Direct and Indirect Effects of Alloantibodies Link Neointimal and Medial Remodeling in Graft Arteriosclerosis

Olivier Thaunat, Liliane Louedec, Jianping Dai, Florence Bellier, Emilie Groyer, Sandrine Delignat, Anh-Thu Gaston, Giuseppina Caligiuri, Etienne Joly, Didier Plissonnier, Jean-Baptiste Michel, Antonino Nicoletti

Objective—Chronic vascular rejection, the main cause of allograft failure, is characterized by the destruction of smooth muscle cells (SMCs) in the media concomitantly with the proliferation of SMCs in the adjacent neointima. We hypothesized that alloantibodies might be responsible for these 2 opposite but coordinated events.

Methods and Results—We used the rat aortic interposition model of chronic vascular rejection. During the rejection process, a neointima composed of proliferating SMCs from the recipient developed, whereas the SMCs in the media, all of donor origin, underwent apoptosis. Alloantibody deposition was detected only in the media. Using in vitro cultures experiments, we observed that alloantibody binding to donor SMCs exerts (1) a rapid upregulation of the transcription of growth factors genes, followed by (2) the induction of apoptosis after 24 hours. The transient production of growth factors by donor SMCs in response to the binding of alloantibodies induced the proliferation of recipient SMCs in culture supernatant transfer experiments. Additional data suggest that among the repertoire of alloantibodies, those directed against major histocompatibility complex I might carry the remodeling effect.

Conclusions—Our data suggest that during chronic vascular rejection, alloantibody binding to donor medial SMCs is a crucial event that links neointimal and medial remodeling. (Arterioscler Thromb Vasc Biol. 2006;26:2359-2365.)

Key Words: anti-MHC antibodies ■ chronic rejection ■ graft arteriosclerosis ■ remodeling ■ smooth muscle cells

During the last 20 years, the half-life of transplants has remained the same as the result of chronic rejection, which represents the main cause of long-term graft failure.1 Excluding organ-specific manifestations, the most common histopathological feature is chronic vascular rejection, also known as graft arteriosclerosis, which is estimated to affect more than 40% of recipients within the first 5 years following transplantation.2

The animal model of aortic transplantation between histoincompatible rat strains reproduces the main characteristics of allograft arteriosclerosis found in the arteries of rejected human grafts.3 In previous studies, we have shown that the rejection process of the aortic graft follows 2 consecutive phases.3 During the first phase (day 0 to 5 posttransplantation), the circulating leukocytes of the recipient target the endothelial cells (ECs) of the graft that expose donor major histocompatibility complex (MHC) molecules. The cellular cytotoxic effectors rapidly destroy the endothelium of the graft but fail to reach the allogenic smooth muscle cells (SMCs) in the media because they are protected by the elastic laminas. The immune system of the recipient then switches from a cellular to a humoral response.3 During this phase, destruction of SMCs in the media results in the shrinkage of this tunica, whereas the same cell type, ie, SMC, proliferates in the adjacent neointima leading to a widespread and diffuse narrowing of the vascular lumen. Because the events taking place in these 2 tunica are tightly coordinated,3 we hypothesized that a single effector, alloantibodies, may paradoxically trigger both the destruction and the proliferation of the SMCs depending on their localization in the vessel.

A seminal work by Russell et al4 has demonstrated that the passive transfer of sera containing alloantibodies was sufficient to promote graft arteriosclerosis. Because MHC molecules are highly polymorphic, they are the main targets of the humoral alloimmune response. Several clinical studies have confirmed that the development of alloantibodies directed against donor MHC molecules is associated with an increased risk of transplant arteriosclerosis after transplantation.5

Interestingly, in addition to their classical role in antigen presentation, MHC molecules also act as signal-transducing molecules, and it has been demonstrated that the binding of antibodies to MHC molecules could modulate the biology of cells.6

Original received March 9, 2006; final version accepted August 6, 2006.

From the Université Pierre et Marie Curie-Paris6 (O.T., F.B., E.G., S.D., A.-T.G., G.C., A.N.), INSERM UMRS 681, Centre de recherche des Cordeliers, Paris; INSERM U698 and Université Denis Diderot (L.L., D.J., J.-B.M.), Hospital Xavier Bichat, Paris; INSERM U563 (E.J.), IFR Claude de Preval, Toulouse; and Department of Vascular Surgery (D.P.), Hôpital Universitaire de Rouen, France.

