

Alterations in the vascular extracellular matrix of granulocyte macrophage colony-stimulating factor (GM-CSF) -deficient mice

GABRIELE PLENZ,^{*,†,‡,1} HEIKE ESCHERT,^{*,†} STEFAN BEISSERT,[§] VOLKER ARPS,^{*} JÜRGEN R. SINDERMANN,^{*,†} HORST ROBENEK,^{*} AND WOLFGANG VÖLKER^{*}

^{*}Institute for Arteriosclerosis Research, 48149 Münster, Germany; [†]Department of Cardiology and Angiology, [‡]Department of Thoracic and Cardiovascular Surgery, University Hospital Münster, 48149 Münster, Germany; and [§]Department of Dermatology, University Hospital Münster, 48149 Münster, Germany

ABSTRACT GM-CSF takes part in the cytokine network regulating the metabolism of extracellular matrix (ECM) during atherogenesis. Since data also point to an effect of GM-CSF on the vascular ECM in general, the vascular collagenous matrix was studied in wild-type and GM-CSF-deficient mice. Histological examination revealed a disorganized vascular ECM in GM-CSF-deficient mice involving the collagenous matrix and elastic fiber system. As shown by electron microscopy, collagen bundles were disrupted and reduced. The diameter of fibrils varied widely. mRNA expression of collagens and related molecules was studied. Fibrillar collagens were markedly reduced, $\alpha 1(I)$ procollagen to 16.5% of control levels $\alpha 1(III)$ procollagen was abolished whereas the expression level of network-forming $\alpha 1(VIII)$ procollagen was not altered. As shown by *in situ* hybridization, the number of collagen-expressing cells was reduced. Matrix metalloproteinases and their inhibitor 1 were not affected by GM-CSF deficiency. Our studies demonstrate that GM-CSF plays a major role in the cytokine network regulating the metabolism of vascular collagens. GM-CSF deficiency leads to an altered composition of the vascular collagenous matrix, i.e., reduced amount of fibrillar collagen, altered ratio of fibrillar and network-forming collagen, and failures in the fibrillogenesis. We suggest that GM-CSF is a basic requirement for the maintenance of vessel wall integrity and resilience.—Plenz, G., Eschert, H., Beissert, S., Arps, V., Sindermann, J. R., Robenek, H., Völker, W. Alterations in the vascular extracellular matrix of granulocyte macrophage colony-stimulating factor (GM-CSF) -deficient mice. *FASEB J.* 17, 1451–1457 (2003)

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GRANULOCYTE MACROPHAGE colony-stimulating factor (GM-CSF) is a 23 kDa glycoprotein cytokine first characterized for its ability to stimulate progenitor hemopoietic cells to proliferate and differentiate into mature granulocytes and macrophages (1). Subsequently it has been shown to have multiple effects in immune activa-

tion and to affect mitogenesis (2, 3), acting in concert with other members of the CSF family as a key mediator in inflammation and host defense (4, 5). Mice carrying a null allele of GM-CSF show regular steady-state hematopoiesis and develop the lung pathology characteristic of alveolar proteinosis (6, 7).

Evidence implicating GM-CSF as a critical player in the processes of atherogenesis has been steadily mounting. GM-CSF is reported to lower plasma cholesterol levels in humans and animals (8, 9) and to enhance uptake and degradation of acetylated low density lipoprotein *in vitro* (10). Vascular endothelial cells (ECs), smooth muscle cells (SMCs), fibroblasts, and monocytes have the capacity to express GM-CSF *in vitro* (11–14). In atherosclerotic lesions, GM-CSF has been immunolocalized to ECs, SMCs, and macrophages (15–17). Characteristic expression and distribution patterns of GM-CSF arise during the development of atherosclerotic lesions in human coronary arteries (15). Apparent similarities in the distribution patterns of GM-CSF and type VIII collagen, a nonfibrillar network-forming collagen, have been observed (16). GM-CSF transiently stimulates the transcription of type VIII collagen by vascular SMCs *in vitro* (16). Studies of GM-CSF-treated Watanabe rabbits provided evidence that GM-CSF takes part in regulating the metabolism of the vascular collagenous extracellular matrix (ECM) (18). Taken together, these findings point to an important role of GM-CSF in the cytokine network regulating the metabolism of the vascular ECM. Nevertheless, in the absence of any direct data on the relation between GM-CSF or GM-CSF deficiency and the vascular ECM, it has not been possible to assess the significance of this cytokine on the metabolism of vascular ECM. Therefore, the aim of this study was to demonstrate the effect of GM-CSF deficiency on the vascular ECM. It represents a histological, ultrastructural and molecular assessment of the

