Vascular Biology, Atherosclerosis and Endothelium Biology

Galectin-3 Gene Inactivation Reduces Atherosclerotic Lesions and Adventitial Inflammation in ApoE-Deficient Mice

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This study has examined the role of galectin-3 (GaL3), a multicompartimented *N*-acetyllactosamine-binding chimeric lectin, on atherogenesis in the ApoE-deficient mouse model of atherosclerosis. Pathological changes consisting of atheromatous plaques, atherosclerotic microaneurysms extending into periaortic vascular channels, and adventitial and periaortic inflammatory infiltrates were assessed in an equal number ($n = 36$) of apolipoprotein (Apo)E-deficient mice and ApoE-GaL3 double-knockout mice. These mice were divided into three age groups, 21 to 23 weeks, 25 to 31 weeks, and 36 to 44 weeks of age. Results of this morphological analysis have shown an age-related increase in the incidence of aorta atheromatous plaques and periaortic vascular channels in ApoE-deficient mice. By contrast ApoE/GaL3 double-knockout mice did not show an increase in pathological changes with age. The 36- to 44-week group of $ApoE^{-/-}/GaL3^{-/-}$ mice had a significantly lower number of atherosclerotic lesions ($P < 0.004$) and fewer atheromatous plaques ($P < 0.008$) when compared with $ApoE^{-/-}/GaL3^{+/+}$ mice of the same age. $ApoE^{-/-}/GaL3^{-/-}$ mice had a lower number of perivascular inflammatory infiltrates and mast cells than those found in $ApoE^{-/-}/GaL3^{+/+}$ mice. The reduced number of perivascular mast cells may have resulted in a low level of interleukin-4 that contributed to the reduction in the morphological parameters of atherogenesis correlated with the lack of GaL3 expression. The effect of GaL3 deficiency on atherogenesis decrease could be related to its function as a multifunctional protein implicated in macrophage chemotaxis, angiogenesis, lipid loading, and inflammation. (*Am J Pathol* 2008, 172:247–255; DOI: 10.2353/ajpath.2008.070348)

Vascular atherosclerotic lesions are considered to be the result of an inflammatory proliferative response to insults to the artery wall in which an excess of modified lipoproteins plays a crucial role. The inflammatory cascade may be triggered by different pathogens,¹ pathogenic molecules and metabolic events,² or be a component of an immune response.³ Inflammation is a major mechanism in the initiation and progression of lesions in atherosclerosis with inflammatory cells releasing a host of enzymes, cytokines, and growth factors that can induce further damage to the vascular wall.⁴

Galectin-3 (GaL3) is one of the many factors suspected to be involved in atherosclerosis. GaL3, an *N*-acetyllactosamine-binding chimeric lectin, is a mediator/modulator of cell-to-extracellular matrix adhesive interactions and signal transduction events, and it plays a role in organogenesis, angiogenesis, inflammation, and autoimmune disorders.^{5–7} As a multifunctional and multicompartimented protein, GaL3 is involved in T-cell apoptosis and proliferation, macrophage chemotaxis, phagocytosis, neutrophil extravasation and NADPH-oxidase activity, and mast cell activation.⁸

The hypothetical role of GaL3 in atherogenesis was suggested by the finding that GaL3 is up-regulated in the aorta of hypercholesterolemic rabbits⁹ and in human atherosclerotic lesions.¹⁰ Support for this hypothesis comes from the demonstration of GaL3 expression in rat post-angioplasty stenotic changes,¹¹ foam cell macrophages from kidneys of apolipoprotein E-deficient mice,¹² myocarditis lesions in virus-infected hypercholesterolemic mice,¹³ and mouse peritoneal macrophages loaded *in vitro* with modified lipoproteins.¹⁴ The demonstration that GaL3 mediates the endocytotic uptake of modified low-density lipoproteins resulting in intracellular accumulation of cholesteryl esters¹⁵ and may serve as an advanced glycation end (AGE) product receptor¹⁶ provided an in-

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Atherosclerosis is a complex disease with a multifactorial etiology related to traditional and nontraditional risk fac-

sight to the mechanisms through which GaL3 may contribute to atherogenesis.

