Induction of Macrophage Chemotaxis by Aortic Extracts of the mgR Marfan Mouse Model and a GxxPG-Containing Fibrillin-1 Fragment

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Background—The primary cause of early death in untreated Marfan syndrome (MFS) patients is aortic dilatation and dissection.

Methods and Results—We investigated whether ascending aortic samples from the fibrillin-1–underexpressing mgR mouse model for MFS or a recombinant fibrillin-1 fragment containing an elastin-binding protein (EBP) recognition sequence can act as chemotactic stimuli for macrophages. Both the aortic extracts from the mgR/mgR mice and the fibrillin-1 fragment significantly increased macrophage chemotaxis compared with extracts from wild-type mice or buffer controls. The chemotactic response was significantly diminished by pretreatment of macrophages with lactose or with the elastin-derived peptide VGVAPG and by pretreatment of samples with a monoclonal antibody directed against an EBP recognition sequence. Mutation of the EBP recognition sequence in the fibrillin-1 fragment also abolished the chemotactic response. These results indicate the involvement of EBP in mediating the effects. Additionally, investigation of macrophages in aortic specimens of MFS patients demonstrated macrophage infiltration in the tunica media.

Conclusions—Our findings demonstrate that aortic extracts from mgR/mgR mice can stimulate macrophage chemotaxis by interaction with EBP and show that a fibrillin-1 fragment possesses chemotactic stimulatory activity similar to that of elastin degradation peptides. They provide a plausible molecular mechanism for the inflammatory infiltrates observed in the mgR mouse model and suggest that inflammation may represent a component of the complex pathogenesis of MFS. (Circulation. 2006;114:1855-1862.)

Key Words: chemotaxis ■ elastin-binding proteins ■ fibrillin ■ Marfan syndrome

Marfan syndrome (MFS) is a dominantly inherited disorder with highly variable clinical manifestations, including aortic dilatation and dissection, ectopia lentis, and skeletal anomalies.1,2 The primary cause of death in untreated individuals with MFS is aortic dissection or rupture as a consequence of progressive dilatation of the aortic root. Mutations in the gene for fibrillin-1 (FBNI) cause MFS and a series of related disorders of connective tissue.3,4 Fibrillin was initially thought to be a purely structural protein in which mutations would exert a dominant negative effect by interfering with the aggregation of wild-type monomers into mature microfibrils. However, subsequent studies on fibrillin-1–underexpressing murine models have emphasized the predominant role of fibrillin-1 in tissue homeostasis.5,6 More recent results have come together to demonstrate that alterations in transforming growth factor (TGF)–β signaling play a major role in the pathogenesis of MFS and related disorders.7–10 In addition, FBNI mutations have been shown to increase the susceptibility of fibrillin-1 fragments to in vitro proteolysis,11–13 and fibrillin fragments can themselves upregulate the expression of matrix metalloproteinases (MMPs).14,15

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Inflammation and elastin fragmentation are prominent characteristics of abdominal aortic aneurysms (AAAs).16 Although fragmentation of elastic fibers is also found in MFS,17,18 the inflammatory reaction is much less intense. One previous study has shown small numbers of macrophages surrounding areas of cystic medial necrosis, with the macrophages and smooth muscle cells in these regions being immunopositive for several MMPs, including MMP-2 and MMP-9, that can degrade elastin.19
Inflammatory infiltrates are an important feature in the mgR MFS mouse model. At birth, the homozygous mgR animals are phenotypically indistinguishable from littermates. However, as early as 8 weeks, monocytes begin to infiltrate the medial layer, followed by adventitial inflammation with fragmentation of the medial elastic network and fibroblast hyperplasia, suggesting that inflammatory infiltration is relevant to the progression of aortic aneurysm in this MFS mouse model, although the biochemical events that trigger the accumulation of inflammatory cells in the vessel wall have been unclear.