¹Correspondence: Institute for Arteriosclerosis Research, Domagkstr. 3, D-48149 Münster, Germany. E-mail: plenz@uni-muenster.de

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vascular collagenous matrix, i.e., types I, III, and VIII collagen and related molecules in the aorta of GM-CSF-deficient mice. Our data clearly demonstrate that GM-CSF deficiency leads to alterations in the transcription of vascular collagens and, as a result, to alterations in the composition and structural organization of the vascular ECM.

MATERIALS AND METHODS

Mice

Female GM-CSF-deficient mice (GM^{-/-}) in BALB/c background and age- and sex-matched controls (BALB/c mice, 12 month) were used (6). All animals were housed under Institutional Animal Care and Use Committee-approved protocols in the animal facility of the Medical Department of the University of Münster.

Tissue preparation, histological staining, and electron microscopy

Mice were killed by CO₂ overdose. The heart and aorta were removed. The aortic sinus area was embedded in cryoprotective (Shandon, Germany) and stored at -70°C. Serial sections (10 μm) were cut and analyzed for collagen and elastin. Total collagenous matrix and the elastic fiber system were demonstrated by trichrome staining and elastica van Gieson staining according to standard laboratory procedures. The collagenous matrix was quantified by computer-based morphometric analysis. Collagen content was determined by measuring the relative area in 10 contiguous fields in each section (n=10).

Small segments of the aorta (adjacent to the sinus area) were dissected, washed in cold phosphate-buffered saline, and fixed in a mixture of formaldehyde (1%) and glutaraldehyde (0.1%). Staining and further fixation were performed with OsO₄ (1%). The specimens were dehydrated in a graded series of ethanol and embedded in epoxy resin. Ultrathin sections were stained by conventional means with uranyl and lead salt solutions. High magnification electron micrographs were used to measure and calculate the diameter of collagen fibrils in vascular tissues of wild-type mice and GM-CSF-deficient mice. Means and standard deviations were calculated and significant differences estimated with Student's *t* test. The remaining part of the aorta was used to isolate total RNA.

Probes, labeling procedure, RNA analysis, and in situ hybridization

For hybridization, the following recombinant cDNA clones were used: pHGM-CSF containing an insert complementary to the human GM-CSF mRNA, pH677 complementary to procollagen α1(I) mRNA, pH37 complementary to procollagen α1(III) mRNA, pBSIIa1Col8 complementary to human procollagen α1(VIII), PA5/2 complementary to human MMP1, phgelA complementary to human MMP2, phgelB complementary to human MMP9, phimp1 complementary to human TIMP1 (19), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Clontech, Heidelberg, Germany) complementary to human GAPDH mRNA. In vitro transcription was performed according to the manufacturer's protocol using digoxigenin-labeled UTP (Roche, Mannheim, Germany).