These data prompted us to investigate the potential contribution of this multifunctional glycoprotein to the development and progression of atherosclerotic lesions in an animal model. To address this question we compared the development and progression with age of atherosclerotic lesions in the presence or absence of GaL3 expression in the ApoE-deficient mouse model.¹⁷ Results of this work demonstrated that in the absence of GaL3 the development and age-related progression of some aortic and periaortic atherosclerotic changes in the ApoE-deficient mouse were reduced. Understanding the participation of GaL3 in atherogenesis could be useful in controlling the pathogenic mechanisms that result in this common disorder.

Materials and Methods

Generation of ApoE^{-/-}/GaL3^{-/-} Mice

ApoE-deficient mice of the strain C57BL/6J-ApoE^{tm1Unc17} were purchased from The Jackson Laboratory (Bar Harbor, ME) and were on the C57BL/6 genetic background. Galectin-3-null mutant mice (ApoE^{+/+}/GaL3^{-/-}) generated from chimeric males derived from MF1 blastocytes injected with WW6 ES cells and mated with 129 c/c females¹⁸ generously provided by Dr. Francoise Poirier (INSERM U426, Paris, France) were crossbred with ApoE^{-/-} mice. Mice obtained from this crossbreeding had a mixed 129/C57 background. Mice were genotyped for GaL3 gene null mutation by polymerase chain reaction analysis of tail DNA. All mice were housed and cared for according to the institutional and the National Institutes of Health guidelines on the care and use of experimental animals. The animals were housed in metabolic cages at 21 to 23°C with a humidity level of 50 to 60% and a 12-hour light/dark cycle and were fed a normal mouse chow diet. Food and water were available *ad libitum*.

Tissue Preparation for Light Microscopy

Mice were anesthetized, and after laparotomy the inferior vena cava was used for obtaining blood. A 1-ml 4% buffered neutral formalin solution was injected into the left ventricle. The heart with the aorta and adjacent adipose tissue were dissected and stored in neutral-buffered formalin until they were processed. Before paraffin embedding the aorta was separated from the heart at the level of the aortic sinus. Sections were stained with Harris hematoxylin and eosin (H&E), Masson trichrome, and Verhoeff's elastic tissue stain. Histochemical staining with toluidine blue was performed to identify mast cells. For toluidine blue staining slides of paraffinized sections of the aorta and periaortic adipose tissue were dewaxed, rehydrated, and incubated with 0.05% w/v toluidine blue and counterstained with 0.01% w/v eosin.

Galectin-3 Immunostaining

Paraffin-embedded sections of aorta and periaortic tissue were processed for staining for GaL3 by the immu-

noperoxidase procedure. The M3/38 monoclonal antibody¹⁹ used in the immunostaining procedure was prepared from the supernatant of the M3/38.1.2.8.HL.2 rat hybridoma TIB 166 obtained from the American Type Culture Collection (Bethesda, MD). Immunohistochemical staining was performed using a nonbiotin amplification system (NBA; Zymed Immunochemicals, South San Francisco, CA). As negative controls, the primary antibody was replaced with either normal rat IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or with phosphate-buffered saline. Sections were sequentially incubated with fluorescein isothiocyanate-conjugated secondary antibody and anti-fluorescein isothiocyanate peroxidase-conjugated tertiary antibody. Next, the sections were overlaid with the 3',3'-diaminobenzidine substrate as the chromogen. The sections were counterstained with Mayer hematoxylin and permanently mounted in Cytoseal XYL (Stephens Scientific, Riverdale, NJ).