Macrophages in foci of inflammation are derived from blood monocytes attracted by chemotactic factors produced in inflammatory foci. Approximately 50 human chemokines have been identified that contribute to monocyte recruitment in vascular and nonvascular diseases. However, chemotactic stimulatory activity is also associated with several components of the extracellular matrix, including collagen and elastin-derived peptides.

A repeated peptide in elastin, Val-Gly-Val-Ala-Pro-Gly (VGVAPG), is known for its chemotactic activity to fibroblasts and monocytes. This effect is mediated by binding of VGVAPG to the 67-kDa elastin binding protein (EBP) present on the surface of mononuclear phagocytes. Elastin-derived peptides released from human AAA tissue can attract mononuclear phagocytes through ligand-receptor reactions with the EBP, which provides a plausible explanation for the inflammatory response that accompanies the development of aneurysms in this disorder.

We hypothesized that the fragmentation of elastin and fibrillin-rich microfibrils characteristic of human MFS and seen in the mgR mouse model could expose otherwise cryptic EBP motifs that could be responsible for recruiting macrophages to the aorta of the mgR MFS mouse model and thus initiate an inflammatory reaction. In this work, we used a chemotaxis chamber system to show that aortic extracts from mutant mgR/mgR mice and a fibrillin-1 fragment containing an EBP binding sequence both significantly increase monocyte chemotaxis. These results demonstrate that abnormalities of the aorta in the mgR model can have deleterious secondary effects that could significantly contribute to the progression of disease.

**Methods**

**Preparation of Murine Aortic Extracts**

Heterozygous mgR fibrillin-1–underexpressing mice were generously provided by Dr Francesco Ramirez. Homozygous (mgR/mgR) mice and their wild-type siblings were included in the study. All animals were genotyped with polymerase chain reaction as described. At 2 and 4 months after birth, the mice were killed and the ascending aortas were prepared. Aortic tissue extracts were prepared using a modification of a previously described protocol. Briefly, immediately after dissection, tissue specimens were washed 2 times in phosphate-buffered saline (PBS) and weighed. Specimens were then incubated in PBS with 2 mol/L NaCl overnight at 4°C with gentle shaking (20 rpm per 1 g tissue). At the end of incubation, aortic tissue was removed, and the incubation solution was centrifuged at 10,000 rpm for 30 minutes to remove particulate debris. Aortic extract solution was stored at 4°C for experiments on the following day (or at –20°C for future repeats).

**Cell Culture**

Mouse macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection. These cells have properties similar to those of mouse resident macrophages and exhibit responsiveness to chemotactic stimuli. Macrophages were cultured in medium containing RPMI 1640 (Biochrom AG, Berlin, Germany), 10% FBS, and penicillin/streptomycin mix (final concentration: penicillin, 100 IU/mL; streptomycin, 100 μg/mL). For harvesting the cells, monolayers of RAW 264.7 cells were washed 2 times with PBS with a nonenzymatic cell dissociation solution (Sigma, St. Louis, Mo). After 25 minutes of incubation at room temperature, cells were suspended in PBS, counted, centrifuged, and finally suspended in the chemotaxis buffer consisting of RPMI 1640 supplemented with 1% bovine serum albumin (Sigma) at 1 × 10^6 cells/mL.

**Recombinant Fibrillin-1 Polypeptides**

Two recombinant polypeptides, rFib47wt and rFib47mt, were generated as described to correspond to amino acids 1 to 250. This region encompasses cbEGF-like motifs 29, 30, and 31 and cbEGF-like motifs 22 and 23. rFib47mt contains a putative EBP recognition motif EGF/EGFPEG at amino acids 2194 to 2199. In rFib47mt, the conserved proline residue at position 2198 is mutated to a serine residue (EGF/EGFPEG→EGF/ESFG), thereby abolishing the EBP consensus sequence.