Isolation of RNA and Northern blot analysis were performed as described (16). mRNAs of ECM molecules below the sensitivity of Northern blot analysis and GAPDH were assessed by RT-PCR. One microgram total RNA was reverse transcribed using Superscript II according to the manufacturer's instructions (Life Technologies GmbH, Heidelberg, Germany). The RT products (2 μL) were brought to a volume of 100 μL containing 1.5 mM MgCl₂, 0.1 mM of each dNTP, 1 × buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), and 2.5 U of Taq Polymerase (Life Technologies GmbH). **Table 1** shows the sequences of the primers set up in the PCRs and the product sizes. For RT-PCR 0.5 μM of each primer was used. Amplification was carried out in a Biometra UNO thermocycler (Biometra, Göttingen, Germany) after an initial denaturation at 95°C for 10 min using the following profile: denaturation at 95°C for 60 s; primer annealing at the indicated temperatures for 70 s; primer extension at 72°C for 60 s; and a final extension at 72°C for 10 min. Aliquots of the PCR reaction products were analyzed using standard agarose gel electrophoresis. To evaluate relative expression, the agarose gels or luminographs on X-ray film were scanned using a laser densitometer (Personal Densitometer, Molecular Dynamics, Dossenheim, Germany). Absorption units were corrected for GAPDH mRNA levels.

Using in situ hybridization experiments (16), the number and distribution pattern of expressing cells were characterized. The number of the respective mRNA-expressing cells was evaluated microscopically by counting all cells per section (ECs, adventitial cells) or in a microscopic area (×400; SMCs). The calculated number of mRNA-expressing cells was related to the total number of cells (expressed in %). Data are expressed as the means ± SD and significant differences were estimated with Student's *t* test.

TABLE 1. RT-PCR primer and PCR characteristics

Product	Size (bp)	Annealing-T (°C)	Cycles	Direction	Sequence 5'-3'
MMP1	149	59	39	Left	CTG GGA GCA AAC ACA TCT GA
				Right	CTG CTT GAC CCT CAG AGA CC
MMP3	154	60	39	Left	CAT CAC CAA TGT GCA GCT CT
				Right	GGA AGA GAT GGC CAA AAT GA
MMP9	150	58	39	Left	TCG CGT GGA TAA GGA GTT CT
				Right	ACC TGG TTC ACC TCA TGG TC
MMP12	149	60	36	Left	CTG GGC TTC TCT GCA TCT GT
				Right	GTG GAA ATC AGC TTG GGG TA
TIMP1	302	54	39	Left	CCA GCG TTA TGA GAT CAA GAT G
				Right	GGA TGG ATA AAC AGG GAA ACA C

^a MMP: matrix metalloproteinase; TIMP: tissue inhibitor of MMP.

RESULTS

Histological and ultrastructural characterization of collagen and related structures in the aorta of GM-CSF-deficient and wild-type mice

Trichrome and elastica van Gieson staining revealed differences in the vascular collagenous matrix and the elastic fiber system between wild-type and GM-CSF-deficient mice. In the aorta of wild-type mice, the medial collagenous matrix homogeneously lined the elastic fibers. Depositions of collagen were found in the adventitia. The elastic compartment of the media comprised several homogeneously thick layers of elastin. The aortas of the GM-CSF-deficient mice stained poorly and diffusely for the collagenous matrix and elastic fiber system as shown by light microscopy (Fig. 1). The characteristic arrangement of layers with bundles of collagen lining the elastic fibers was disturbed. In general, the amount of collagen was reduced and some areas were completely devoid of collagenous matrix (Fig. 1A, B). As demonstrated by computer-based morphometry, the area of the collagenous component was reduced by 44% in GM-CSF-deficient mice compared with wild-type mice (area in total: $21.9 \pm 7.3 \mu\text{m}^2$ vs. $39.3 \pm 10.0 \mu\text{m}^2$, $P < 0.005$). The thickness of the remaining elastic fibers strongly varied, indicating pro-

