventitia and an extensive intimal thickening at the site of the granulomas, but the medial smooth muscle cells are preserved. In both types of transplants with an intact endothelium as determined by light microscopy, adventitial inflammatory cell proliferation was accompanied by smooth muscle cell replication in the media and thickening of the intima. We therefore propose that an adventitial proliferative response is a prerequisite for intimal thickening to occur. In the allograft but not in starch-exposed syngeneic grafts there was also a notable lymphoid activation in the adventitia, which was accompanied by medial necrosis. We suggest that the medial necrosis in the allograft is linked to a toxic effect of activated lymphoid cells on medial myocytes and is not a prerequisite for intimal proliferation. Instead, intimal proliferation and medial necrosis in the allograft seem to be independently regulated.

(Arteriosclerosis and Thrombosis 1993;13:1019-1025)

KEY WORDS • chronic rejection • arteriosclerosis • smooth muscle cells • adventitial inflammation

A common feature in all allografts undergoing chronic rejection is persistent perivascular inflammation and intimal thickening, called allograft arteriosclerosis. In contrast to classic atherosclerosis, which is mostly focal and eccentric, allograft arteriosclerosis is concentric and generalized. In ordinary atherosclerosis the internal elastic lamina is entirely disrupted at the site of the lesion, whereas in allograft arteriosclerosis it is mostly preserved, with occasional breaks only.\(^1,2\)

The vascular wall changes in long-surviving, nonimmunosuppressed, rat aortic allografts are virtually identical with those observed in human allografts. There is one notable exception, however\(^3\): in contrast to human allografts, in which only some thinning and occasional myocyte necrosis are observed in the vascular media,\(^1,2\) rat vascular allografts undergo a complete loss of smooth muscle cell nuclei in the media, thus indicating medial necrosis.

Tritiated thymidine (\(^3\)H-TdR) labeling studies in rat aortic allografts have demonstrated that in chronic rejection, smooth muscle cell replication begins in the media but is thereafter rapidly extended to the intima.\(^4\) In human allografts, the morphological orientation of the smooth muscle cells near the intimal elastic lamina suggests that they are “poised” to exit the media.\(^2\) A plausible explanation for the loss of smooth muscle cells in the media would thus be that myocytes have left the media and migrated into the intima. This, however, is not the case.

Recently, we fortuitously found that some syngeneic controls of rat aortic allografts displayed focal intimal thickening. Under normal conditions, the syngeneic controls do not display any intimal changes. It appeared that the surgeon who had performed the microsurgery on these control animals had used surgical gloves and that the focal thickening in the intima was invariably associated with starch granulomas in the adventitia. Closer examination of these specimens revealed two changes, which are important in understanding the mechanism of allograft arteriosclerosis: first, that lymphocytes within the adventitial granuloma, in contrast to the allograft, displayed only a low level of lymphoid activation, and second, that the focal intimal lesion in the granuloma-associated arteriosclerotic change was not linked with medial necrosis.

Therefore, we performed a systematic study to investigate the interrelation between medial necrosis and intimal thickening and to explore the reasons for medial necrosis in rat aortic allografts.

Methods

Experimental Animals

Inbred rat strains WF (AG-B2, RTV1\(^r\)) and DA (AG-B4, RT1\(^a\)) were used for transplantation. All ani-
mals were purchased from the Zentralinstitut für Versuchstierzucht GmbH, Hannover, FRG. Male rats weighing 200 to 300 grams and 1 to 3 months of age were used as donors and recipients.

All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the Institute of Laboratory Animal Resources and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985).

Aortic Transplantations

A segment of the descending thoracic aorta approximately 3 cm long was excised, perfused with saline, and used as a transplant. The rats were anesthetized with intraperitoneal chloral hydrate (6 mL/kg of body weight) and received 0.3 mg/kg buprenorphine (Temgesic) SC for postoperative pain relief. The graft was transplanted into a heterotopic position below the renal arteries and above the bifurcation, thereby forming a "loop" in the abdominal cavity. The cranial suture line was made as close to the renal arteries as technically possible to minimize the difference in vessel diameter. An end-to-end anastomosis was performed by using a 9-0 continuous nylon suture. The DA-to-WF-strain combination was used for the allografts. Syngeneic control grafts were made from the DA to the DA strain. The grafts from at least five animals at each time point were removed at various times after transplantation and processed for histology, autoradiography, and frozen-section immunohistochemistry. Both in situ fixation, via infusion of 10% neutral formaldehyde to the left atrium before the anesthetized animal was killed, and regular fixation were employed with similar results. Specimens for immunohistochemistry were immersed in Tissue-Tek from Miles, Elkhart, Ind, and snap-frozen.