**Chemotaxis Assay**

Chemotaxis was assayed in a 48-well chemotaxis chamber (Neuro-Probe). The bottom wells were filled with 25 μL of attractant solution. An 8-μm-pore-diameter polycarbonate-free polycarbonate filter (NeuroProbe, Gaithersburg, Md) was placed on the bottom plate. A silicon gasket and the top plate with 48 holes were then mounted, forming the top wells. The cells were added in a volume of 50 μL. In parallel experiments, extraction buffer (PBS plus 2 mol/L NaCl) or chemotaxis buffer was loaded into the bottom wells as a negative control. After 2 hours of incubation at 37°C and 5% CO2, the filter sheet was removed, and nonmigrated cells were wiped off the top side. The filter was then fixed in 70% methanol for 30 seconds and stained in Wright-Giemsa (Sigma). All assays were done in triplicate during the same experiment. In each well, 4 random fields were chosen with a microscope set at ×200 magnification. The fields were observed and stored with the Leica DC viewer program. Fields were scored by 2 observers blinded to the chemotactic stimulus (or control) using an in-house Java program.

**Experimental Design**

For each time interval, 3 or 4 wild-type and mutant mice were killed, and all aortic extracts were investigated at least 3 times. Polypeptide samples were diluted in chemotaxis buffer to a concentration of 100 μg/mL and were tested for their ability to act as chemotactic stimuli for macrophages. Checkerboard analysis was used to distinguish chemotaxis from chemokinesis. To investigate a potential interaction with EBP, cells were exposed to 1 mol/L lactose or glucose or 0.1 mol/L VGVAPG for 1 hour incubation at 37°C before the chemotaxis assays were started. As a further control, samples were preincubated for 30 minutes at room temperature with the mouse monoclonal antibody (mAb) BA4 derived from immunization against bovine α-elastin (Sigma) or with murine IgG (1 mg/mL; Sigma), both at a dilution of 1:1000.

**Immunohistochemistry of Human Aortic Specimens**

Human aortic segments were obtained from the surgical pathology records of the Johns Hopkins Hospital. Samples from 28 persons with MFS and from 4 control subjects (3 heart donors, 2, 9, and 24 years of age and 1 sample from a 30-year-old man who had died after a motor vehicle accident) were analyzed. Slides were cut from paraffin-embedded, formalin-fixed tissues. After deparaffinization, antigen retrieval was performed in a Tris buffer (pH 9.5) for 30 minutes. Slides were incubated with CD68 (KP-1) antibody (a macrophage marker) for 32 minutes at room temperature. Slides...
were then blocked from endogenous peroxidase for 4 minutes with inhibitor solution (Ventana Medical Systems Inc) before secondary antibody and diaminobenzidine incubations of 8 minutes, respectively. Slides were then counterstained with hematoxylin, and a coverslip was placed. All steps were performed on a Ventana BenchMark XT automated IHC stainer using standard reagents (Ventana Medical Systems Inc, Tucson, Ariz).

CD68^+ cells were counted only in the tunica media. CD68^− cells naturally occur in the underlying adventitia without relation to vascular disease and were not counted. CD68^+ cells along the intima were not included because atherosclerotic plaques or fatty streaks could potentially influence the cell counts.

**Statistical Analysis**

Data are expressed as mean±SE. The value for the mean macrophage chemotactic activity (number of migrated cells) of each group was compared using the Student’s t test. Statistical significance was accepted at P<0.05. The bar charts show mean and SEs.

The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

**Results**

**Aortic Extracts Induce Macrophage Migration**

We investigated whether aortic specimens from homozygous mutant mgR mice can act as a chemotactic stimulus for macrophages. The mgR mouse is a well-characterized mouse model for MFS.6 Two- and 4-month-old homozygous mgR/mgR mice were compared with age-matched wild-type (wt) mice. As previously described,6,31 aortic extracts of 2-month-old homozygous mutant mgR mice can act as a chemotactic stimulus for macrophages. The mgR mouse is a well-characterized mouse model for MFS.6 Two- and 4-month-old homozygous mgR/mgR mice were compared with age-matched mouse model for MFS.6 Two- and 4-month-old homozygous mutant mgR mice can act as a chemotactic stimulus for macrophages. The mgR mouse is a well-characterized mouse model for MFS.6 Two- and 4-month-old homozygous mutant mgR mice can act as a chemotactic stimulus for macrophages.