Morphological Quantitation of Atherosclerotic Lesions

To evaluate the extent of lesions in the aorta and periaortic tissue, 20 longitudinal sections of the aorta, each 6- μ m thick, were collected at 30- μ m intervals. Video images from the tissue sections were captured directly from a SPOT camera (Diagnostic Instruments, Inc., Sterling Heights, MI) attached to a Axiophot light microscope (Zeiss, Thornwood, NY) and displayed on a MultiSync E900 monitor (NEC, Irving, TX). The number of aortic lesions was recorded in each animal. Mast cell density was quantified by counting the number of toluidine blue-positive mast cells per microscopic field ($\times 250$). The number of mast cells in 20 microscopic fields was recorded for each mouse aorta.

Statistical Analysis

The data were analyzed with commercial software (SigmaStat, Chicago, IL). Statistical analysis consisted of analysis of variance with Newman Keuls post hoc tests to examine individual group differences. Significance was set at $P < 0.05$.

Results

Generation of ApoE^{-/-}/GaL3^{-/-} Mice Colony

ApoE^{-/-} and GaL3^{-/-} mice were intercrossed to produce the F1 generation, and ApoE^{-/-}/GaL3^{-/-} mice were obtained from the F2 generation. Genotyping of offspring from the F2 generation showed the expected distribution of genotypes for both males and females. An ApoE^{-/-}/GaL3^{-/-} colony was established from the F2 generation. Plasma cholesterol levels were comparable for ApoE^{-/-}/GaL3^{-/-} (405 ± 85 mg/dl) and ApoE^{-/-}/GaL3^{+/+} (418 ± 27 mg/dl) mice and were significantly different ($P < 0.001$) from that of ApoE^{+/+}/GaL3^{-/-} mice (81 ± 6 mg/dl).

Figure 1. Cross section through an *ApoE*^{-/-}/*GaL3*^{+/+} mouse aorta; an atheromatous plaque shows intense immunostaining for GaL3 appearing as brown deposits in foam cells.

Expression of Galectin-3 in Atherosclerotic Lesions

To ascertain the expression of GaL3, atherosclerotic lesions of the aorta from both *ApoE*^{-/-}/*GaL3*^{+/+} and *ApoE*^{-/-}/*GaL3*^{-/-} mice were immunostained for GaL3. Intense immunostaining for GaL3 was found in the foam cells that were present in the atheromatous plaques in the artery wall and in the microaneurysm-derived channels expanding into the periaortic adipose tissue in sections from *ApoE*^{-/-}/*GaL3*^{+/+} mice (Figure 1). Few cells in the adventitial inflammatory infiltrates showed moderate positive staining. Aorta specimens from C57/BL/6 mice as wild-type controls or *ApoE*^{+/+}/*GaL3*^{-/-} mice were consistently negative for GaL3 immunostaining (data not shown).

Atherosclerotic Lesions of the Aorta and Periaortic Tissue

This study analyzed and compared atherosclerotic and inflammatory lesions in the aorta artery and periaortic tissue from 36 *ApoE*^{-/-}/*GaL3*^{+/+} mice and 36 *ApoE*^{-/-}/*GaL3*^{-/-} mice. To assess the influence of age on the development of pathological changes, the 36 male mice of each of the two mouse strains that were the object of this study were divided in three age groups: young mice, 21 to 23 weeks of age (*ApoE*^{-/-}/*GaL3*^{+/+}, *n* = 11; *ApoE*^{-/-}/*GaL3*^{-/-}, *n* = 13), middle age mice, 25 to 31 weeks of age (*ApoE*^{-/-}/*GaL3*^{+/+}, *n* = 16; *ApoE*^{-/-}/*GaL3*^{-/-}, *n* = 14), and old mice, 36 to 44 weeks of age (*ApoE*^{-/-}/*GaL3*^{+/+}, *n* = 9; *ApoE*^{-/-}/*GaL3*^{-/-}, *n* = 9).

