Chronic Rejection of Rat Aortic Allografts

III. Synthesis of Major Eicosanoids by Vascular Wall Components and Effect of Inhibition of the Thromboxane Cascade

Ari Mennander, Sinikka Tiisala, Jarkko Ustinov, Anne Raisanen, Timo Paavonen, and Pekka Häyry

We have previously demonstrated that rat aortic allografts from the DA (RT1') to the WF (RT1") strain develop chronic arteriosclerotic changes in the vascular wall after a short spontaneously reversible acute rejection episode. These changes, which are lacking in syngeneic DA-to-DA control grafts, are virtually identical with those observed in human allografts during chronic rejection. In this study we have investigated whether eicosanoids are involved in the generation of arteriosclerotic changes. Incubation of aortic wall rings in vitro and immunochemical assays demonstrated that the arteriosclerotic allografts synthesize significantly more thromboxane B2 (TxB2) but not 6-ketoprostaglandin F1α (6-keto-PGF1α) or leukotriene B4. The increased synthesis of TxB2 in the allografts persisted for at least 5 months after transplantation. Separate incubation of the two major components of the vascular wall, after microdissection of the intima (media plus) adventitia, demonstrated that most of the synthesis of TxB2 during chronic rejection was due to the outer layer of aorta, presumably the inflammatory cells of the adventitia. In contrast, most of the 6-keto-PGF1α was synthesized by the inner layer of the aorta, presumably the endothelial cells and the smooth muscle cells of the intima. Administration of 1 mg·kg⁻¹·day⁻¹ of a specific TxA2 receptor inhibitor, GR32191B, to the recipient rat reduced the proliferative response of inflammatory cells in the adventitia by 30%, as detected by in vivo tritiated-thymidine (³H-TdR) labeling and autoradiography, but did not reduce the proliferative response of smooth muscle cells in the media and intima. The drug also delayed the intimal thickening and the generation of allograft arteriosclerosis by 1 month but was unable to inhibit it indefinitely. We conclude that TxA2 is involved in allograft arteriosclerosis, although the impact is relatively small. (Arteriosclerosis and Thrombosis 1992;12:1380-1386)

KEY WORDS • chronic rejection • arteriosclerosis • eicosanoids • GR32191B

A basic feature in all allografts undergoing chronic rejection is persistent perivascular inflammation and allograft arteriosclerosis. In contrast to classical atherosclerosis, which is usually focal and asymmetric, allograft arteriosclerosis is concentric and generalized. Allograft arteriosclerosis particularly affects the first- and second-order intragraft arteries to the level of the arterioles. The regulation of arterial smooth muscle cell proliferation in chronically rejecting allografts is probably complex. Cytokines produced by inflammatory cells as well as growth factors produced by both these and graft parenchymal cells may be involved. Several lines of evidence indicate that arachidonic acid metabolites also participate in the regulation of smooth muscle cell proliferation in rejecting allografts.

In clinical studies patients who experience chronic allograft rejection may have increased levels of thromboxanes in their blood and urine while the levels of prostaglandins and prostacyclin are the same or lower than in control subjects. Prostaglandins are known to inhibit the proliferation of smooth muscle cells in vitro, whereas the leukotrienes LTβ, LTα, and LTD4 initiate DNA synthesis of growth-arrested arterial smooth muscle cells.

We have recently developed an animal model for allograft arteriosclerosis. In short, aortic allografts are transplanted without immunosuppression between histoincompatible rat strains. After a short, spontaneously reversible acute episode of rejection, these allografts develop vascular wall changes that are virtually identical to those observed in human tissue transplants undergoing chronic rejection.

In this communication we demonstrate that aortic allografts undergoing chronic rejection exhibit an increased synthesis of thromboxane B2 (TxB2) but only a small increase in the synthesis of 6-ketoprostaglandin F1α (6-keto-PGF1α) and no increase in LTβ. Blocking of the thromboxane pathway by a specific inhibitor of the TxA2 receptor, although inhibitory to inflammatory cell proliferation, only temporarily attenuates the development of arteriosclerotic alterations in these transplants.
Methods