Twenty DA-to-DA syngeneic aortic transplants were then performed by smearing starch particles around the adventitia with surgical gloves. The transplants were removed as above, and quantitative histology was performed as above. The success rate was >95%, and no heparin was used.

Autoradiography

Some of the rats received 250 μCi of ³H-TdR (NEN, Boston, Mass) by intravenous injection 3 hours before they were killed. Histological specimens were processed from paraffin sections, emulsion autoradiography (Ilford L4; Ilford, Mobberley, UK) was performed, and the labeling of the nuclei in the transplanted aortic wall was compared with the labeling index in the recipient aorta. On some occasions autoradiography was performed on immunohistochemically stained slides to identify the type of ³H-TdR-labeled cells.

Histological Specimens and Staining

For evaluation of morphological changes, paraffin sections were stained with Mayer's hematoxylin and eosin, and orcein was used for elastic fibers. Longitudinal sections were also prepared, for which the vessel wall on both the graft and host sides of the suture line could be observed, as well as cross sections for quantification of circular changes in the graft. Quantitative histology (morphometry) was always done from the middle third section of the transplant. Aortas from normal nontransplanted rats and the thoracic aorta of the recipient rat were used as controls.

Immunohistochemistry

For immunohistochemistry, 3-to-4-μm-thick frozen sections were stained by the immunoperoxidase tech-

Fig 1. Photomicrographs of cross sections of a syngeneic graft (left panel), an allograft (middle panel), and a syngeneic graft with starch-induced granulomas (right panel) 3 months after transplantation. Small letters indicate the main anatomical structures of the aortic wall: a, adventitia; m, media; i, intima. Note the granulomas in the adventitia, preservation of smooth muscle cells in media, and intimal thickening in the right panel.
technique by using monoclonal anti-rat antibodies to the interleukin-2 (IL-2) receptor, interferon gamma (IFN-γ), leukocyte common antigen (LCA; OX1), and class II major histocompatibility complex antigen (OX4). Rat anti-class II antibody and rat anti-LCA were obtained from Sera Lab, Sussex, England; rat anti-IFN-γ antibody was from Dr Peter van der Meide, TNO, Rijkskowisk, The Netherlands; rat anti-IL-2 receptor was from Dr J. Kupiec-Weglinski, Harvard Medical School, Boston, Mass; and anti-smooth muscle α-actin was from Biomakor, Rehovot, Israel. The cryosections were stained by using the two-layer indirect immunoperoxidase technique described in detail elsewhere. Briefly, the sections were incubated with diluted monoclonal antibody (usually 1:20; =1:5000 for α-actin), washed, and consecutively incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulin and goat anti-rabbit immunoglobulin (Dako Immunoglobulins a/s, Glostrup, Denmark), followed by treatment with a substrate solution containing the chromogen 3-amino-9-ethylcarbazole. Hydrogen peroxide was added to the 3-amino-9-ethylcarbazole solution immediately before use. The samples were counterstained with Mayer’s hemalum solution and mounted.

Quantification of Histology and Immunohistochemistry

The morphological changes were quantified according to standard morphometric principles and expressed as point score units, ie, the mean number of points falling over a given anatomic area defined by straight cross-sectional lines and a 0.02-mm grid (for details, see Reference 3). The following variables were evaluated: the number of nuclei and the thicknesses of different layers of the aorta, ie, adventitia, media, and intima, separated pairwise from each other by external and internal elastic laminae, respectively, as well as the number of antibody-positive cells in immunoperoxidase sections. A minimum of five technically successful transplantations were made for each time point unless otherwise noted, and their means (±SEM) were used as final scores.

Statistics

The Mann-Whitney U test was used for statistical analysis. Values of P<.05 were considered statistically significant.

Results

DA-to-WF allografts demonstrated an early inflammatory episode in the allograft adventitia that was
significantly smaller in syngeneic transplants. The allograft media showed a gradual loss of nuclei, focal breaks in the otherwise-intact internal elastic lamina, and in the intima, a gradual accumulation of nuclei in the subendothelial space and an increase in intimal thickness. These changes were nonexistent in the syngeneic control animals (Figs 1 and 2). We have previously demonstrated that most of the nuclei in the adventitia represent those of inflammatory cells, whereas the nuclei in the media and the intima are exclusively those of smooth muscle cells.

In syngeneic grafts where granulomas were induced experimentally by starch contamination, the following features were observed. There was an accumulation of white blood cells in the adventitia, but they were grouped around starch particles in the typical “granuloma” fashion, with some epithelial and giant cells surrounding them. In corresponding areas in the intima, focal breaks in the internal elastic lamina and a prominent intimal thickening were observed: phenotypically myocyte-like cells appeared in the subendothelial space in the starch-treated syngeneic control animals even faster than in ordinary allografts. In very marked contrast, however, the nuclear density in the media remained at the level of the untreated syngeneic controls (Figs 1 and 2).