The atherosclerotic lesions consisted of atheromatous plaques, microaneurysms, and periaortic vascular channels (Figure 2). Atheromatous plaques were predominantly of the fatty type consisting mainly of foam cells. Atherosclerotic microaneurysms were commonly found often beneath an atheromatous plaque with their lumen filled with atheromatous material (Figure 2A). Vascular channels were frequently found within the periaortic tis-

sue at a variable distance from the aorta wall (Figure 2, B and C). Serial sectioning of aorta specimens demonstrated that these channels were an extension of the atherosclerotic microaneurysms (data not shown). These channels were lined by endothelial cells and frequently included two layers of elastic tissue (Figure 2C). They were circumscribed by a layer of acellular fibrotic tissue and frequently by an inflammatory infiltrate. Mast cells were commonly present at the periphery of the fibrotic tissue.

In *ApoE*^{-/-}/*GaL3*^{+/+} mice the average number of atherosclerotic lesions increased significantly in 36- to 44-week-old mice (*P* < 0.01) (Figure 3). This increase did not occur in *ApoE*^{-/-}/*GaL3*^{-/-} mice, and the overall incidence of atherosclerotic lesions in older mice (the 36- to 44-week group) was significantly lower when compared with *ApoE*^{-/-}/*GaL3*^{+/+} mice of the same age group (*P* < 0.004) (Figure 3, asterisk). The significantly increased incidence of atherosclerotic changes in *ApoE*^{-/-}/*GaL3*^{+/+} indicates the continued progress of this pathological process with age. The lower rate of atherosclerotic lesions in 36- to 44-week-old *ApoE*^{-/-}/*GaL3*^{-/-} mice suggests that in the absence of GaL3 expression age-related progression of atherosclerotic aorta lesions was diminished.

To assess age-related differences of pathological changes between the two strains of mice, we examined separately the incidence of each type of atherosclerotic lesion in different age groups. In *ApoE*^{-/-}/*GaL3*^{+/+} mice the average number of plaques increased significantly from the 21- to 23-week group to the 25- to 31-week group (*P* < 0.001) and was even higher in the 36- to 44-week age group (*P* < 0.003) (Figure 4). By contrast, in *ApoE*^{-/-}/*GaL3*^{-/-} mice there was an insignificant increase within each age group. Therefore there were significantly fewer plaques in the 36- to 44-week age group than in *ApoE*^{-/-}/*GaL3*^{+/+} mice of similar age (*P* < 0.008) (Figure 4C, asterisk). These results show that in *ApoE*-deficient mice the process of atheromatous plaque formation progresses with age and the lack of GaL3 expression hinders this process.

The incidence of atherosclerotic microaneurysms and periaortic vascular channels increased significantly in the 36- to 44-week-old *ApoE*^{-/-}/*GaL3*^{+/+} mice when compared with the 21- to 23-week-old and the 25- to 31-week-old group of the same strain (*P* = 0.037 and *P* = 0.02, respectively) (Figure 5A, asterisk). This increase was accounted for primarily by a higher incidence of periaortic vascular channels in the 36- to 44-week-old group of *ApoE*^{-/-}/*GaL3*^{+/+} (*P* = 0.04) (Figure 5C, asterisk). The incidence of microaneurysms did not increase significantly with age in either of the two mouse strains. The frequency of periaortic vascular channels stayed at the same level in each age group of *ApoE*^{-/-}/*GaL3*^{-/-} mice and did not differ from that found in the 21- to 23-week-old and the 25- to 31-week-old group of the *ApoE*^{-/-}/*GaL3*^{+/+} mice. These results show that, in the absence of GaL3 expression, progression of periaortic vascular channels was prevented. No atherosclerotic microscopic changes were found in the aortas of C57BL/6 (*n* = 10)

