Network of Vascular-Associated Dendritic Cells in Intima of Healthy Young Individuals


Abstract—In earlier studies, our group has established a new “immunological” hypothesis for atherogenesis supported by experimental and clinical studies showing that inflammatory immunological reactions against heat shock protein 60 initiate the development of atherosclerosis. In the present study, we describe the discovery of a so-far-unknown network of dendritic cells in the innermost layer of arteries, the intima, but not veins of healthy humans and rabbits. The number of these dendritic cells is comparable to that of Langerhans cells in the skin, and dendritic cells show a similar phenotype (CD1a+/S-100−/CD31+/CD34−/CD83−/CD86− and no staining for von Willebrand factor or smooth muscle cell myosin). These vascular-associated dendritic cells accumulate most densely in those arterial regions that are subjected to major hemodynamic stress by turbulent flow conditions and are known to be predisposed for the later development of atherosclerosis. These results open new perspectives for the activation of the immune system within the arterial wall. (Arterioscler Thromb Vasc Biol. 2001;21:503-508.)

Key Words: atherosclerosis • intima • arteries • immunofluorescence • dendritic cells

Atherosclerosis is a multifactorial disease depending on various exogenous factors that become effective on an appropriate genetic background. For many years, atherosclerosis research has concentrated on lipid accumulation and the proliferation of smooth muscle cells. However, more recently, the results of numerous studies have emphasized the pivotal role of the immune system in atherogenesis.1–3 Clinical and experimental data from our own group have provided evidence that atherosclerosis starts as an inflammatory immunological disease that is due to an (auto)immune reaction based on humoral and cellular immunity against microbial-human cross-reactive epitopes of heat shock protein 60.4–6 Immunohistochemical studies on frozen sections of arteries revealed mononuclear cell infiltrations not only in early and late atherosclerotic lesions but also in the arteries of healthy infants and children at sites subjected to major hemodynamic (turbulent) stress that are known to be predisposed for the development of atherosclerotic lesions later in life. These accumulations consist of activated T cells (most of them carrying the αβ T cell receptor and a considerable number expressing γδ T cell receptor as well), macrophages, dendritic cells (DCs), some scattered mast cells, and a very few natural killer cells or B cells.7 In analogy to the mucosa-associated lymphoid tissue, we tentatively called these accumulations of mononuclear cells in healthy children “vascular-associated lymphoid tissue” and assumed a similar function for these as a system of local defense of the vascular system.8

During the last decade, great progress has been made in the field of DC research.9 Because it is now generally accepted that the immune system plays an important role in atherogenesis, it was deemed of interest to perform a critical study on the occurrence and distribution of DCs in the vascular system with special emphasis on the sites of the newly discovered vascular-associated lymphoid tissue.

One of the typical features of DCs is their presence at the borderline of the body to its environment. At these locations, they are present in an immature stage, characterized by high endocytotic activity and low T-cell stimulatory potential because of the lack of costimulatory molecules, such as CD40, CD54, and CD86.10 Thus, DCs are present in the skin,11 in the intestine (mainly in the Peyer’s patches),12 and in the respiratory tract.13 But only recently have there been suggestions that atherosclerotic lesions may also be populated by DCs.14–16 DCs in the atherosclerotic vessel wall express human lymphocyte antigen-DR, CD1a, and S-100 protein14,16 and are positive for intercellular adhesion molecule-117 and vascular cell adhesion molecule-1.18

Previous studies investigating vascular DCs concentrated only on atherosclerotic altered arteries, but there were no data available concerning the presence of DCs in the healthy intima, as we had demonstrated to be the case in earlier preliminary work.7,8

We have now focused our attention on the exact distribution and abundance of the vascular-associated DCs.
Methods

Arterial Specimens

Rabbits

Five female New Zealand White rabbits between 8 and 12 weeks of age were obtained from Savo/Charles River Co, Kisslegg im Allgäu, Germany. They were housed individually under conventional conditions in wire-bottomed cages at 22°C with a relative humidity of 60% in the Central Experimental Animal Facilities of the University of Innsbruck, Medical School. They received water ad libitum and were fed a standard diet (T775, Tagger & Co). All animal experiments were performed according to the institutional guidelines.

The animals were euthanized under ketamine (25 mg/kg) and xylazine (5 to 10 mg/kg) anesthesia by heart puncture. The aortas were carefully removed from the surrounding tissue from the beginning of the aortic arch to the bifurcation into the 2 iliac arteries. Then, they were immersed in ice-cold PBS, pH 7.2, and immediately processed for further immunohistochemical or immunofluorescence studies.

Humans

Human arterial samples from 14 infants and children aged 8 months to 16 years were obtained from the Department of Forensic Medicine, University of Innsbruck, Medical School. Causes of death were sudden infant death syndrome, polytraumata, intoxication, drowning, pulmonary embolism, and aspiration pneumonia.