The antibodies to LCA and anti-smooth muscle α-actin were applied to the vascular granulomas, and the staining pattern was compared with that of the allografts. It appeared that the anti-LCA-positive white blood cells, found predominantly in the allograft adventitia, were also localized within the starch-induced granulomas of syngeneic grafts (Fig 3). Both medial and intimal cells of the syngeneic grafts with starch-induced granulomas were stained with anti-smooth muscle α-actin antibodies, whereas only intimal cells in the allograft (Fig 4) had such staining.

To investigate the mechanisms leading to intimal thickening under these two conditions, ie, in the allograft and the granuloma-induced syngeneic graft, we performed the following staining procedures from frozen-section specimens: anti-IL-2 receptor, indicating the activation level of the inflammatory lymphocytes; anti-IFN-γ, as an example of a major lymphokine derived from activated lymphocytes; and anti-class II expression, as an end point for the IFN-γ effect. As shown in the Table, there was a very high level of IL-2 receptor expression in the allograft that was lacking in
syngeneic controls, regardless of whether the allografts had been exposed to starch or not. IFN-γ was associated primarily with the allograft and was mostly lacking in both types of syngeneic grafts. Class II expression, as expected, was significantly more prominent in the allograft.

Finally, autoradiograms were made from paraffin sections to quantify the rate of cell proliferation in the three layers of the aorta. We have previously shown that the replicating cells in the allograft adventitia are primarily anti-LCA-positive white blood cells (mainly lymphoid cells), whereas in the media and intima they are mainly α-actin-positive smooth muscle cells. A high level of proliferation of white blood cells in the adventitia and of smooth muscle cells in the media and the intima was observed in the allografts but not in unexposed syngeneic grafts (Fig 5). In clear contrast, the starch-exposed syngeneic transplants displayed a prominent white blood cell proliferation in the adventitia 3 and 7 days postoperatively. Similarly, a smooth muscle cell proliferative burst was observed in the media, coinciding with the aforementioned, but declining to zero level at 1 month. No significant proliferation of smooth muscle cells was observed in the intima (Fig 5).

**Expression of Various Molecules in the Adventitial Inflammatory Infiltrate**

<table>
<thead>
<tr>
<th>Time after transplantation (mo)</th>
<th>Anti-IL-2 receptor</th>
<th>Anti-IFN-γ</th>
<th>Anti-class II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Syn</td>
<td>Allo</td>
<td>Syn + granuloma</td>
</tr>
<tr>
<td>1</td>
<td>0.4±0.1</td>
<td>2.9±0.6</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>2</td>
<td>0.4±0.1</td>
<td>2.8±1.0</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>0.4±0.1</td>
<td>2.2±0.2</td>
<td>0.3±0.0</td>
</tr>
<tr>
<td>5</td>
<td>0.3±0.1</td>
<td>0.8±0.2</td>
<td>ND</td>
</tr>
</tbody>
</table>

IL, interleukin; IFN-γ, interferon gamma; syn, syngeneic; allo, allograft; ND, not determined.

*Antibody-positive cells in the adventitia.

†In point score units, three to six determinations per time point (mean±SEM).

‡Significance at P<.05 by the Mann-Whitney U test between adjacent values.
Adventitia

Media

Intima

FIG 5. Semilog plots of frequency of proliferating nuclei as detected by $^3$H-thymidine uptake and autoradiography in the syngeneic grafts (open squares), allografts (closed squares), and syngeneic grafts with starch-induced granulomas (open triangles) at various times after transplantation in the adventitia, media, and intima. The results are expressed as total labeled cell number per cross section in each layer of the aorta.

Discussion

Taken together, our observations are the following: Aortic allografts, with a prominent adventitial inflammation and a high level of lymphoid activation, demonstrate intimal thickening and medial necrosis, whereas experimentally induced starch granulomas in syngeneic transplants, with an equally prominent adventitial inflammation but a low level of lymphoid activation, demonstrate similar intimal thickening but no medial necrosis.

Medial changes are also present in human allografts undergoing chronic rejection, although they are far less conspicuous: the common finding in human allografts is medial thinning and focal myocyte necrosis. These observations suggest that in human allografts also, there is a toxic effect on the media, although it is far less pronounced than in our rat aorta model.