Correspondence to Dr Olivier Thaunat, INSERM UMRS 681, Institut Biomedical des Cordeliers, 15 rue de l’école de médecine, 75006 Paris, France. E-mail olivier.thaunat@free.fr

© 2006 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org

DOI: 10.1161/01.ATV.0000241980.09816.ac

2359
In the present work, we have tested whether alloantibodies binding to MHC molecules on graft medial smooth muscle cells could promote the events leading to the development of graft arteriosclerosis.

Materials and Methods

Murine Experimental Model

Age-matched male Brown–Norway (BN) (RT1<sup>+</sup>) and Lewis (LEW) (RT1<sup>-</sup>) rats were obtained from Charles River (l’Arbresie, France). LEW rats were used as recipients and syngeneic donors, BN rats as allogeneic donors. The aorta transplantation was performed as previously described.<sup>7</sup> (Please see the online data supplement.) Serum was collected and aortic grafts were removed after saline perfusion from the Lewis recipients under anesthesia. Fresh aortic samples were dissected and embedded in paraffin, in Epon resin or by perfusion from the Lewis recipients under anesthesia. Fresh aortic phometry was used to quantify the intensity of IgG and IgM fluorescence (Leica Microsystemes SAS; France). Computer-assisted mor- counterstained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescence tests and with an avidin-fluorescein isothiocyanate or avidin-ALEXA 555 or by detection by a biotinylated anti-mouse antibody that was revealed with avidin-fluorescein isothiocyanate or avidin-ALEXA 555 or by

Titration of Alloantibody in the Serum

Serum alloantibodies were titrated by flow cytometry using Lewis (recipient) fibroblasts expressing BN (donor) MHC molecules as previously described.<sup>7</sup> (Please see the online data supplement.)

Immunohistochemistry Analysis

Immunohistochemistry for rat IgG (biotinylated rabbit anti rat IgG; DAKO, Trappes, France), IgM (purified mouse anti rat IgM; MARM4), rat pan MHC I (purified mouse anti-RT1.A; OX-18), BN-specific MHC I (purified mouse anti-RT1.A'; OX-27), and rat pan MHC II (purified mouse anti-RT1.B; OX6) was performed on 5-μm thick cryostat cross-sections of aortic allograft. The biotinylated primary antibody was revealed with avidin-ALEXA 555. The other purified mouse anti-rat antibodies were detected by a biotinylated anti-mouse antibody that was revealed with avidin-fluorescein isothiocyanate or avidin-ALEXA 555 or by an anti-mouse IgG1 conjugated with ALEXA 350. The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescence was examined with a microscope equipped with epifluorescence (Leica Microsystems SAS; France). Computer-assisted morphometry was used to quantify the intensity of IgG and IgM deposition in the medial (Figure I in the online data supplement).

In Situ Apoptosis Detection

The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique was used to detect apoptosis on 5-μm thick paraffin-embedded cross-sections as previously described. Cell survival was evaluated using the tetrazolium salt reduction (MTT) assay<sup>11</sup> according to the instructions of the manufacturer (Roche Diagnostics, Indianapolis, Ind). (Please see the online data supplement.)

Transmission Electron Microscopy

Electron microscopy analysis was performed on aortic grafts 5 days (5 grafts) and 2 month (5 grafts) after transplantation, as previously described.<sup>7</sup> (Please see the online data supplement.)

In Vitro Experiments

Rat Vascular SMC Isolation and Culture

The technique of tissue processing and culture has been previously described.<sup>8</sup> (Please see the online data supplement.) Cells were used at passage 3 to 6. After passage 3, SMCs acquire a myofibroblastic phenotype<sup>6</sup> and therefore represent an appropriate in vitro model of neointima.

Generation and Purification of Polyclonal Alloantibodies

Polyclonal anti-BN alloantibodies were generated by the skin graft method.<sup>10</sup> Briefly, 1-cm<sup>2</sup> full-skin patch was removed from a BN rat and grafted orthotopically onto a LEW recipient. Four successive skin grafts were performed every 14 days to each of the 10 sensitized LEW rats.

Presence of anti-BN alloantibodies in sensitized LEW rats was assessed using flow cytometry as previously described.<sup>7</sup> (Please see the online data supplement.) Serum IgG were purified by chromatography on a protein G-Sepharose column, followed by immediate size-exclusion chromatography on a superose-12 column. (Please see the online data supplement.)

SMC Survival Assay

LEW and BN SMCs were plated (10<sup>4</sup> cells/well) in flat bottom 96-well plates and cultured until confluence. After a starvation period of 24 hours, 0.1 mg of LEW anti-BN alloantibodies (from sensitized LEW rats) or control IgG (from naive LEW rats) was added to the culture. Cell survival was evaluated using the tetrazolium salt reduction (MTT) assay<sup>11</sup> according to the instructions of the manufacturer (Roche Diagnostics, Indianapolis, Ind). (Please see the online data supplement.)