Figure 2. **A:** Longitudinal cross section through the aorta from an *ApoE*^{-/-}/*Gal3*^{+/+} mouse showing large atheromatous plaques protruding into the lumen. Beneath the upper plaque a microaneurysm filled with atheromatous material is penetrating deep into the periaortic adipose tissue and is surrounded by inflammatory cells (arrow). H&E staining. **B:** Vascular channel extending from a microaneurysm into the periaortic adipose tissue (arrow). H&E staining. **C:** Cross section through a vascular channel located at a distance from the aorta wall, with the lumen partially filled with foam cells and the wall displaying two layers of elastic tissue (arrow). The channel is surrounded by fibrotic tissue (blue). Masson trichrome staining. **D:** Cross section through a nodular inflammatory infiltrate in the vicinity of aorta adventitia (arrow). Masson trichrome staining.

and *ApoE*^{+/+}/*Gal3*^{-/-} (*n* = 10) mice of similar age included as controls.

Inflammatory Changes in the Aorta Adventitia and Periaortic Tissue

Inflammatory infiltrates were defined by clusters of at least 20 mononuclear cells per high-power microscopic field (×400), found in at least three sections from one animal. They were located in the tunica adventitia and the periaortic adipose tissue and consisted of lymphocytes, plasma cells, and macrophages organized as attenuated bands in the adventitial fibrous tissue or as nodules distributed either around vasa vasorum or a vascular channel expanding into the periaortic adipose tissue (Figure 2D).

The incidence of adventitial and periaortic tissue inflammatory infiltrates in *ApoE*^{-/-}/*Gal3*^{+/+} mice showed an average of inflammatory infiltrates/mouse significantly higher than that found in *ApoE*^{-/-}/*Gal3*^{-/-} mice (*P* = 0.003). The average number of adventitial and periaortic

inflammatory infiltrates/mouse in *ApoE*^{-/-}/*Gal3*^{+/+} mice increased with each age group whereas in *ApoE*^{-/-}/*Gal3*^{-/-} mice the incidence of inflammatory infiltrates was approximately the same for all three age groups (Figure 6). *ApoE*^{-/-}/*Gal3*^{-/-} mice had a significantly lower number of inflammatory infiltrates than those found in *ApoE*^{-/-}/*Gal3*^{+/+} mice in the 25- to 31-week-old group (*P* = 0.024) (Figure 6, asterisk). These data show that in each of the three age groups there were fewer inflammatory infiltrates and that the steady increase in the frequency of adventitial and periaortic inflammatory infiltrates seen in *ApoE*^{-/-}/*Gal3*^{+/+} did not occur in mice lacking *Gal3* expression.

Mast Cells Density in Aorta Adventitia and Periaortic Tissue

In *ApoE*^{-/-}/*Gal3*^{+/+} and *ApoE*^{-/-}/*Gal3*^{-/-} mice one or two mast cells were present at rather regular intervals along the aorta artery wall in the segments that were not affected by atherosclerotic lesions. These cells were sit-

Figure 3. The incidence of aortic and periaortic atherosclerotic lesions in three age groups of *ApoE*^{-/-}/*GaL3*^{+/-} (21 to 23 weeks, *n* = 11; 25 to 31 weeks, *n* = 16; 36 to 44 weeks, *n* = 9) and *ApoE*^{-/-}/*GaL3*^{-/-} (21 to 23 weeks, *n* = 13; 25 to 31 weeks, *n* = 14; 36 to 44 weeks, *n* = 9) mice. Statistically significant increase for these pathological changes was found in the 36- to 44-week-old *ApoE*^{-/-}/*GaL3*^{+/-} mice compared to the younger groups of the same strain and with the same age group of *ApoE*^{-/-}/*GaL3*^{-/-} mice (star).

uated at the periphery of the adventitia at the interface with the periaortic adipose tissue. In *ApoE*^{-/-}/*GaL3*^{+/-} mice clusters of 8 to 10 mast cells were seen in the vicinity of periaortic vascular channels and microaneurysms commonly located at the periphery of the fibrotic tissue surrounding these pathological changes (Figure 7A) or in the proximity of atheromatous lesions (Figure 7B). In *ApoE*^{-/-}/*GaL3*^{+/-} mice such clusters were much less common and consisted of fewer cells (Figure 7, C and D). Mast cell density in *ApoE*^{-/-}/*GaL3*^{+/-} mice (*n* = 10) was 59.60 ± 6.92 mast cells/aorta compared to 30.10 ± 5.50 mast cells/aorta in *ApoE*^{-/-}/*GaL3*^{-/-} mice (*n* = 10) (*P* = 0.013). This significant reduction was mainly attributable to fewer mast cells occurring around the atherosclerotic vascular and perivascular changes.