Our observations indicate that medial necrosis and intimal thickening in aortic allografts, although they occur together, are independently regulated. Adventitial cell proliferation was a prerequisite for intimal thickening, since these were not observed in syngeneic controls not exposed to starch. On the other hand, medial necrosis was associated only with lymphoid cell activation in the adventitia, as a high level of adventitial activation (IL-2 receptor expression) was observed only in the allograft but not in syngeneic control animals exposed or not to starch. We believe that medial necrosis is due to either a direct toxic effect of the adventitial inflammatory cells on medial myocytes and/or an indirect effect by compromising the vasa vasorum. The cytokines delivered at the site of lymphoid activation and the activated lymphocytes themselves display toxic effects to neighboring cells when immunosuppression is inadequate, and the microvascular endothelial cells are among the most vulnerable targets.

The far-less-prominent medial damage in human transplants compared with those in the rat also deserves comment. Human transplants are immunosuppressed, whereas the rat transplants were not. The immunosuppression strictly curbs any detectable level of lymphoid activation in the human graft except during acute rejection, whereas a high level of lymphoid activation and IL-2 receptor expression in the rat allograft is the rule. Our interpretation that lymphoid activation is a prerequisite for medial damage is further substantiated with our previous finding that if the allograft recipient is immunosuppressed with cyclosporine, a compound that suppresses adventitial inflammation and entirely eliminates lymphoid activation, there is strong intimal proliferation but no medial necrosis.

Allograft arteriosclerosis is obviously under the control of the anti-allograft immune response, as no such changes are recorded in syngeneic transplants unless they have been exposed to a long ischemic time (B. Fellström, MD, personal communication, September 1992). We have also demonstrated that the intimal thickening in allograft arteriosclerosis is invariably associated with persistent perivascular inflammation, elevated levels of lymphoid activation, expression of the IL-2 receptor, and release of various cytokines and thromboxane from the adventitia, as well as the release of several growth factors including epidermal growth factor (EGF), insulin-like growth factor–1 (IGF-1), and platelet-derived growth factor (PDGF) from the vascular intima. The question arises whether all these molecular components are necessary to continue the process after induction.

These results demonstrate that adventitial lymphoid activation, although a central feature in acute rejection, is not a prerequisite for intimal proliferation. Starch granulomas containing lymphocytes and macrophages were sufficient to induce the intimal lesion at the granuloma site in the absence of any detectable IL-2 receptor expression. Thus, in the case of the starch granuloma, the intimal lesion is induced via an alternative pathway in the absence of lymphoid cell activation, and the presence of activated lymphoid cells displaying
the IL-2 receptor is not needed for progression of the lesion.

A surprising finding in this study was that prominent intimal thickening may be induced, as in the case of starch granulomas, by very little proliferative activity in the intima. On the other hand, a prominent though short-lasting burst of smooth muscle cell replication was seen in the media immediately before intimal thickening occurred. At this stage the intima still consisted of a single endothelial cell layer. In the carotid balloon-injury model Clowes et al\(^1\) showed proliferative peaks in blue-stained regions in the intima and media at 4 and 2 days after operation, respectively. In our case, the myocytes of the intima in syngeneic grafts exposed to starch must have been derived from the media by migration. Thus, the extent of smooth muscle cell replication is not directly proportional to the extent of intimal thickening.

Adventitial inflammation induced by placement of a Silastic collar around the carotid artery has previously been linked to neointimal thickening.\(^13\) Our results indicate that the type of inflammation may be linked with such thickening whether medial necrosis occurs concomitantly or not. Considering the molecular mechanisms in the allograft model, the following types of molecules may be involved: immune cytokines, eicosanoids, and growth factors secreted from the damaged endothelium. In the nonallogeneic model, the following growth factors have been proposed: basic fibroblast growth factor\(^14,15\) and PDGF A-chain.\(^16\) We have recently shown that in rat aortic allografts at least EGF, PDGF B-chain, and IGF-1 are prominently expressed both in mRNA and at the protein level, whereas the eicosanoids, the thromboxane B\(_2\) level is elevated but 6-ketoprostaglandin F\(_{1\alpha}\) and leukotriene B\(_4\) remain at control levels. As yet, all these studies are only descriptive, because blocking experiments have not been done to demonstrate the relevance of each molecule to the process.

The role of T-cell activation in intimal thickening is a controversial issue. Jonasson et al\(^17\) and Hansson et al\(^18\) have reduced the process in the carotid denudation model with cyclosporine or T-cell depletion, whereas Ferns et al\(^19\) have been unable to show any effect of cyclosporine. In the allograft, cyclosporine is clearly antiinflammatory and proatherosclerotic.\(^10\)

Acknowledgments

This study was supported by grants from the Finnish Academy, the Sigrid Juselius Foundation, the University of Helsinki, and the Finnish Medical Society Duodecim, Helsinki, Finland.

References

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*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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