Quantification of In Vitro Apoptosis

Apoptotic cell death of BN or LEW (control) SMCs was measured after 8 and 24 hours of culture with LEW anti-BN alloantibodies. The Cell Death Detection sandwich ELISA<sup>10,13,15</sup> kit (Roche Diagnostics, Mannheim, Germany), which determines cytoplasmic histone-associated DNA fragments was used according to the instructions of the manufacturer.

Production of Growth Factors by SMCs Incubated With Alloantibodies

Transfer of Culture Supernatant

Supernatants from LEW (control) or BN SMCs stimulated with LEW anti-BN alloantibodies were harvested after 8 or 24 hours of culture. The supernatants were then added to 5.10<sup>4</sup> serum-starved LEW SMCs for which the proliferation was assessed by the MTT assay as described above after 48 hours of culture.

Semiquantitative Polymerase Chain Reaction

Transcription levels of 4 growth factors (platelet-derived growth factor [PDGF]-A and -B, fibroblast growth factor [FGF]-2, insulin-like growth factor [IGF]-1, transforming growth factor [TGF]-β) and a housekeeping gene (GAPDH) were analyzed by semiquantitative RT-PCR in BN SMC pellet (10<sup>5</sup> cells/ well; flat bottom 24-well plates) after 2 or 4 hours of culture with alloantibodies. RT-PCR was performed as described in the online data supplement.

Statistical Analysis

Data were analyzed using the Statview 5.0 software (Abacus Concept Inc). Statistical significance of results was determined by 1-way ANOVA, followed by Fischer’s partial least-squares difference tests and with a Mann–Whitney nonparametric test. Probability values of less than 0.05 were considered statistically significant.

Results

Arterial Wall Is Profoundly Reshaped During Chronic Rejection

Morphological changes of EC and medial SMCs of aortic allograft were tracked during the chronic rejection process. Five days posttransplantation, transmission electron microscopy analysis showed donor leukocytes bound to the surface of graft ECs (Figure 1A). This feature was associated with the necrosis of ECs and their shedding in the lumen (Figure 1B), indicating that graft ECs are targeted early by the rejection process. In contrast, no leukocyte was detectable beyond the internal elastic lamina, suggesting that medial SMCs that displayed a normal phenotype were spared by the early
cell-mediated injury stage of rejection. Two months posttransplantation, a neointima consisting of SMCs intermingled with mononuclear cells had developed. Medial SMCs had lost their normal contractile phenotype and were either apoptotic (Figure 1C) or displayed a synthetic phenotype (Figure 1D). At this time point, we were still unable to detect any leukocyte infiltration in the media.

TUNEL staining of aortic allografts collected 2 months posttransplantation confirmed that medial SMCs underwent massive apoptosis, whereas SMCs in the adjacent neointima were spared by this process (Figure 1G and 1H). Proliferation pattern of SMCs analyzed at the same time point by proliferating-cell nuclear antigen staining revealed an inverse situation, ie, neointimal SMCs but not medial SMCs, were stained (Figure 1E and 1F).

Kinetics of Alloantibody Production and Deposition in the Medial Layer of Allografts

Alloantibody levels in the serum of grafted animals peaked 45 days after transplantation and dropped at 90 days (Figure 2A). IgM deposits in the media of the graft were detected as early as 15 days. Fifteen days later (at day 30), IgG were also present and peaked at 45 days (Figure 2A and supplemental Figure I). Alloantibody binding was followed by a decreased in medial thickness (Figure 2A). These kinetics suggested that alloantibodies may be involved in the destruction of the medial SMCs.

The pattern of immunoglobulin deposition within chronically rejected aortic grafts was reproducible. Immunoglobulin deposits were bound to the remaining medial SMCs of the donor, whereas adjacent neointima was devoid of detectable immunoglobulin deposition (Figure 2B).

Alloantibody binding to medial SMCs is likely unable to trigger the activation of the complement cascade because immunofluorescence staining for the C5b9 lytic complex was negative (Figure 2C).

Chronically Rejected Aortic Allografts Are Chimeric for SMCs

The origin of SMCs in aortic grafts 2 months posttransplantation was analyzed by immunohistochemistry. We observed that SMCs from both the media and neointima expressed MHC I molecules, as assessed by the staining with the pan-MHC I OX-18 antibody. However, only medial SMCs were stained with the OX-27 antibody (Figure 2D), which is specific for BN MHC I molecules. This demonstrates that chronically rejected aortic grafts are chimeras in which the medial SMCs are from the donor, whereas neointimal SMCs are from recipient origin.