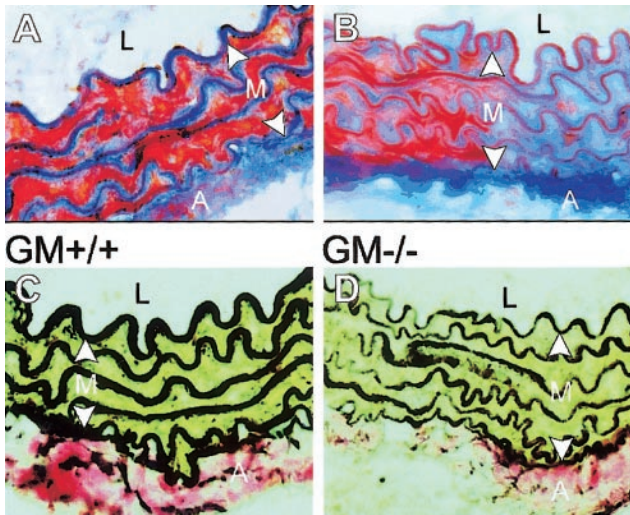


Figure 1. Histological features of the vascular matrix of wild-type and GM-CSF-deficient mice. A, B) Trichrome staining showing the collagenous matrix (blue). C, D) Elastica van Gieson staining demonstrating the elastin fiber system (black). A) In the aorta of wild-type mice (GM+/+), the medial collagenous matrix homogeneously lined the elastic fibers. Depositions of collagen appear in the adventitia. B) Aortas of the GM-CSF-deficient mice (GM-/-) stained poorly and diffusely for the collagenous matrix. The collagenous compartment is reduced and some areas are completely devoid of collagenous matrix. C) In the aorta of wild-type mice, several homogeneously thick layers of elastin are observed. D) In GM-CSF-deficient mice the thickness of the remaining elastic fibers varies. Ruptures in the internal and external elastic laminae are occasionally found. Original magnification: $\times 200$.