We hypothesized that the increase in chemotactic activity could be related to activation of the elastin-laminin receptor resulting from binding of elastin degradation products to the EBP. It has been previously described that extracts from surgical AAA specimens and elastin degradation products, including the hexapeptide VGVAPG, increase macrophage chemotaxis.25 We confirmed that the chemotaxis by RAW 264.7 cells is also stimulated by VGVAPG. Stimulation with 10^-5 mol/L VGVAPG led to an increase in migrated cells from 8.0±1.4 to 50.4±2.2 cells per field.

Incubation of macrophages in lactose causes them to shed the EBP, and this can be used to demonstrate specificity of a chemotactic stimulus on ligand-receptor interactions involving the EBP.25,29 We therefore repeated the experiments with preincubation of macrophages in lactose for 1 hour. This treatment significantly reduced chemotaxis in 2-month-old mutant mice (Figure 1B). A similar difference was observed for 4-month-old mice (data not shown). No inhibitory effect was observed after pretreatment with glucose (data not shown), as has been previously described for stimulation with AAA extracts.25

We performed checkerboard analysis28 to determine whether the macrophage migration was related to directed chemotaxis or undirected chemokinesis. Macrophages showed migration only in the presence of mutant aortic extracts in the lower well; adding mutant aortic extracts to the upper well greatly reduced the observed cell counts (see the Table). This result indicates that the observed cell migration is caused by chemotaxis rather than by (undirected) chemokinesis.

**A Recombinant Fibrillin-1 Fragment Containing a Putative EBP Binding Site Acts as a Chemotactic Stimulus**

We have recently shown that incubation with a recombinant fibrillin-1 fragment (rFib47wt) increases MMP-1 expression and production 7- to 9-fold; the effect was abrogated by...
introducing a mutation of the EBP recognition sequence (GxxPG→GxSG). We therefore investigated whether this fibrillin fragment could also affect macrophage chemotaxis. The fibrillin-1 fragment rFib47mt increased chemotaxis 4-fold compared with control (buffer) samples (Figure 2). The corresponding mutant construct was not significantly different from control (Figure 2A), indicating specificity of the reaction for EBP. Additionally, preincubating macrophages with lactose also reduced the chemotactic effects of the GxxPG polypeptide (Figure 2B). In a separate experiment, macrophages were pretreated with 0.1 mmol/L VGVAPG hexapeptide for 1 hour, and then the chemotaxis experiment was performed as above. Preincubation with VGVAPG blocked the chemotactic response, reducing the mean number of migrated cells from 10.8±2.0 to 1.7±0.3 cells per field. These results indicate that a fibrillin-1 fragment containing a putative EBP ligand GxxPG sequence can act as a chemotactic stimulus and that its effects are specific to the interaction with EBP.

BA4 Inhibits Chemotaxis to mgR/mgR Aortic Extracts or the GxxPG Fibrillin-1 Fragment

The monoclonal antibody BA4 was raised against bovine α1-elastin but has been shown to react against VGVAPG and several other xGxxPG hexapeptides that interact with EBP. Pretreatment of mgR/mgR aortic extracts from 4-month-old mice or rFib47wt with BA4 significantly reduced the chemotactic response (Figure 3).

Macrophage Infiltration Is Observed in Surgical Aortic Specimens of Marfan Patients

To investigate a potential role of macrophage infiltration and inflammation in human MFS, we have investigated surgical ascending aorta specimens from 28 persons with MFS who underwent aortic surgery and 4 control samples. Four MFS patients had identified FBN1 mutations; the remainder all met clinical diagnostic criteria for MFS. The presence of macrophages was assessed with immunostaining with an anti-CD68 antibody. Monocytes and macrophages are not typically seen in the intimal and medial layers of the arterial wall except in the setting of an inflammatory process such as atherosclerosis, vasculitis, or trauma. Monocytes and macrophages can be seen in the underlying adventitia of vessels in both MFS and control samples in a nonspecific fashion. A collection of macrophages was noted in the tunica intima of a single MFS patient, which may be related to the disease or likely to a fatty streak; otherwise, no CD68+ cells were noted in the tunica intima of any other sample.