Alloantibody Binding Exerts a Sequential Biphasic Effect on the Survival of Cultured SMCs

The presence of alloantibodies in purified IgG was tested by measuring by flow cytometry their binding to LEW fibroblasts transfected or not with BN MHC molecules. IgG from nonimmune controls did not bind to the 2 types of LEW fibroblasts (Figure 3A). In contrast, purified IgG from all sensitized LEW rats contained alloantibodies that bound to LEW fibroblasts transfected with BN MHC molecules (Figure 3A). Importantly, no binding was detected when the same IgG were tested on nontransfected LEW fibroblasts.

Adjunction of LEW anti-BN alloantibodies induced a sequential biphasic effect on the survival of cultured BN SMCs. We observed an initial short phase, during which BN
SMC survival seems unaffected by alloantibodies, followed by a rapid drop in their survival (Figure 3B). As anticipated, LEW anti-BN alloantibodies had no effect on the survival of LEW SMCs (Figure 3B).

BN SMCs cultured during 24 hours with alloantibodies displayed an apoptotic phenotype (Figure 3B). Accordingly, after 8 hours, i.e., during the initial phase, BN SMCs cultured with LEW anti-BN alloantibodies displayed a level of cytoplasmic histone-associated DNA fragments reduced by 30% as compared with BN SMCs alone (Figure 3C). In contrast, when the same measure was performed after 24 hours of culture, i.e., when BN SMCs started to undergo apoptosis, this level was increased by 50% (Figure 3C). No difference was observed at the 2 time points, when the level of cytoplasmic histone-associated DNA fragments of LEW SMCs cultured alone was compared with that of LEW SMCs cultured with LEW anti-BN alloantibodies (data not shown).

### Conditioned Medium From Donor SMCs Cultured With Alloantibodies Induces Proliferation of Recipient SMCs

Supernatants from BN SMCs cultured with IgG from naive LEW rats had no effect on the cell growth when transferred onto LEW SMCs. In contrast, when supernatants from BN SMCs cultured 8 hours with alloantibodies were transferred on LEW SMCs, a significant proliferation was detected (Figure 4A). Interestingly, this effect was not detected any longer when the same experiment was performed with the supernatants from BN SMCs cultured 24 hours with alloantibodies (Figure 4A).

Given that we had shown that LEW anti-BN alloantibodies did not bind to LEW SMCs and that they had no effect when added directly to LEW SMCs, we assumed that the proliferation of LEW SMCs cultured with conditioned medium from BN SMCs treated for 8 hours with alloantibodies was triggered by promitotic factors produced transiently by BN SMCs in response to alloantibodies.

### Transcription of Growth Factors Is Upregulated in SMCs Exposed to Alloantibodies

The transcription levels of PDGF, FGF-2, IGF-1, and TGF-β, all involved in chronic vascular rejection, were assessed by semiquantitative RT-PCR. As controls, BN SMCs were also exposed to IgG from naive LEW rats. The transcription levels were measured after 2 and 4 hours of incubation.

We were unable to find detectable levels of TGF-β mRNA in SMCs exposed or not to alloantibodies at the 2 time points. In contrast, BN SMCs cultured with alloantibodies significantly increased their level of expression of PDGF, FGF-2, and IGF-1 mRNA as compared with the same cells cultured with control IgG (Figure 4B). Interestingly, the kinetics of expression of these 3 growth factors were different. PDGF mRNA level was transiently augmented after 2 hours and preceded the increase in FGF-2 and IGF-1 mRNA levels observed at 4 hours. This may indicate, as recently reported, that PDGF induces the production of other growth factors that act synergistically to promote the survival and the proliferation of SMCs in autocrine/paracrine mode.
Anti–MHC I Alloantibodies Carry the Remodeling Effect

Anti–MHC alloantibodies are predominant during the humoral alloimmune response. We hypothesized that alloantibodies responsible for the remodeling effect during chronic rejection were directed against donor MHC. We found that medial SMCs from the rejected aortic graft did not express MHC II molecules (supplemental Figure II). Strikingly, LEW anti-BN alloantibodies did induce apoptosis of LEW fibroblasts only if transfected with the BN RT1.A1n MHC I molecules (F-LEW/F+H11001A1n cells; Figure 3D), indicating that anti–MHC I alloantibodies can induce cell death of donor SMCs.