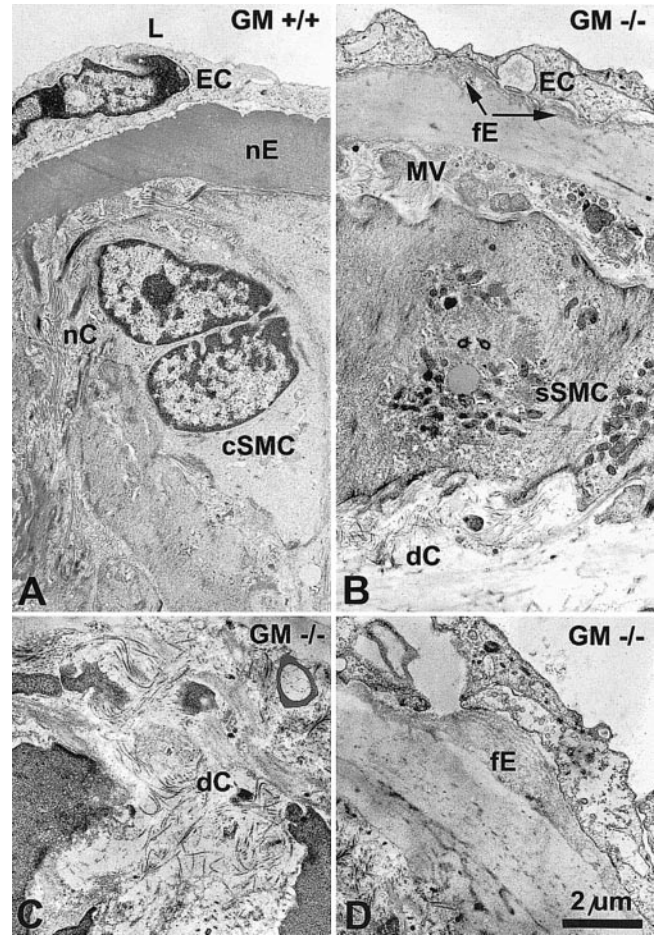


Figure 2. Ultrastructural alterations in the aorta of GM-CSF-deficient mice (GM-/-) compared with wild-type mice (GM+/+). Overview demonstrating the ultrastructural features of elastin, extracellular matrix and cells in wild-type (A) and GM-CSF-deficient mice (B). C) In GM-CSF-deficient mice the intimal space outlining the elastica interna appears to be widened and is filled with fibrous material proliferating from the amorphous elastin. D) Elastic layers in the space between medial SMCs appear swollen and fuzzy. The SMCs of GM-CSF-deficient mice show the typical synthetic phenotype. Collagen bundles are loosely arranged and even disintegrated. Bar: $2 \mu\text{m}$.

cesses of degradation and/or insufficient fiber formation (Fig. 1C, D). Ruptures mainly in the internal and external elastic laminae occasionally were observed.

To evaluate the morphologic differences between wild-type and GM-CSF-deficient mice in more detail, electron microscopic studies were performed. Differences were seen in the elastic layers between the two groups of animals (Fig. 2A, B). In GM-CSF-deficient mice the intimal space outlining the elastica interna appeared widened and filled with fibrous material proliferating from the amorphous elastin. Elastic layers in the space between medial SMCs appeared swollen and fuzzy. Osmium staining of elastin was less intense than in normal arteries.

Most striking were the alterations found in the collagenous matrix. Collagen bundles appeared to be loosely arranged and even disintegrated. Cell fragments

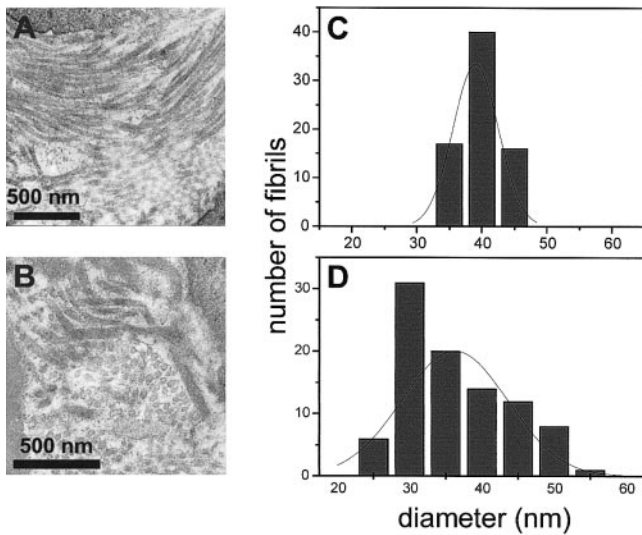


Figure 3. Lack of GM-CSF affects the fibrillogenesis of collagens. Bundles of collagen fibers in the media of the aorta of wild-type mice (A) and GM-CSF-deficient mice (B). Bars: 0.5 μ m. Compared with wild-type mice (C), the average thickness of collagen fibrils is reduced and their diameter varies widely in GM-CSF-deficient mice (D).

were associated with an irregularly organized collagen network (Fig. 2A–D). **Figure 3** demonstrates the effect of GM-CSF deficiency on the medial collagen fiber system in more detail. In addition to the obvious alterations in the structural organization of the collagen fiber bundles, electron microscopy revealed alterations on the collagen fiber level (Fig. 3A, B). Compared with the collagen fibers of wild-type mice, the average thickness of collagen fibrils of GM-CSF-deficient mice was reduced (GM $^{-/-}$: 36.25 \pm 7.33 nm; WT: 39.03 nm; $P < 0.005$). The diameter of the fibrils varied more widely (GM $^{-/-}$: 23–57 nm; WT: 32–46 nm) in GM-CSF-deficient mice (Fig. 3C, D). The SMCs of GM-CSF-deficient mice showed the typical synthetic phenotype featuring numerous intracellular vesicles and mitochondria.