Discussion

To determine the role of GaL3 in atherogenesis, we compared the development and age-related progression of aorta and periaortic atherosclerotic changes in *ApoE*-*GaL3* double-knockout mice and *ApoE*-deficient mice.

Figure 4. The incidence of atheromatous plaques in *ApoE*^{-/-}/*GaL3*^{+/-} and *ApoE*^{-/-}/*GaL3*^{-/-} mice. The frequency of plaques increased with age in *ApoE*^{-/-}/*GaL3*^{+/-} mice but not in *ApoE*^{-/-}/*GaL3*^{-/-} mice. Compared to *ApoE*^{-/-}/*GaL3*^{+/-} mice of the same age, significantly fewer plaques developed in the 36- to 44-week-old *ApoE*^{-/-}/*GaL3*^{-/-} mice (*P* > 0.008) (star).

Figure 5. The incidence of microaneurysms and periaortic vascular channels combined (A) and separated (B, C) in *ApoE*^{-/-}/*GaL3*^{+/-} and *ApoE*^{-/-}/*GaL3*^{-/-} mice. Statistically significant differences were found in the 36- to 44-week group in the *ApoE*^{-/-}/*GaL3*^{+/-} mice in the incidence of combined (A, star) and vascular channels (C, star) compared with the younger groups. No such age-related increase was seen in *ApoE*^{-/-}/*GaL3*^{-/-} mice.

This work has produced the following results. 1) The age-related increase in the incidence of aorta atherosclerotic lesions that occurred in *ApoE*-deficient mice was not observed in *ApoE*-*GaL3* double-knockout mice, a difference accounted mostly by a reduction in the development and progression of atheromatous plaques. 2) The lack of *GaL3* expression did not alter the development of aorta atherosclerotic microaneurysms but delayed the age-related progression of periaortic vascular channels. 3) In the absence of *GaL3* expression, the incidence of adventitial and periaortic inflammatory infiltrates and perivascular mast cell density was significantly lower.

These results have demonstrated that the absence of *GaL3* expression reduced the incidence and age-related progression of some of the pathological changes affecting the aorta and periaortic tissue in *ApoE*-deficient mice. The lower incidence of atheromatous plaques in the absence of *GaL3* that was strongly expressed in the foam cells of aorta atheromatous plaques of *ApoE*-deficient mice suggests that this multifunctional protein may play a role in the transformation of macrophages into foam cells.

Figure 6. Age-related distribution of the incidence of aortic and periaortic inflammatory infiltrates in *ApoE*^{-/-}/*Gal3*^{+/+} and *ApoE*^{-/-}/*Gal3*^{-/-} mice. The incidence of inflammatory infiltrates was significantly lower in the 25- to 31-week-old *ApoE*^{-/-}/*Gal3*^{-/-} mice compared with the *ApoE*^{-/-}/*Gal3*^{+/+} mice of same age (star).

The expression of GaL3 is highly up-regulated when monocytes differentiate into macrophages,²⁰ a step in atherogenesis that follows monocyte recruitment from the

peripheral blood to the artery wall²¹ and also when *in vivo* or *in vitro* macrophages¹⁴ or aortic smooth muscle cells²² were loaded with lipids and transformed into foam cells. Like the activated macrophages,²³ plaque foam cells may secrete GaL3, a potent chemoattractant for monocytes and macrophages,²⁴ and thus enhance the recruitment of these cells to the artery wall. GaL3 has been shown to induce chemotaxis of monocytes and macrophages at micromolar concentrations.²⁴ Because GaL3 is known to reach relatively high concentrations in the cytosol of many cell types,²⁵ of which a high percentage is secreted,²⁶ such local chemotactic concentrations might be reachable.