Discussion

The hallmark of graft arteriosclerosis is the development of a thick neointima composed mainly of myofibroblasts. The origin of these cells has been extensively debated. Earlier studies proposed that these cells migrated from the media of the graft14 suchlike in the balloon-injured arteries. Recent data rather favor a recipient origin, either from bone marrow–derived precursors15 or from SMCs migrating from the edge.

Figure 3. Effect of alloantibody binding on the survival of cultured SMCs. Flow cytometry was performed on LEW fibroblasts transfected or not with BN MHC molecules (A) to analyze the binding of alloantibodies present in purified IgG from naïve LEW rats (gray line) and sensitized LEW rats (black line) (filled histograms, fluorescence intensity obtained with the secondary antibody alone). The time-dependent survival of LEW and BN SMCs cultured with 0.1 mg of LEW anti-BN alloantibodies was evaluated using the MTT assay (B). Results are expressed as the ratio of the optical density (OD) (570 to 630 nm) obtained with SMCs cultured with and without alloantibodies. *P<0.05 LEW SMCs vs BN SMCs. Representative micrographs of LEW and BN SMCs cultured 24 hours with LEW anti-BN alloantibodies are shown. Apoptotic cell death of BN SMCs was quantified after 8 and 24 hours of culture with LEW anti-BN alloantibodies using ELISA-kit (C). Results are expressed as the ratio of the OD (405 to 490 nm) obtained with BN SMCs cultured with and without alloantibodies. **P<0.01 with vs without alloantibodies. Figures are representative of 3 individual experiments. The proapoptotic effect of the LEW anti-BN alloantibodies was analyzed using colorimetric MTT assay. F-LEW-F or F-LEW+F+H11001A1n were cultured with 0.1 mg of purified LEW anti-BN alloantibodies (D). Cell survival was evaluated after 36 hours and is expressed as the ratio of the OD (570 to 630 nm) obtained with fibroblasts cultured with and without alloantibodies. ***P<0.001 LEW-F vs LEW-F+F+H11001A1n.

Figure 4. Proliferation of SMCs and growth factor transcription levels. Supernatants from BN SMCs cultured for 8 hour and 24 hour with IgG from naïve LEW rats or with LEW anti-BN alloantibodies were transferred on LEW SMCs. Proliferation of LEW SMCs was measured by the MTT assay after 48 hours (A). *P<0.05 with IgG from naïve LEW rats vs with LEW anti-BN alloantibodies. Levels of the mRNA for PDGFA and B, FGF-2, IGF-1, and TGF-β were measured by semiquantitative RT-PCR in BN SMCs cultured for 2 or 4 hours with IgG from naïve LEW rats, or with LEW anti-BN alloantibodies, or without addition of IgG. The results are normalized to the GAPDH mRNA level (B). *P<0.05, **P<0.01 LEW anti-BN vs LEW naïve and SMCs alone.
of the graft. The remodeling process characterizing chronic rejection also affects the medial layer because SMCs progressively disappear from grafted arteries.

In the present study, we propose to consider alloantibodies as among the triggering factors for the reshaping process of the vessel during graft arteriosclerosis. In an experimental model of chronic vascular rejection, we have shown that medial SMCs switch toward a synthetic or apoptotic phenotype, whereas SMCs in the neointima proliferate subsequently to the rapid destruction of the endothelium of the graft. Therefore, SMCs have opposite fates depending on their localization within grafted arteries. We have linked these differentiated events with the pattern of deposition of alloantibodies. Indeed, alloantibody deposits are detected within the media, whereas the neointima remains devoid of IgG deposition. Because alloantibodies are directed against antigens that are polymorphic and thus distinct between the donor and the recipient, we hypothesized that the pattern of deposition was a witness to the fact that chronically rejected arteries were chimeras. We showed indeed that whereas MHC I was expressed by SMCs both in the media and the neointima, only medial SMCs expressed donor (BN) MHC I molecules. From this, we can deduce that (1) the proliferating SMCs in the neointima are from recipient origin, and (2) therefore anti-donor alloantibodies can directly target only SMCs in the media. These observations have however to be reconciled with previous studies in which passive transfer of alloantibodies to a recipient of an allograft was sufficient to trigger the full remodeling of grafted arteries, including the shrinkage of the media but also the development of the neointima. Conversely, cyclosporin therapy was shown to be efficient to prevent against the development of graft arteriosclerosis in the rat aortic interposition model. In this setting, reduced titer of alloantibodies correlated with an increased survival of medial SMCs as well as a reduced accumulation of host-derived SMCs in the neointima. This implies that despite their exclusive deposition in the media, alloantibodies could have an effect both on the media and the neointima.