Expression of collagens and related molecules in the aorta of GM-CSF-deficient mice

To evaluate the effect of GM-CSF deficiency on the expression of vascular collagens, *in vitro* RNA analyses and *in situ* hybridization for the most prominent vascular collagens, i.e., fibrillar α 1(I) and α 1(III) procollagen and network forming α 1(VIII) procollagen, were performed. We looked for the expression of molecules related to the catabolism of collagens such as collagenase (MMP1), gelatinase A and B (MMP2 and 9), elastases (MMP3 and 12), and TIMP1. Northern blot analyses demonstrated a strong expression of fibrillar and network-forming collagen and MMP2 mRNAs in the aorta of wild-type mice. In comparison, in the aorta of GM-CSF-deficient mice expression of fibrillar collagens was strongly reduced (CI: 0.19 \pm 0.02 vs. 1.15 \pm 0.17; CIII: not detectable vs. 0.72 \pm 0.24). The relative expres-

sion of procollagen α 1(I) was reduced to \sim 16.5% of the wild-type level ($P < 0.001$), whereas the expression of procollagen α 1(III) mRNA was completely abolished ($P < 0.001$). The level of α 1(VIII) procollagen mRNA was slightly but not significantly decreased (CVIII: 0.65 \pm 0.19 vs. 0.73 \pm 0.11). Expression of MMP2 was not affected by the lack of GM-CSF.

By using Northern blot analyses, MMP1, 3, 9, and 12 and TIMP1 mRNAs were not detected in the aortas of wild-type mice and GM-CSF-deficient mice. However, RT-PCR revealed expression of MMP3, MMP9, MMP12, and TIMP1. Differences in the mRNA levels of GM-CSF-deficient and wild-type mice were not found. Despite enhanced sensitivity of RT-PCR amplification, MMP1 was not detectable.

To demonstrate the distribution patterns of collagen-expressing cell types *in situ*, hybridization was performed for the α 1 procollagen mRNAs of types I, III, and VIII collagen (**Table 2** and **Fig. 4**). In wild-type mice all three procollagens were expressed by medial SMCs and adventitial cells. Some type III and VIII collagen mRNA-expressing cells were also found in the endothelium. In the aorta of GM-CSF-deficient mice the number of α 1(I) procollagen mRNA-expressing cells markedly decreased in the media (4.4-fold) and adventitia (15-fold); cells expressing α 1(III) procollagen mRNA were not found. Whereas the number of type VIII collagen mRNA-expressing cells slightly increased in the media and endothelium, type VIII collagen-expressing cells were reduced in the adventitia (2.3-fold).

DISCUSSION

To demonstrate the influence of GM-CSF on the structural organization and metabolism of the vascular ECM, the present study evaluated the expression of collagens and related molecules in the aorta of GM-CSF-deficient and wild-type mice. Our results show that GM-CSF deficiency leads to disturbances in the arrangement of the vascular ECM and is associated with alterations in the transcription of collagens.

TABLE 2. Types I, III, and VIII collagen-expressing vascular cell types in the aorta of wild-type (GM $^{+/+}$) and GM-CSF-deficient (GM $^{-/-}$) mice as demonstrated by *in situ* hybridization

	CI mean \pm SD	CIII mean \pm SD	CVIII mean \pm SD
GM $^{+/+}$			
EC	0	4.13 \pm 4.16	16.5 \pm 8.8
SMC	54.63 \pm 12.48	21.5 \pm 1.48	22.00 \pm 3.89
adC	99.25 \pm 11.75	93 \pm 18.73	74.38 \pm 24.27
GM $^{-/-}$			
EC	0	0**	20.89 \pm 8.85
SMC	12.38 \pm 8.09*	0*	26.25 \pm 7.67
adC	6.63 \pm 7.63*	0*	34.00 \pm 33.52**

* CI, CIII, CVIII: procollagen α 1 mRNAs; EC: endothelial cells; SMC: smooth muscle cells; adC: adventitial cells. * $P < 0.0001$; ** $P < 0.01$.