Experimental Animals
Rat strains WF (AG-B2, RT1) and DA (AG-B4, RT1) were used for transplantation. All animals were purchased from the Zentralinstitut fur Versuchstierzucht GmbH, Hannover, FRG. Male rats weighing 200–300 g and 1–3 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) was excised, perfused with saline, and used as a transplant. The experimental animals were anesthetized with intraperitoneal chloral hydrate (6 ml/kg body wt). The graft was perfused and transplanted into a heterotopic position below the renal arteries and above the bifurcation, thereby forming a “loop” in the abdominal cavity. This modification of the original technique allowed us to obtain more material for both histological and biochemical determinations. The histological results did not differ from those originally reported. The cranial suture line was completed as close to the renal arteries as technically possible to minimize the difference in diameter. An end-to-end anastomosis was performed by using a 9-0 continuous nylon suture. The DA-to-WF-strain combination was made from the DA to the DA strain. The grafts were removed at 10 and 20 days and at 1, 3, and 6 months after transplantation and processed for histology, autoradiography, frozen-section immunohistochemistry, and biochemical determinations. 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, Miles, Elkhart, Ind., and snap-frozen.

Medication
GR32191B, which is a potent and specific TxA2 receptor antagonist,8 was a gift from the Glaxo Group Research Ltd., Greenford, UK. The drug was dissolved in water and administrated orally (forced administration) at the rate of 1.0 mg · kg⁻¹ · day⁻¹ until the end of the experiment. This dose is approximately 10 times higher than the dose that inhibits accumulation of platelets onto denuded guinea pig carotid arteries.

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 (one 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, 3–4-µm-thick frozen sections were stained by the immunoperoxidase technique using monoclonal anti-rat antibodies to leukocyte common antigen (LCA) and a-smooth muscle actin. Anti-LCA (OX-1) antibody was obtained from Sera Lab, Sussex, UK, and anti-smooth muscle actin antibody was from Bio-Makor, Rehovot, Israel. The cryosections were stained by the two-layer indirect immunoperoxidase technique described in detail elsewhere.9 Briefly, the sections were incubated with appropriately diluted mononclonal antibody (usually 1:20), washed, and consecutively incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulin and goat anti-rabbit immunoglobulin (Dakko 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 in Rat Aortic Transplants
The morphological changes were quantified according to standard morphometric principles10 and expressed as point score units (PSUs), i.e., the mean number of points falling over a given anatomic area defined by straight cross-sectional lines and a 0.02-mm grid. The following variables were evaluated: the number of nuclei and the thicknesses of different layers of the aorta, i.e., adventitia, media, and intima, separated pairwise from each other by external and internal elastic laminae, respectively. 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.

Autoradiography
Some of the rats received 250 µCi of tritium-labeled thymidine (3H-TdR, NEN, Boston, Mass.) by intravenous injection 3 hours before they were killed. Histological specimens were processed from paraffin sections, emulsion autoradiography (Ilford L.4, 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.

Eicosanoid Measurements From Rat Aortas
Aortic allografts and thoracic and abdominal aortas of the recipient or of a normal rat were removed and sliced. Aortas were first incubated for 30 minutes in an ice bath with 0.1 M sodium phosphate buffer, pH 7.4, in 0.9% NaCl supplemented with 1% bovine serum albumin, 200 nM calcium ionophore (Sisnten), and 0.1% NaN3 and thereafter for another 30 minutes at 37°C in the same buffer (1–3 ml). Exclusion of NaN3 and/or calcium ionophore from the incubation medium (which was present in the preliminary experiments in Table 1)
TABLE 1. Synthesis of Major Eicosanoids 1 Month After Transplantation by the Aortic Allograft, the Syngeneic Graft, and the Recipient Thoracic Aorta

<table>
<thead>
<tr>
<th></th>
<th>6-Keto-PGF(_{1\alpha}) (ng/mg dry wt)</th>
<th>(\text{TxB}_2) (pg/mg dry wt)</th>
<th>LTB(_4) (pg/mg dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allograft</td>
<td>47.9±26.9</td>
<td>3,597±469*</td>
<td>2</td>
</tr>
<tr>
<td>(n=15)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syngeneic graft</td>
<td>18.9±3.2</td>
<td>846±127*</td>
<td>&lt;2</td>
</tr>
<tr>
<td>(n=16)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal aorta</td>
<td>14.5±0.5</td>
<td>309±33</td>
<td>&lt;2</td>
</tr>
<tr>
<td>(n=22)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