We therefore first asked whether alloantibodies could be responsible for the disappearance of SMCs of the medial donor. We observed that alloantibodies deposition in the media failed to activate the complement cascade. We found that the effector mechanism leading to cell destruction relies rather on a proapoptotic effect triggered by the binding of alloantibodies on SMCs.

We next addressed whether alloantibodies could trigger the proliferation of SMCs of the recipient, leading to the development of the neointima. Because we observed that they have no direct effect on SMCs of the recipient, we hypothesized that the effect of alloantibodies on the development of the neointima may be a consequence of their binding to SMCs of the medial donor. To model in vitro the influence of events taking place in the media on the development of the neighboring neointima, we performed culture supernatant transfer experiments. We demonstrated that binding of alloantibodies to SMCs of the donor induces an early and transient production of growth factors that in turn is responsible for the proliferation of SMCs of the recipient. Therefore, alloanti-body binding to SMCs of the donor in the media transduces a signal that exerts a sequential biphasic effect able to trigger both the remodeling of the media and the development of the neointima during graft arteriosclerosis. Unraveling the intracellular events following alloantibody binding may help to explain this biphasic sequential effect on SMCs. The finding that medial SMCs participate to the production of growth factors promoting neointimal proliferation does not preclude that other cells could do so. For instance, recipient inflammatory cells infiltrating the neointima are likely participating to this process.

One limitation of this study is that we did not explore the role of the early deendothelialization in the development of the neointima. Indeed, the mechanical destruction of ECs is sufficient to trigger the development of a neointima in a nonalloimmune setting. Of note, we have shown in an alloimmune system that deendothelialization followed by immediate reendothelialization with recipient SMCs was able to prevent only marginally the development of neointima, suggesting that alloimmune response is the main trigger for intimal SMC proliferation in graft arteriosclerosis.

Another limitation of this study is that we did not directly identify the specificity of alloantibodies carrying the remodeling effect. Indeed, to model in vitro the in vivo situation as closely as possible, we used nonpooled polyclonal alloantibodies purified from individual animals containing multiple specificities (mostly anti–MHC I and anti–MHC II antibodies). However, neither SMCs from the donor nor from the recipient expressed the MHC II molecules as assessed by immunohistochemistry with a mouse anti-rat MHC II antibody (OX-6; supplemental Figure I). Furthermore, we showed that polyclonal alloantibodies induced apoptosis of recipient fibroblasts when they were transfected to express a single alloantigen, the donor MHC I molecules.

In conclusion, we propose that anti–MHC I alloantibodies play a key role in the arterial remodeling during graft arteriosclerosis. Significantly, we have recently reported that lymphoid neogenesis takes place in the adventitia of grafted arteries and leads to the development of ectopic germinal centers in which B cells produce anti–MHC I alloantibodies locally. In the present work, we demonstrate that the binding of anti–MHC I alloantibodies to the SMCs of the medial donor exerts a sequential biphasic effect. First, they induce a transient production of growth factors that promote an inappropriate response to injury of the intima. These growth factors act in a paracrine fashion to promote the proliferation of SMCs of the recipient that may contribute to the development of an obstructive neointima. In a second phase, the binding of anti–MHC I alloantibodies drives the apoptosis of SMCs of the donor, resulting in the shrinkage of the media. This work ultimately supports a vision in which the remodeling features specific to each tunica are not independent but rather linked to each other in a chain reaction initiated by the production of anti–MHC I alloantibodies.

Acknowledgments
We are grateful to Dr Srini Kaveri for critical reading of the manuscript.


Disclosure(s)

None.

References

Direct and Indirect Effects of Alloantibodies Link Neointimal and Medial Remodeling in Graft Arteriosclerosis

Olivier Thaunat, Liliane Louedec, Jianping Dai, Florence Bellier, Emilie Groyer, Sandrine Delignat, Anh-Thu Gaston, Giuseppina Caligiuri, Etienne Joly, Didier Plissonnier, Jean-Baptiste Michel and Antonino Nicoletti

Arterioscler Thromb Vasc Biol. 2006;26:2359-2365; originally published online August 17, 2006;
doi: 10.1161/01.ATV.0000241980.09816.ac

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/26/10/2359

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2006/08/21/01.ATV.0000241980.09816.ac.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
SUPPLEMENTAL DATA – ONLINE

MATERIALS AND METHODS

Aorta transplantation

All animal experimentation was undertaken in compliance with the European Community standards (authorization n° 75-214).