For investigations on vascular DCs in adults, we used human arterial samples from the carotid arteries, aortas, and iliac arteries of 20 young adults between 17 and 34 years of age, whose death was due to suicide, homicide, or accident (obtained from the Department of Forensic Medicine, University of Innsbruck, Medical School) or who were candidates for organ transplantation (obtained from the Department of Transplant Surgery, University of Innsbruck, Medical School) and did not suffer from any clinical manifestation of cardiovascular disease.

In 5 of the above-mentioned cases, veins were collected in addition to the arteries (either the jugular vein or the iliac vein as a pendant to the respective arterial sample).

The samples for frozen sections were collected, immersed in transport buffer as described by Michel et al., and transported to the laboratory on ice. Samples for the preparation of intimal sheets were immersed in ice-cold PBS instead of transport buffer. The surrounding connective tissue was removed, and each artery was filled with freezing medium (O.C.T. Tissue Tek, Miles Inc, Diagnostic Division), shock-frozen, and stored in liquid nitrogen until being cut into 4-µm-thick frozen sections.

Staining Procedures

Immunohistochemistry on Frozen Sections

Slides with frozen sections were air-dried for 30 to 60 minutes at room temperature. Blocking of nonspecific antibody binding was achieved by incubation with 10% normal human serum (heat inactivated at 56°C for 30 minutes) in blocking reagent (No. 1096176, Boehringer-Mannheim) for 15 minutes. Excess serum was blotted off; the primary antibody was diluted in Tris-buffered saline (TBS), pH 7.4, and applied directly without any washing procedure; and the sections were incubated for 30 minutes. Optimal dilutions for all antibodies were determined in pilot studies. Incubation took place in a humidified chamber at room temperature. The sections were then rinsed 3 times in TBS, the secondary antibody was applied, and the sections were incubated for another 30 minutes. They were rinsed as described above, and incubation with the alkaline phosphatase/anti–alkaline phosphatase (APAAP) complex followed at room temperature for 30 minutes. The incubation step with the secondary antibody and the APAAP complex was repeated to increase the staining intensity. After 3 further changes of TBS, visualization with Fast Red Naphthol (Sigma) and counterstaining with Mayer’s hemalaun was performed. Slides were finally mounted in Kayser’s glycerol gelatin (Merck). The Table shows the source and dilution of the antibodies used.

Double Staining on Frozen Sections

Double staining was achieved by combination of the APAAP technique and the DAKO animal research kit. Immunohistochemistry was performed as described above for single staining. After visualization with Fast Red, the second antibody was treated with the biotinylation reagent according to the manufacturer’s instructions and developed with horseradish peroxidase-labeled streptavidin contained in the test kit.

En Face Immunofluorescence on Human or Rabbit Intimal Sheets

Fresh arterial samples were washed in cold PBS to remove blood. They were opened longitudinally, cut into 2 halves, and dissected into small pieces of ~0.7 x 0.7 cm, followed by incubation in 0.5 mol/L NH₄SCN (Merck) at 37°C for 60 minutes. After a wash in PBS at room temperature, the intima was gently lifted off from the rest of the vessel wall with delicate forceps. After they were rinsed in PBS for 30 minutes, the samples were ready to undergo the staining procedure.

Sheets were acetone-fixed for 10 minutes at room temperature (with the exception of those that were to be stained for CD1a) and rinsed in PBS for 20 minutes thereafter. Then incubation in the optimally diluted primary antibody took place overnight at 4°C. Washing was performed in PBS/1% BSA (Sigma) for 4 hours at room temperature on a multiaxle rotator, and the sheet was incubated in diluted FITC-labeled secondary antibody for the next 4 hours at room temperature. Finally, the sheets were washed in PBS/1% BSA on the rotator at room temperature overnight and mounted the following morning. Incubation in NH₄SCN, acetone fixation, and incubation with antibodies were performed in micro–test tubes (No. 0030120.086, Eppendorf-Netheler-Hinz), whereas the washing took place in 50-mL tubes (No. 252070, Falcon, Becton-Dickinson Labware) to guarantee excess washing fluid. The specimens were examined with a laser scanning confocal fluorescence microscope (Zeiss), with magnifications ranging between 100- and 630-fold.

Negative Controls

For all experiments, negative controls were carried out either by omitting the first antibody or by using an irrelevant isotype-matched antibody.

Photographic Documentation

Stained sections were examined by light microscopy (Optiphon-2, Nikon), and pictures were taken on the same microscope with use of...
automatic exposure equipment (UFX-DX, Nikon) and daylight films (Fujichrome Velvia-50).

Intimal sheets were analyzed by confocal microscopy, and pictures were stored and printed on a laser printer.