Investigation of the 4 control samples confirmed this finding, showing an average of only 0.25±0.14 CD68+ cells per ×200 microscopic field. In contrast, the samples from all MFS patients showed an average of 3.7±0.6 CD68+ cells per field (mean±SE; range, 0 to 15). The difference was statistically significant. There was a tendency for samples from MFS patients >25 years of age to have more CD68+ cells that did not reach statistical significance. Figure 4 summarizes our results. Figure 5 presents a representative case study.

**Discussion**

In this work, we have demonstrated that aortic extracts of the mgR MFS mouse model induce chemotaxis and that this effect is mediated by EBP. Additionally, we have shown that a recombinant fibrillin-1 fragment containing a GxxPG EBP recognition sequence is able to induce chemotaxis in a comparable fashion. Macrophage infiltration and inflamma-
tion play a well-known role in AAAs but have not previously been investigated in MFS. In AAAs, soluble elastin-derived peptides released from AAA tissue act as chemoattractants for mononuclear phagocytes, providing a plausible explanation for the intense inflammatory response seen in that disorder. Macrophage-released proteases, including MMP-12, could be responsible for further destruction of elastin-rich tissues in the aorta.

To the best of our knowledge, no previous study has specifically focused on the role of macrophages in MFS, although the presence of macrophages was noted in surrounding areas of cystic medial necrosis in a study about changes in MMP immunohistochemistry in aortic specimens from MFS patients. In the present study, we have identified a statistically significant increase in the number of macrophages in the tunica media of 28 persons with MFS. We note that although histological analysis of patient specimens can provide us with important information, the results of such an analysis need to be interpreted with caution for 2 reasons. First, a surgical specimen is a snapshot taken years after the initiation of aortic disease, and it is not possible to know whether the initiating factors are still present or visible in the specimen. Multiple stages of aortic disease have been recognized in AAAs, each of which shows significant differences in parameters such as MMP-12 activity. Surgical specimens obtained during prophylactic or acute aortic root replacement in patients with MFS are likely to show the result of long-term aortic disease and will not necessarily reflect the initiating processes. Second, it is not possible to differentiate causative factors and epiphenomena on the basis of the appearance of a histological analysis. Nonetheless, we have presented definitive evidence that macrophage infiltration can be observed in aortic specimens of MFS patients and have presented the first specific demonstration of CD68+ macrophages in aortic specimens of MFS patients. Together with our experimental results, these findings support the notion that inflammation could play a role in the pathogenesis of MFS. We are planning further investigations to address the question of whether EBP ligands are present in increased concentrations in samples from MFS patients.

Although intense inflammatory changes are not routinely observed in patients with MFS, loss of elastin, disorganization of tissue architecture, and fragmentation of fibrillin-rich microfibrils are well-known characteristics of the disorder. Fragmentation and disarray of elastic fibers is a focal and acquired event that occurs in association with abnormal production of matrix-degrading enzymes by resident vascular smooth muscle cells and ultimately vessel wall inflammation in the mgR mouse model. Fragments of extracellular matrix may have signaling activities that the corresponding intact molecules do not have, so fragments may alter feedback regulation and contribute to progression of tissue damage.

It is therefore possible that early alterations in the extracellular matrix of the mgR mouse themselves have secondary deleterious effects. For instance, fragmentation of the aortic media could release soluble elastin degradation products or fibrillin-1 fragments that recruit macrophages into the aorta; if these macrophages release proteolytic enzymes that cause further fragmentation of tissues, a vicious cycle could ensue. A similar model has been
proposed for cigarette smoke–induced emphysema in a mouse model; cigarette smoke could induce macrophages to produce macrophage elastase, which cleaves elastic tissue, producing elastin-derived peptides that are chemotactant to monocytes. Thus, a positive feedback loop could perpetuate macrophage accumulation and lung destruction.38,39 Indeed, the paradigm of limited elastic fiber degradation progressing to intense elastolysis in association with inflammatory infiltration of the vessel wall and increased expression of MMPs is emerging as a common theme in the pathogenesis of abdominal aortic aneurysms.16,40,41