Rats were anesthetized with 50mg/Kg of pentobarbital injected intraperitoneally. Two animals were operated simultaneously, one as the donor of aortic graft and the other as the recipient, with the aid of an operating microscope. A 1 cm long segment of the donor abdominal aorta was excised, perfused with normal saline and small collateral arteries that originated from the graft were ligated. The donor aorta was transplanted in orthotopic position by end-to-end anastomosis in the recipient aorta below the renal arteries and above the iliac bifurcation. We used the 38 animals that survived to the surgical procedure. No immunosuppressive or anticoagulant treatment was used. Aortic grafts were removed at indicated time-points from the Lewis recipients under anesthesia and perfused with saline. Fresh aortic samples were dissected and embedded in paraffin or in OCT medium (Tissue-Tek, Agar Scientific Ltd, UK) and snap frozen immediately in liquid nitrogen.

Titration of alloantibody in the serum

Two hundreds µl of serum harvested at various time points from grafted animals were incubated with 200,000 Lewis (recipient) fibroblasts expressing Brown-Norway (donor)
MHC molecules cells (LEW-F+A1n) for 30 min at 4°C. The binding of antibodies on the cell surface was then determined with a FITC-conjugated rabbit anti-rat Ig secondary antibody (PARIS; France), by measuring the mean fluorescence intensity (MFI) with a LSR II flow cytometer (BD Biosciences; France).

**Morphometry**

Labeling of IgG and IgM was quantified from images acquired on the same field within the green, red, and blue channels of a fluorescent microscope. Image analysis was performed using a customized program (Leica Qwin). Elastin autofluorescence (green channel) was subtracted from the fluorescence detected on the blue channel while no elastin signal was detected in the red channel in the acquisition condition used for image capture. The external and internal elastic lamina were used to delineate the media and to calculate its surface area and its thickness.

**In-situ apoptosis detection**

*In situ* 3-end labeling of apoptotic DNA was performed using Apotag Peroxidase Kits (Oncor, USA) following the manufacturer's instructions. Briefly, after dewaxing, rehydration, and blocking of endogenous peroxidase, 3-hydroxy-DNA strand breaks in permeabilized tissue sections were enzymatically labeled with digoxigenin-nucleotides, by using terminal deoxynucleotidyl transferase (TdT). The labeled DNA was then bound with antidigoxigenin antibody peroxidase conjugate, and the peroxidase color reaction was developed with a 3-amino-9-ethyl carbazole substrate.
Transmission electron microscopy

Aortic graft specimens were dissected, immediately fixed in 2.5 % glutaraldehyde in PBS buffer, post-fixed in 4% osmium tetroxide and embedded in Epon resin. Ultrathin sections (50 to 80 nm thick) were prepared, stained with lead citrate and uranyl acetate and observed with a Zeiss EMI transmission electron microscope.

Rat vascular SMC isolation and culture

Rat (LEW and BN) aortic SMC were isolated after microdissection of the adventitia from the media and the endothelium. The media was cut into 1 mm-long rings and subjected to an enzymatic digestion with a mixture of collagenase and elastase (Gibco/Invitrogen). Cell suspensions were plated and cultured to confluence at 37°C, 5% CO₂, in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum (FCS), 100U/ml penicillin and 0.1 mg/ml streptomycin (complete medium).

Polyclonal alloantibodies generation and purification

Ten µl of serum collected 10 days after the 4th skin graft were incubated 20 min at 4°C with 2.5 \(10^5\) LEW fibroblasts expressing or not BN MHC molecules. The binding of antibodies on the cell surface was detected with a FITC-conjugated rabbit anti-rat Ig secondary antibody (PARIS; France) by measuring the mean fluorescence intensity in a LSRII flow cytometer (Becton Dickinson Biosciences).

A two-step procedure for IgG purification was applied to avoid the contamination of the IgG preparation with other serum proteins. Fourteen days after the 4th skin graft, 10 ml of blood was collected from LEW rats sensitized or not (source for control IgG) and serum
was prepared. Serum IgG were purified by chromatography on a protein G-Sepharose column, followed by immediate size-exclusion chromatography on a superose-12 column. The IgG-containing fraction was dialyzed against PBS at 4°C for 24h, adjusted to a concentration of 10 mg/ml, aliquoted and stored at –80°C until analysis.