Results

Immunohistochemistry on Frozen Sections

CD1a⁺ Cells Are Present in All Human Arterial Specimens

CD1a⁺ DCs were found in all human arterial samples. In arterial specimens showing incipient atherosclerotic development, this finding was expected; therefore, these served as positive controls for all further investigations.

CD1a⁺ Cells Are Present in Intima of Children

In children, DCs were found in all arterial specimens of the intima in the subendothelial location, showing the typical morphological features of DCs with long cytoplasmic processes. They are found in higher density in areas of bifurcation and more sparsely distributed in regions without a deviating flow pattern. The best staining impressions were obtained in tangential sections of the intima, displaying the intimal area more broadly (Figure 1).

After demonstration of the presence of DCs in the intima of healthy children by staining for CD1a, further analysis concerning the characterization of the vascular-associated DCs was performed to determine the subset of DCs. We stained for CD86 (B7.2) as a marker for mature DCs, CD83, S-100 (Figure 2), lag (a marker for Birbeck granules that are specific for Langerhans cells), and CD31. von Willebrand factor as an endothelial cell–specific molecule not expressed on any subset of DCs was included as a control.

To exclude a type of cell showing smooth muscle cell or macrophage phenotype with additionally acquired DC markers, we also stained for smooth muscle cell myosin and CD68, respectively, in double-staining experiments or on serial sections. These experiments showed that vascular-associated DCs have the following phenotype: CD1a⁺ S-100⁺ lag⁺ CD31⁺ CD68⁺ CD83⁺ CD86⁺, and they are negative for von Willebrand factor and smooth muscle cell myosin. This pattern of marker expression is similar to that of immature Langerhans cells in the epidermis.

En Face Immunofluorescence on Intimal Sheets

Major Histocompatibility Complex Class II⁺ Cells With DC Morphology Are Found Abundantly in Intima of Normal Rabbits

Rabbit intimal sheets were analyzed by immunofluorescence for Ia expression. Ia⁺ cells were found spread over the intima in all stained specimens. Considering the shapes of their cell bodies and their long processes, they were primarily recognized as either macrophages or DCs (Figures 3 and 4), and they formed a network in areas of dense accumulation, particularly areas of Ia⁺ cell accumulation, such as those parts of the rabbit aorta at which arteries branched off. Ostia of branching arteries were not surrounded by stained cells, but...
they were concentrated at those parts of the branch at which the mechanical drag is most severe (Figure 5). Optical sections in the laser scanning microscope showed that all Ia\(^+\) cells were lying under the endothelium and that none was adherent to the endothelium. Endothelial cells did not stain for Ia.

Closer scrutiny showed that Ia\(^+\) cells were orientated longitudinally within the aorta except at those sites where turbulent predominates over laminar blood flow.

**CD1a\(^+\) Cells Are Found in Human Intima in Amounts Equivalent to Those in Ia\(^+\) Cells in the Rabbit**

Because no specific marker exists for rabbit DCs, identification of these cells was performed on human arterial intimal sheets from individuals without macroscopic atherosclerotic lesions. CD1a\(^+\) cells were found in most parts of the arterial specimens examined. As already shown in the rabbit by expression of major histocompatibility complex (MHC) class II, CD1a\(^+\) DCs are not distributed regularly, but they aggregate in hemodynamically stressed areas, where they form a network-like structure similar to Langerhans cells in the skin, extending their processes in all directions (Figure 6).

A very striking finding was that DCs do not exist in veins that can therefore be considered to be an integrated negative control for the staining procedures.

**Discussion**

In the present study, we show that DCs are present in the normal intima of children. In contrast to all present investigations, we prove not only that DCs appear in the vessel wall on inflammatory stimuli during atherogenesis but also that they preexist under the endothelium, even in arteries of young individuals still unaffected by any form of atherosclerosis. The staining pattern for various markers used in DC characterization suggests a similarity between vascular-associated DCs and Langerhans cells, inasmuch as both of them are CD1a\(^-\) S-100\(^+\) lag\(^+\) CD31\(^-\) CD68\(^-\) CD83\(^-\) and CD86\(^-\) in the immature state. In contrast to other investigations involving atherectomy specimens with atherosclerotic lesions in which stellate cells\(^{21}\) or subsets of smooth muscle cells\(^{22}\) are found to display some of these markers, we (1) used exclusively unaffected arteries of children and adolescents that showed no myxomatous tissue, (2) showed by double staining and by use of serial sections that these CD1a\(^+\) cells are negative for myosin and CD68, and (3) showed evidence that these cells are DCs by staining for lag, a unique marker for Langerhans cells.\(^{23}\)

The highest density of vascular DCs was observed at areas in which turbulence predominates over laminar hemodynamics. This is true for human arteries as well as arteries from rabbits, which are a generally accepted experimental animal model for atherosclerosis research into the role of lipids as well as immunological processes. The location of vascular DCs corresponds exactly to the sites prone to the later...
development of atherosclerotic lesions. Turbulent flow results in complex secondary flows with flow reversal and dynamic stagnation points, resulting in prolonged endothelial resident times for large atherogenic particles (e.g., LDLs) or blood cells. An increased contact time between leukocytes and endothelial cells could lead to increased transendothelial migration and, therefore, explains the higher density of all different cell types, including DCs, at these sites.