6-Keto-PGF\(_{1\alpha}\), 6-ketoprostaglandin \(\text{F}_{1\alpha}\); \(\text{TxB}_2\), thromboxane \(\text{B}_2\); LTB\(_4\), leukotriene \(\text{B}_4\). Values are mean±SEM. Significances by the Mann-Whitney \(U\) test at *\(p<0.001\); all other probability values were greater than 0.05, i.e. nonsignificant.

did not affect the eicosanoid determinations. The incubation was stopped by reimmersing the aortas in the ice bath. Liquids were collected from the incubation tubes for radioimmunoassay, and the aortas were dried overnight at 37°C and weighed. Commerically available radioimmunoassay kits (Amersham, Amersham, UK) for the stable end products, 6-keto-PGF\(_{1\alpha}\) for prostacyclin, \(\text{TxB}_2\) for \(\text{TxA}_2\), and LTB\(_4\), were employed. Normal nontransplanted aortas were used as controls. The results were expressed as nanograms or picograms per milligram of aorta (dry weight).

Results

Synthesis of Major Eicosanoids by the Arterial Wall

DA rat thoracic aortas were transplanted to WF or DA recipients and removed 1 month after transplantation. The middle part (one third) of the transplant was cut into slices and incubated in serum-free medium in vitro, and the incubation medium was investigated for the presence of 6-keto-PGF\(_{1\alpha}\), \(\text{TxB}_2\), and LTB\(_4\) by using specific radioimmunoassays. As shown in Table 1, the synthesis of \(\text{TxB}_2\) increased significantly from about 300 pg/mg to about 3,500 pg/mg dry wt in the allografts; there was also a modest increase in the synthesis of \(\text{TxB}_2\) to approximately 850 pg/mg in the syngeneic grafts. In contrast, there was no significant increase in the synthesis of 6-keto-PGF\(_{1\alpha}\) in the allografts or in syngeneic grafts when compared with nontransplanted aortas (not shown) or to the recipients' own aortas; neither was there any measurable increase in the synthesis of LTB\(_4\).

Time Profile of Thromboxane Response

Another set of experiments was performed to investigate the time dependence of the thromboxane response by comparing allografts to syngeneic grafts up to 5–9 months after transplantation. As shown in Figure 1, at 1 week after transplantation, increased amounts of \(\text{TxB}_2\) were measured from both the allografts and the syngeneic grafts. Thereafter, the synthesis of \(\text{TxB}_2\) rapidly declined to a level of approximately 500–1,000 pg/mg dry wt in syngeneic grafts but remained at a high level of approximately 5,000–6,000 pg/mg dry wt in allografts up to the end of the observation period of 5 months.

Synthesis of 6-Keto-PGF\(_{1\alpha}\) and \(\text{TxB}_2\) by Different Layers of the Vascular Wall

For some reason that we do not know, the cohesion between the newly formed intima and media was relatively weak in advanced arteriosclerosis. This made it possible for us to dissect these two components by standard microdissection techniques (Figure 2), which thereby permitted the analysis of eicosanoids separately from the intima and (media plus) adventitia.

In the third experiment, therefore, we investigated the origin of 6-keto-PGF\(_{1\alpha}\) and \(\text{TxB}_2\) in regard to the major components of the aortic wall, i.e., the intima and the (media plus) adventitia. The wall components of the aortic allografts were microdissected along the internal elastic lamina (Figure 2), and the resulting two components were investigated separately.

As shown in Table 2, both vascular wall components synthesized prostanoids. However, more 6-keto-PGF\(_{1\alpha}\) was synthesized in the intima, whereas more of the \(\text{TxB}_2\) synthesis occurred in the outer layers of the aortic wall, i.e., in the component consisting of media and inflammatory adventitia.

Proliferation of Inflammatory and Smooth Muscle Cells in the Vascular Wall and Effect of \(\text{TxA}_2\) Antagonist GR32191B on the Proliferative Response

Aortic allografts were performed from the DA to the WF rat strain or, for comparison, from the DA to the DA strain. One group of allograft recipients received 1 mg·kg\(^{-1}\)·day\(^{-1}\) GR32191B. This dose is 10 times more than the minimum dose that produces maximal inhibition of platelet deposition onto denuded guinea pig carotid arteries.