The EBP is 1 of the 3 protein subunits that make up the elastin-laminin receptor.42 This protein has previously been implicated in a variety of signaling processes mediated by binding to elastin-derived peptides, such as mechanotransduction,43 induction of MMP expression,44 and chemotaxis.44 We have demonstrated specificity for EBP of the chemotactic response toward mgR/mgR aortic extracts and rFib47wt by showing inhibition of the response by pretreatment of macrophages by lactose, which is known to cause shedding of the EBP from the cell surface,42 and by pretreatment with BA4 or VGVAPG. Additionally, in vitro mutation of the EBP consensus site in rFib47wt from EGFEPG to EGFESG severely inhibited the chemotactic effect. This mutation was introduced to demonstrate the specificity for the EBP and does not represent a mutation found in MFS.

Although elastin-derived fragments are presumably quantitatively more important than fibrillin in inducing chemotaxis in the mgR/mgR mouse, our finding that a GxxPG-carrying fibrillin-1 fragment can also induce chemotaxis shows that chemotactic effects are not limited to elastin. We have provided evidence that the effects of the fibrillin-1 fragment are mediated by interaction with EBP. The fact that the same fibrillin-1 fragment can both induce increased MMP-1 expression and act as a chemotactic stimulus is consistent with the finding that elastin-derived peptides have both properties.24,44 We note that the present experiments were not designed to differentiate between elastin- or fibrillin-derived EBP ligands in the mgR/mgR aortic extracts. Fragmentation of aortic tissue could potentially affect both elastin- or fibrillin-derived EBP ligands.

Conclusions

Our results highlight the possibility that the fragmentation of the ECM seen in the mgR MFS mouse model is itself an important contributory factor toward the initiation of the inflammatory reaction in the aortic wall. Although it is not possible to conclude that inflammation plays a role in initiation or progression of aortic dilatation in MFS, we have provided evidence that increased numbers of macrophages are found in surgical aortic specimens from MFS patients. This suggests the possibility that recruitment of inflammatory cells by elastin-derived peptides and fibrillin fragments may contribute to the complex pathogenesis of MFS. Because suppression of immune signaling by agents such as rapamycin45 and of MMP production by doxycyclin46 effectively prevents the occurrence of AAAs in animal models, future research along these lines could be fruitful with respect to the development of novel therapies for human MFS.

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Disclosures
None.

References
Marfan syndrome (MFS) is a common inherited disorder of connected tissue often associated with progressive dilatation of the ascending aorta, leading to acute aortic dissection. The molecular pathogenesis of MFS is complex, with alterations in transforming growth factor-β metabolism and a number of other factors thought to play some role in the initiation and progression of disease. In this work, we have shown that aortic extracts of the mgR mouse model for MFS elicit macrophage chemotaxis. The effects were inhibited by pretreatment with lactose or with a monoclonal antibody directed against an elastin-binding protein (EBP) interaction sequence in elastin, indicating involvement of EBP in mediating the effects. We have also shown that a recombinant fibrillin-1 fragment containing an EBP interaction sequence can elicit chemotaxis of macrophages by interaction with EBP. In a clinical study of aortic specimens from 28 persons with MFS, we demonstrate a significant increase in CD68+ macrophage counts in the tunica media compared with control subjects. Although this study was not designed to investigate the role of elastin or fibrillin-1 fragments in human MFS, our clinical and experimental findings support the notion that inflammation induced by elastin or fibrillin-1 fragments could play some role in the pathogenesis of MFS. This raises the possibility of novel therapeutic options, such as matrix metalloproteinase inhibitors to block fibrillin/elastin degradation or antiinflammatory agents to inhibit invasion of macrophages into aortic tissue.
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