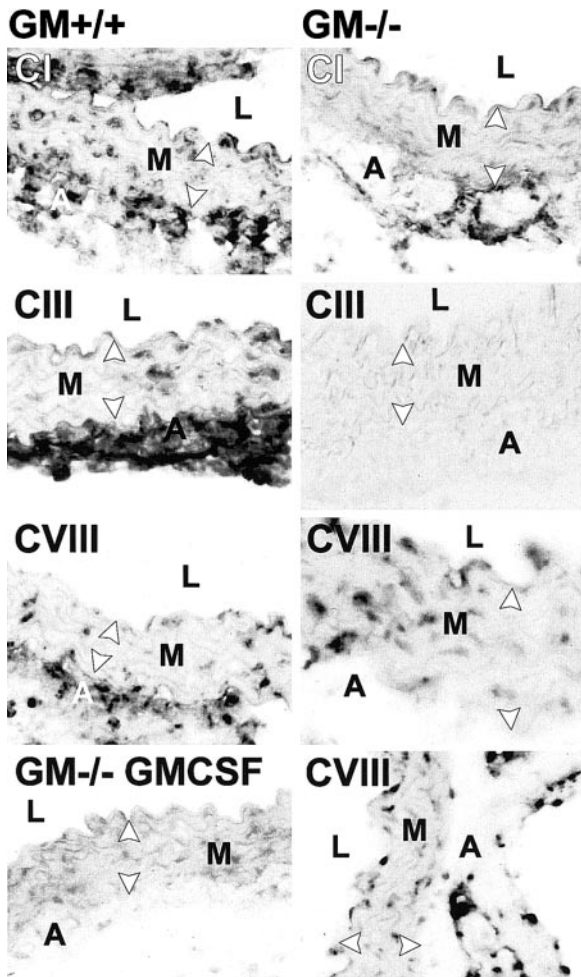


Figure 4. In situ hybridization demonstrating the distribution of collagen mRNA-expressing cells types in the aorta of wild-type (GM^{+/+}) and GM-CSF-deficient mice (GM^{-/-}). (GM^{+/+}): In wild-type mice procollagen $\alpha 1$ (I) mRNA (CI), procollagen $\alpha 1$ (III) mRNA (CIII), and procollagen $\alpha 1$ (VIII) mRNA (CVIII) are expressed by medial SMC and adventitial fibroblasts. CIII and CVIII are also expressed by some endothelial cells. (GM^{-/-}): In the aorta of GM-CSF-deficient mice the number of CI-expressing cells is markedly reduced in the tunica media and adventitia. CIII-expressing cells were not detected. The number of CVIII-expressing cells is increased in the media and reduced in the adventitia. To evaluate for unspecific background, a control hybridization was performed with GM-CSF sense probes on GM^{-/-} aorta. Nonspecific background was not observed. Original magnification: $\times 200$.

The present study demonstrates for the first time that the lack of GM-CSF leads to alterations in the vascular collagenous matrix, i.e., reduced amounts, irregularly distributed collagen fibrils, and failures in the assembly of collagen fibrils. These alterations were accompanied by degraded and in large areas rather amorphous vascular ECM. To elucidate the molecular mechanisms underlying the structural alterations observed in the vascular ECM of GM-CSF-deficient mice, we evaluated the transcription of collagens (types I, III, and VIII) (20, 21) and of molecules involved in the catabolism of collagens (MMPs and TIMP1) (22, 23). GM-CSF defi-