![Figure 1. Semilog plot showing recovery of thromboxane B\(_2\) (\(\text{TxB}_2\)) from allografts (○) and syngeneic grafts (○) as a function of time after transplantation. Three to seven determinations in each time point with SEM indicated by vertical bars. Shaded area indicates \(\text{TxB}_2\) recovery from normal nontransplanted control aortas (mean±SD).](http://atvb.ahajournals.org/DownloadedFrom/cthwala.journals.org/global/figures/figure1.jpg)
FIGURE 2. Photomicrograph showing microdissection of the allograft intima. Note the inflammation in the adventitia, the loss of nuclei in the media, and the thickening of the intima. Here, the intima is separated along the internal elastic lamina. e, endothelium; i, intima; m, media; a, adventitia. Hematoxylin-eosin staining, ×225.

The allograft recipients received an intravenous bolus of 250 μCi 3H-TdR 3 hours before they were killed, and autoradiograms were made from the paraffin sections.

In untreated allograft recipients there was a rapid increase in the number of 3H-TdR-incorporating cells in the adventitia (Figure 3). Combined autoradiography and staining for anti-α-smooth muscle actin and/or anti-LCA demonstrated that most of the labeled cells in the adventitia were LCA-positive inflammatory white cells, but occasional labeled cells expressing α-smooth muscle actin were also found (not shown). The remaining labeled cells were double-antibody-negative. A high level of white cell proliferation in the adventitia lasted for 4 months after transplantation, after which time it declined. Only occasional 3H-TdR-incorporating cells were seen in the adventitia of syngeneic control rats. Administration of 1 mg·kg⁻¹·day⁻¹ GR32191B significantly reduced the proliferative response of inflammatory white cells in the allograft adventitia from approximately 200 cells to 50–130 cells per cross section but did not block it entirely.

There was also a rapid increase in the number of 3H-TdR-incorporating cells in the media of the allografts, lasting 1–3 months after transplantation, after which time the proliferative response in the media declined (Figure 3). Counterstaining with monoclonal antibodies demonstrated that the proliferating cells in the media were all α-smooth muscle actin-positive smooth muscle cells (not shown). Very few 3H-TdR-incorporating cells were seen in the media of syngeneic

TABLE 2. Synthesis of 6-Keto-PGF₁α and TxB₂ by Major Layers of the Vascular Wall 2 Months After Transplantation

<table>
<thead>
<tr>
<th>Layer</th>
<th>6-Keto-PGF₁α (ng/mg dry wt)</th>
<th>TxB₂ (pg/mg dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intima (n=4)</td>
<td>22.9±5.6*</td>
<td>2,979±802</td>
</tr>
<tr>
<td>Media+adventitia (n=4)</td>
<td>12.4±2.3</td>
<td>8,369±1,064*</td>
</tr>
<tr>
<td>Ratio</td>
<td>1.8</td>
<td>0.3</td>
</tr>
</tbody>
</table>

6-Keto-PGF₁α, 6-ketoprostaglandin F₁α; TxB₂, thromboxane B₂. Values are mean±SEM.
Significances by the Mann-Whitney U test at *p<0.001.
controls. Administration of GR32191B did not reduce the proliferative response of smooth muscle cells in the media.

The first 3H-TdR-incorporating cells were seen in the allograft intima at 0.5–1 month after transplantation (Figure 3), and contrary to the results cited above, the cell proliferation in the intima did not decline but continued at a reasonable level until the end of the observation period of 5 months. Counterstaining with monoclonal antibodies demonstrated that most of the 3H-TdR-incorporating cells in the intima were again smooth muscle cells (not shown). In addition there were some double-antibody-negative cells, but no LCA-positive white cells incorporating 3H-TdR were observed in the intima. Practically no smooth muscle cell proliferation was seen in the intima of syngeneic controls. Administration of GR32191B did not reduce the number of 3H-TdR-incorporating smooth muscle cells in the allograft intima.

Quantification of Vascular Wall Changes in Allografts, Syngeneic Grafts, and Allografts Treated With GR32191B

Aortic allografts were exchanged between the same strain combinations as above, and one group of the allograft recipients received 1 mg · kg⁻¹ · day⁻¹ GR32191B. The drug-treated animals were killed 1–4 months postoperatively, and the control rats were killed at 5, 6, and 12 months. Morphometric quantification was done for the inflammatory cell nuclei in the adventitia, for the smooth muscle cell nuclei in the media, and for the nuclear content and thickness of the intima by using paraffin sections from the middle third of the transplant.