In addition to this potential role in macrophage recruitment, GaL3 may actually participate in lipid loading of artery wall macrophages, the basic mechanism of plaque formation in *ApoE*-deficient mice.²⁷ GaL3 was shown to mediate the endocytotic uptake of modified lipoproteins¹⁵ and to be a component of the AGE-receptor complex expressed in macrophages.¹⁶ The functional interaction of GaL3 (AGE-R3) with AGE-R1 and AGE-R2

Figure 7. **A:** Longitudinal cross section of aorta from an *ApoE*^{-/-}/*Gal3*^{+/+} mouse stained for mast cells showing a periaortic vascular channel (yellow star) with several darkly stained mast cells (red arrows) in its surrounding area. **B:** Longitudinal cross section through the aorta of an *ApoE*^{-/-}/*Gal3*^{+/+} mouse showing a cluster of mast cells (red arrows) along the adventitia next to intimal lipid deposition (yellow arrow). **C:** Longitudinal section through the aorta of an *ApoE*^{-/-}/*Gal3*^{-/-} mouse showing a periaortic vascular channel (yellow star) surrounded by very few mast cells. **D:** Longitudinal section through the aorta of an *ApoE*^{-/-}/*Gal3*^{-/-} mouse showing few isolated mast cells (red arrows) in the periaortic tissue in the vicinity of a complicated atheromatous plaque (yellow arrow) and periaortic vascular channels (yellow star). Scale bars: 50 μ m (A, B); 100 μ m (C, D).

receptors²⁸ suggested the possible interaction with AGE-binding proteins.²⁹ Thus a putative sequence of events may start with the migration of monocytes into the artery wall, triggering GaL3 up-regulation in these cells. GaL3 may function as a chemotactic factor increasing local monocyte migration and also as a receptor binding and internalizing oxidized lipoproteins to artery wall macrophages, thus contributing to the foam cell transformation. GaL3 chemotactic activity,²⁴ binding and internalization of AGE products,¹⁶ endocytotic uptake of modified lipoproteins resulting in intracellular cholesterol accumulation,¹⁵ and binding to lipopolysaccharides³⁰ could explain its multifunctional role in atherogenesis.

The finding of vascular channels embedded in the periaortic adipose tissue revealed a complication of the atherosclerotic lesions that can be detected only when longitudinal sections of aorta include the periaortic tissue. Taking into account that *ApoE*^{-/-} C3H/HeJ mice exhibit more destruction of the aorta elastic media with dilatation than *ApoE*^{-/-} C57BL/6 mice,³¹ differences between *ApoE*^{-/-}/*GaL3*^{+/+} and *ApoE*^{-/-}/*GaL3*^{-/-} mice might have been influenced by the different genetic backgrounds of these two strains of mice. However, it should be noted that the incidence of microaneurysms was similar in both strains, which may suggest that the absence of GaL3 expression has reduced the higher destructive activity conferred by the genetic background of *ApoE*^{-/-}/*GaL3*^{-/-} mice possibly related to an increased proteolytic activity.³¹ On the other hand, the development of periaortic vascular channels may depend on angiogenesis triggered by the interaction between microaneurysms and the periaortic adipose tissue. Fat tissue has been reported to induce neovascularization,³² and tissue lipids have been shown to possess angiogenic activity.³³ The reduction in the incidence of periaortic microvessels in *ApoE*-GaL3 double-knockout mice could be related to the role of GaL3 as an angiogenic factor,³⁴ possibly through its interaction with NG2, a transmembrane chondroitin sulfate proteoglycan that promotes neovascularization,³⁵ and with Sp1 transcription sites that modulate the expression of vascular endothelial growth factor in atherosclerotic lesions.³⁶⁻³⁸ The morphogenesis and pathogenic role of these microvessels and the role of GaL3 represent an important area of research because artery wall destruction and expansion into surrounding tissue has highly significant clinical consequences.