ciency led to a marked down-regulation of the transcription of type I collagen in SMCs and adventitial fibroblasts, whereas the expression of type III collagen was abolished. The collagen fibril is mainly composed of the collagen types I and III (24–26) and its supramolecular structure depends on the ratio between these different collagens, particularly on the content of type III collagen (27, 28). Thus, the lack of type III collagen might explain the disorganized structure of the collagen fibers, i.e., resulting in failures in the fibrillogenesis or errors in the assembly of collagen fibers and bundles. Compared with the fibrillar collagens, the average mRNA level of the nonfibrillar network-forming type VIII collagen was not significantly affected. Nevertheless, alterations in the cellular expression pattern were observed. Whereas the expression of type VIII collagen by SMCs was not altered, the number of type VIII collagen-expressing adventitial fibroblasts was markedly decreased. As previously shown, GM-CSF modulates the expression of type VIII collagen mRNA in SMCs, endothelial cells, and macrophages in vitro (16). Taken together, our observations indicate a differential influence of GM-CSF on fibrillar collagens and network-forming type VIII collagen in SMCs and fibroblasts and suggest that a certain level of GM-CSF is necessary to maintain regular synthesis (composition and amount) of collagens in the vessel wall.

Only a few studies deal with the relation between GM-CSF and the ECM. Although nothing has been reported yet about the consequences of GM-CSF deficiency, effects of elevated GM-CSF levels on both the anabolism and catabolism of collagen have been discussed. Gingival fibroblasts derived from patients with denture fibromatosis showed simultaneously enhanced synthesis of collagen and increased levels of GM-CSF (29). Enhanced hydroxyproline levels in response to GM-CSF, indicating stimulated synthesis of collagen and post-translational modification of procollagens, have been demonstrated for wound healing in diabetic rats (30). However, treatment with GM-CSF led to reduced deposition of collagenous matrix in atherosclerotic plaques of Watanabe rabbits (18) and prevented bleomycin-induced synthesis of collagen in lung fibroblasts (31). The reduced deposition of collagen has been discussed as an indirect effect, potentially mediated through the differential influence of GM-CSF on the synthesis of collagens by SMCs and the secretion of collagen-degrading enzymes by macrophages. Compared with the effect of increased GM-CSF levels, the lack of GM-CSF resulted in decreased expression of collagen in vascular SMCs and fibroblasts. Combining the results of the present study and those of others, one can assume that GM-CSF is an important player in cytokine network regulating the synthesis of collagens.

So far, our findings about connective tissue abnormalities in GM-CSF-deficient mice can only be interpreted as indicators of a weakened stability of the arterial wall. Although no data related to GM-CSF are known yet, there might be some evidence for vascular diseases. Recent observations in humans suffering from

spontaneous cervical artery dissection associated with bleeding into the carotid vessel wall and stroke revealed structural defects in the elastic and collagenous fibers (32). Such observations elucidate the necessity of the integrity of the ECM for vascular stability and resilience.

Taking into account that GM-CSF stimulates the expression and release of MMPs by fibroblasts and macrophages (33, 34), mechanisms related to the catabolism of collagens could be held responsible for the vascular phenotype of GM-CSF-deficient mice. Our data clearly demonstrate that transcription of collagen-degrading enzymes was not altered in the aorta of GM-CSF-deficient mice compared with wild-type mice. The expression of TIMP1 was not affected by the lack of GM-CSF. Nevertheless, minor effects at the post-translational level cannot be excluded. Thus, our molecular data demonstrate alterations in the anabolism of collagens and point to a regular catabolism of collagens in the vasculature of GM-CSF-deficient mice.

In conclusion, the present study suggests that reduced anabolism and regular catabolism of collagens both contribute to the structural changes observed in the vascular collagenous matrix of GM-CSF-deficient mice. On the molecular level, these changes lead to an altered composition of the vascular collagenous matrix, i.e., reduced fibrillar collagen, altered ratio of fibrillar and network-forming collagen, and failures in fibrillogenesis. One might speculate that basal levels of GM-CSF constitutively expressed by SMCs and adventitial fibroblasts might be a basic requirement for the maintenance of a regular anabolism of collagens in the vessel wall and therefore might be a prerequisite for the maintenance of vessel wall integrity and resilience. **FJ**

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