As shown in Figure 4, there was a rapid increase in the nuclear density in the adventitia of DA-to-WF allografts. Staining with anti-LCA anti-α-smooth muscle cell actin antibodies previously demonstrated that most of these were nuclei of LCA-positive inflammatory cells.⁷ The inflammation peaked at the second month after transplantation and gradually declined thereafter (Figure 4). In syngeneic control rats, only a modest adventitial inflammatory response was observed. Administration of GR32191B to the allograft recipients did not reduce the intensity of the inflammation in the allograft adventitia (Figure 4).

The nuclear density of the media rapidly declined in the allografts (Figure 4), indicating medial necrosis. In syngeneic grafts, the smooth muscle cell nuclei were preserved in the media. Administration of GR32191B to the allograft recipients did not delay the loss of smooth muscle cells from the media, i.e., medial necrosis, and was definitely unable to inhibit it (Figure 4).

The nuclear density of the allograft intima rapidly increased. As previously shown,⁷ most of the cells invading the subendothelial space are α-actin–containing smooth muscle cells. No such influx of smooth muscle cells into the intima was observed in syngeneic control rats (Figure 4). At 1 month after transplanta-
tion, significantly \((p<0.01)\) fewer smooth muscle cell nuclei were recorded in the intima of GR32191B-treated allografts (1.2±0.2 PSUs) compared with untreated allografts (2.3±0.6 PSUs); however, the difference disappeared at 2–3 months after transplantation (Figure 4).

This was also the case when the intimal response was quantified as intimal thickness; there was a rapid increase in the cross-sectional thickness of the intima in allografts, which was not observed in syngeneic control rats (Figure 4). Administration of GR32191B delayed the intimal response by approximately 1 month but was unable to inhibit it indefinitely.

**Discussion**

Disregarding the organ-specific manifestations in the kidney, liver, and heart transplants, the common denominator of chronic rejection in all of these allografts is persistent periarterial inflammation and allograft arteriosclerosis.

We have previously demonstrated that with longer observation times, histo-compatible rat aortic allografts develop vascular wall changes that are comparable with those seen in chronic rejection. Persistent perivascular inflammation, gradual loss of smooth muscle cells in the media, fragmentation of the internal elastic lamina, and appearance of proliferating smooth muscle cells in the intima, all of which were observed in these experimental allografts, are virtually identical with the vascular changes observed in human heart and kidney allografts during chronic rejection.

To understand the molecular mechanism in allograft arteriosclerosis, it is, therefore, crucial to understand how the perivascular inflammation regulates the smooth muscle cell proliferation in the allograft vascular wall. Here the picture becomes more complex. The cells in both the inflammatory infiltrate (including platelets) and the vascular wall itself are able to produce multifunctional cytokines and growth factors that can potentially induce these morphological alterations.

In addition to lymphokines and growth factors, another group of molecules that can potentially modify the arteriosclerotic process is the lipid mediators of inflammation. Prostaglandins have cytoprotective effects and inhibit the proliferation of smooth muscle cells in vitro, whereas leukotrienes may initiate DNA synthesis of subcultured rat arterial smooth muscle cells. Treatment of these cells with cyclooxygenase inhibitors, acetosalicylic acid, or indomethacin blocks the induction of DNA synthesis, suggesting that the effect is mediated by a cyclooxygenase product. Thus, one potential candidate for this effector molecule would be TXA2. Thromboxane has not yet been considered a vascular smooth muscle cell growth-promoting substance per se. On the other hand, thromboxanes cause local platelet activation and vasoconstriction, and they are also considered to be "pro-inflammatory" cytokines.

This study demonstrates a series of findings that are compatible with this suggestion. When the release of the major eicosanoid end products, i.e., 6-keto-PGF\(_{1\alpha}\), TxB\(_2\), and LTB\(_4\), was quantified after short-term incubation of aortic slices in vitro, there was a significant increase in the production of TxB\(_2\) in the allografts but only a small increase in the production of 6-keto-PGF\(_{1\alpha}\) and no increase in LTB\(_4\). The increase in TxB\(_2\) produc-
tion in organ allografts. At present we consider that the lipid inflammatory mediators of inflammation play an intermediary role, which may be bypassed by some other currently unidentified mechanisms.

References
Chronic rejection of rat aortic allografts. III. Synthesis of major eicosanoids by vascular wall components and effect of inhibition of the thromboxane cascade.
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