Inasmuch as our experimental rabbits were normocholesterolemic, these results emphasize that predisposed areas can be recognized from their cellular makeup even without any lipid deposition.

The absence of vascular DCs in veins could be another explanation (besides the difference in blood pressure) of why we develop arteriosclerosis and not venosclerosis. Another striking finding from the present study concerns the localization of DCs in the arterial intima. They are oriented longitudinally with the blood stream in areas of laminar flow conditions, but they change their distribution pattern in areas of turbulent flow. So far, we can only speculate that extracellular matrix proteins in the intima are involved in this process. Further studies on intimal extracellular matrix patterns will show whether they are distributed differentially in areas subjected to different hemodynamic stress.

Surprisingly, en face staining for DCs revealed a network formed by vascular DCs similar to that seen with Langerhans cells in the skin, and by this technique, we were able to show that the vessel wall seems to be protected from blood-borne pathogens in the same manner as other surfaces of the body in close contact with the environment. The fact that the vascular wall is a huge surface and a possible site of antigen processing and presentation and our present demonstration of DCs in the arterial intima open new perspectives in this respect. This prospect is certainly due to the general belief that blood is a sterile fluid not requiring immunosurveillance, because possible noxious substances have already passed the gate control of the skin or the mucosa. However, a closer look shows that even in the blood, invasions of bacteria can occur. Bacteremia not only occurs during septic diseases but also is an intermittent physiological condition in healthy organs, triggered by minitrauma (e.g., tooth brushing), turning pathological only when it lasts longer than a few hours.

This amount of professional antigen-presenting cells in the arterial intima opens new perspectives to immune reactions taking place in the vessel wall itself.

To date, it has been speculated that initialization of the immune response leading to development of atherosclerosis might take place in the para-aortic lymph nodes, but our studies implicate local initiation of the priming of naive lymphocytes. Macrophages, which are always found in atherosclerotic lesions, were proposed as possible candidates because they are highly positive for MHC class II molecules. Endothelial cells are not able to present antigens in the normal unaffected intima because they do not express MHC class II molecules constitutively. Endothelial cells are MHC II positive essentially only in those areas in which activated T cells have accumulated subendothelially but not in the normal intima. This phenomenon can be explained by the release of interferon-γ by infiltrating T cells. For this reason, endothelial cells can be excluded as initiators of an immune reaction by presenting antigen to lymphocytes, whereas they could play a role in the promotion of immune reactions and in the activation of memory T cells. We now disclose the possibility that resident intimal DCs may be effective as local antigen-presenting cells and could perhaps also initiate (auto)immune processes in the vessel wall. Their subendothelial position is very advantageous to this scenario, inasmuch as the DCs can come in close contact with blood-borne antigens as well as with lymphocytes that adhere to the endothelial layer and migrate through the vessel wall. However, whether arteries need be of certain size to be populated by DCs or whether DCs are present even in arterioles remains to be investigated. The arterial surface is a huge area when calculated for the whole body; therefore, our data identify a new “immunological space” at which important immunological reactions could take place and influence physiological as well as pathological processes.

In the present study, we describe the natural habitat of vascular DCs: functional investigations will follow on the basis of the present morphological data.

From the present study, it is not yet clear whether these DCs play an atherosclerosis-promoting or -inhibiting role. Both mechanisms are possible, i.e., induction of tolerance or stimulation of an immune response to the antigen triggering atherogenesis. However, our morphological data suggest an atherosclerosis-enhancing function by the accumulation of DCs in areas of the vascular tree that are predisposed to later atherosclerotic development.

Acknowledgments

This study was supported by the Austrian Science Fund, project No. P-14741/MED (to G.W.), the Hans und Blanca Moser-Stiftung (to G.M.), and a “Förderungsstipendium” of the University of Innsbruck (to G.M.). We would like to thank Dr Hermann Dietrich and Ernst Rainer for expert help with animal experiments and Dr Heidrun Rechelis for confocal microscopic analyses of the rabbit specimens.

References

Network of Vascular-Associated Dendritic Cells in Intima of Healthy Young Individuals

doi: 10.1161/01.ATV.21.4.503

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 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/21/4/503

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/