In the absence of GaL3 expression the incidence of adventitial and periaortic inflammatory infiltrates was significantly lower. GaL3 has been shown to have a proinflammatory role³⁹ contributing to the phagocytic function of macrophages,⁴⁰ control of T-cell receptor complex clustering,⁴¹ and the regulation of T-cell proliferation and death.^{42,43} The participation of GaL3 in the development of inflammatory changes is also suggested by its role as an intracellular mediator of cell survival and interleukin-4-induced B-cell commitment toward a memory phenotype.⁴⁴

Although the role of inflammation in atherogenesis has been widely recognized, the mechanisms involved in this process and the role played by GaL3 is still unclear. The lower incidence of adventitial and periaortic inflammatory

infiltrates may be the result of a process similar to the reduction of peritoneal inflammation in GaL3-null mutant mice.^{45,46} The decrease in the incidence of inflammatory infiltrates seen in the middle age group preceding the decrease in atherosclerotic lesions, plaques, and perivascular channels in the older group suggests a potential pathogenic mechanism. In this respect the significantly lower number of periaortic mast cells in the GaL3-deficient mice compared to *ApoE*-deficient mice may point to one of the mechanisms through which GaL3 intervenes in the development of these lesions. GaL3 is expressed by mast cells^{47,48} and under appropriate conditions has the potential to activate mast cells culminating in augmentation of an inflammatory response.⁴⁹ GaL3-deficient mice exhibited reduced passive cutaneous anaphylaxis and on activation by FcεRI cross-linkage, their bone marrow-derived mast cells secreted a significantly lower amount of histamine and interleukin-4, compared with GaL3^{+/+} bone marrow-derived mast cells.⁵⁰ Because interleukin-4 has been detected in atherosclerotic lesions in mice with pronounced hypercholesterolemia⁵¹ and its deficiency has reduced the development of aorta atherosclerotic lesions in low-density lipoprotein receptor-deficient mice fed a high-fat diet,⁵² the lower incidence of atherosclerotic lesions found in *ApoE*/GaL3-deficient mice could be explained by a lower mast cell number and/or activity resulting in a low level of interleukin-4. Greater numbers of mast cells were associated with atherosclerotic plaques⁵³ and carotid artery adventitia in a balloon injury model.⁵⁴ The importance of perivascular mast cell activation in plaque progression and destabilization has been demonstrated in *ApoE*-deficient mice.⁵⁵ Our study has shown the extent of mast cell involvement in the perivascular inflammatory response, and this mechanism may have significant clinical consequences.

The morphology of the inflammatory changes found in human atherosclerotic lesions⁵⁶ and *ApoE*-deficient mice aorta⁵⁷ suggested that immune mechanisms might be implicated in atherogenesis. Changes in the immune response in GaL3-null mutant mice infected with *Toxoplasma gondii*⁵⁸ indicated that GaL3 plays a regulatory role in both the adaptive and innate immune response to pathogens. Therefore the reduction in the incidence of inflammatory infiltrates in *ApoE*^{-/-}/*GaL3*^{-/-} mice may reflect the role of GaL3 as an immune modulator in the innate and adaptive mechanisms that appear to be involved in atherogenesis⁵⁹ and also as a proinflammatory mediator. Further studies are necessary to investigate these potential mechanisms.

GaL3 deficiency was found associated with significant reductions in the development and rate of progression of some types of aortic and periaortic atherosclerotic lesions in *ApoE*-deficient mice, in particular the atheromatous plaques and the adventitial and periaortic inflammatory infiltrates. These results may be explained by a multifunctional role that GaL3 could play in different steps of atherogenesis. Therapeutic reduction in the expression of GaL3 may provide a new approach in controlling the development and progression of atherosclerotic lesions and their complications.

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