**SMC survival assay**

LEW and BN SMC were plated (10⁴ cells/well) in flat bottom 96-well plates and cultured at 37°C, 5% CO₂, in 100 µl of complete medium (DMEM supplemented with 10% fetal calf serum, 100U/ml penicillin, and 0.1 mg/ml streptomycin) until confluence. Complete medium was then replaced with 90 µl of starvation medium (DMEM supplemented with 2% bovine serum albumin, 100U/ml penicillin, and 0.1 mg/ml streptomycin). After a starvation period of 24h, 10 µl (0.1 mg) of LEW anti-BN alloantibodies (from sensitized LEW rats) or control IgG (from naïve LEW rats) were added to the culture.

Cell survival was evaluated using the tetrazolium salt reduction (MTT) assay according to the manufacturer’s instructions (Roche Diagnostics; IN, USA; please see http://atvb.ahajournals.org). The MTT assay (Roche Diagnostics; IN, USA) is based on the colorimetric measurement of a formazan derivative of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide that is formed by live cells. Briefly, 10 µl of MTT labeling solution (5 mg/ml) were added to the culture at various time-points after addition of antibodies (4h, 8h, 24h, 36h, and 48h). After an incubation period of 4h at 37°C and 5% CO₂, formazan crystals were dissolved by adding 100µl of the solubilization solution (10%SDS in 0.01 M HCl) and the absorbance was measured using a scanning multiwell spectrophotometer at dual wavelength 570–630 nm.
All measurements were performed in duplicates and the experiments were repeated 3 times.

_Semi-quantitative PCR_

Messenger RNAs were extracted with 500 µl of TRIzol (Invitrogen, France) after 2 or 4 h of culture. Reverse transcription reaction was performed with 2 µg of RNA using Oligo dT, random hexamers, and Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen). All oligonucleotide primers were synthesized by Eurogentec (France) (Table I). The PCR reaction was performed in capillaries with 5µl cDNA, 10µl 2x SYBR Green master mix (Qiagen, France) and 0.5 µM of sense and antisense primer. Real time PCR was performed in LightCycler (Roche Diagnostics, France) starting with 15 min of pre-incubation at 95°C followed by 55 cycles of 10 sec at 95 °C, 20 sec at annealing temperature depending on primers used (Table 1), and 30 sec at 72 °C. Amplification of expected PCR product was confirmed by agarose gel electrophoresis (2%) and melting curve analysis.

The threshold cycle (Ct) was determined with the maximum-second-derivative function of the LightCycler software. Results are expressed as the ratio between the Ct of the gene tested and the Ct of GAPDH.
### Table I: Primer list and characteristics of polymerization

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences</th>
<th>Fragment size (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Upper: GTGAAGGTCGGAGTCAACG</td>
<td>299</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Lower: GGTGAAGACGCCAGTGGACTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDGFA &amp; B</td>
<td>Upper: GTAACACCACGCAGTCAAGT</td>
<td>195</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Lower: CTCACCTCACATCCGTCTCCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF-2</td>
<td>Upper: GCACTGAAACGAATGAGGCAGTAT</td>
<td>344</td>
<td>59.7</td>
</tr>
<tr>
<td></td>
<td>Lower: CGCGTCCTTCACCATCAACTTCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-1</td>
<td>Upper: GTACCAAAATGAGCGCACCTC</td>
<td>165</td>
<td>58.1</td>
</tr>
<tr>
<td></td>
<td>Lower: TTGGTCACACACGAACTGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFβ-1</td>
<td>Upper: CTGCCGCTCTGCTCCCACTC</td>
<td>624</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Lower: GCCCTGTATTCCGTTCCTTGT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure I

The computer-assisted morphometric program used to quantify the labeling of IgG and IgM in the media was run on images acquired on the same field within the green, red, and blue channels of a fluorescent microscope (Original magnification x40). Elastin autofluorescence (green channel, Left inset) was subtracted from the fluorescence detected on the blue channel (Right inset) while no elastin signal was detected in the red channel (Middle inset) in the acquisition condition used for image capture. The main picture represents an overlay of the surface areas calculated from each of the three channels on a transversal section of an allograft harvested 30 days post-transplantation. The external and internal elastic lamina were used to delineate the media and to calculate its surface area and its thickness.
Figure II
Six aortic allografts were harvested 10 days (n = 2), 1 month (n = 2) and 2 months (n = 2) post-transplantation. We analyzed by immunohistology the expression of MHC class II by medial SMC of the chronically rejected allografts. Five-micrometer thick transversal sections of aortic allografts were stained using the alkaline phosphatase anti-alkaline phosphatase technique. Primary monoclonal antibodies used were anti pan-MHC class II OX6. A representative photograph obtained on an aorta harvested 10 day post-transplantation is shown (Original magnification x5). MHC class II expression was detected at every time points in the intima and the adventitia of the graft but not in the media.