Overexpression of Transforming Growth Factor-β Is Associated With Increased Hyaluronan Content and Impairment of Repair in Marfan Syndrome Aortic Aneurysm

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**Background**—Marfan syndrome (MFS), a condition caused by fibrillin-1 gene mutation is associated with aortic aneurysm that shows elastic lamellae disruption, accumulation of glycosaminoglycans, and vascular smooth muscle cell (VSMC) apoptosis with minimal inflammatory response. We examined aneurysm tissue and cultured cells for expression of transforming growth factor-beta1 to -beta3 (TGFβ1 to 3), hyaluronan content, apoptosis, markers of cell migration, and infiltration of vascular progenitor cells (CD34).

**Methods and Results**—MFS aortic aneurysm (6 males, 5 females; age 8 to 78 years) and normal aorta (5 males, 3 females; age 22 to 56 years) were used. Immunohistochemistry showed increased expression of TGFβ1 to 3, hyaluronan, and CD34-positive microcapillaries in MFS aneurysm compared with control. There was increased expression of TGFβ1 to 3 and hyaluronan in MFS cultured VSMCs, adventitial fibroblasts (AF), and skin fibroblasts (SF). Apoptosis was increased in MFS (VSMC: mean cell loss in MFS 29%, n of subjects = 5, versus control 8%, n = 3, P < 0.05; AF: 28%, n = 5 versus 7%, n = 5, P < 0.05; SF: 29%, n = 3 versus 4%, n = 3, not significant). In MFS, there was a 2-fold increase in adventitial microcapillaries containing CD34-positive cells compared with control tissue. Scratch wound assay showed absence of CD44, MT1-MMP, and β-3 integrin at the leading edge of migration in MFS indicating altered directional migration. Western blot showed increased expression of TGFβ1 to 3 in MFS but no change in expression of CD44, MT1-MMP, or β-3 integrin compared with controls.

**Conclusions**—There was overexpression of TGF-β in MFS associated with altered hyaluronan synthesis, increased apoptosis, impaired progenitor cell recruitment, and abnormal directional migration. These factors limit tissue repair and are likely to contribute to aneurysm development. (Circulation. 2006;114[suppl I]:I-371–I-377.)

Key Words: aneurysm ■ cell migration ■ circulating progenitor cells ■ hyaluronan ■ Marfan syndrome ■ transforming growth factor-β

Marfan syndrome (MFS) is an inherited connective tissue disorder involving multiple organ systems and results from a mutation in the fibrillin-1 gene. The most serious complication, often occurring in young adults, is the development of aortic aneurysm. Histopathological features of MFS aortic aneurysm include elastic lamellae disruption, excessive vascular smooth muscle cell (VSMC) apoptosis with minimal inflammatory cell infiltrate, cystic medial degeneration (CMD) associated with the accumulation of glycosaminoglycans, and increased expression of matrix metalloproteinases (MMP)-2 and MMP-9.

Recent studies in the fibrillin-1–deficient mouse model of MFS suggest that defective fibrillin-1 alters targeting and sequestration of latent transforming growth factor (TGF)-β, leading to increased TGF-β activation. TGF-β has been reported to regulate hyaluronan secretion by its action on hyaluronan synthase mRNA in cultured human skin fibroblasts (SF). Hyaluronan is a glycosaminoglycan widely distributed in the body and may be important in progression of vascular disease by regulation of extracellular matrix organization, cellular apoptosis, migration, differentiation, and tissue repair. Hyaluronan interacts with CD44 receptor and hyaluronan-mediated motility receptor to influence cell behavior and extracellular matrix homeostasis. Interaction between CD44 and membrane type 1 (MT1)-MMP leads to relocation of MT1-MMP to the leading edge of cell migration, directing cell movement. CD44 is known to partly mediate integrin signaling and activity. This interaction augments transendothelial migration of tumor cells and has been shown to play a critical role in inflammation.
absence of available CD44 caused by truncation, blocking, or high concentration of exogenous hyaluronan results in failure of CD44 to bind integrin and MT1-MMP and hinders cell migration and attachment.11 Transgenic mice expressing antisense CD44 cDNA display abnormal hyaluronan accumulation in superficial dermis and cornal stroma with decreased skin elasticity and impaired local inflammatory responses and tissue repair.11 The model shows striking similarities with MFS, where excessive skin stretch marks, lens dislocation, and absence of inflammatory cells in the developing aortic aneurysm are indicative of reduced tissue elasticity and impaired tissue repair responses. The level of hyaluronan in MFS aortic aneurysm tissue has never been reported. However, in MFS cultured SF a 12-fold increase in hyaluronan accumulation compared with control fibroblasts12 was caused by increased synthesis but normal rate of breakdown.13 Vascular injury stimulates recruitment of circulating and tissue progenitor cells (CPCs) into damaged tissue. CPCs have the potential to exhibit phenotypic plasticity and differentiate into monocytes or other cell types including VSMCs and endothelial cells and may be involved in the maintenance of normal vascular wall function.14,15 Inflammatory cell infiltration would be expected to follow at sites of angiogenesis associated with injury.16

In this report we show that in subjects with MFS, there is increased expression of TGF-β and increased tissue hyaluronan content. Altered cellular protein secretion associated with fibrillin gene mutation, activation of MMPs, VSMC apoptosis, and abnormal cell migration arising from these changes appear to be associated with muted inflammatory responses, limited angiogenesis, slow vascular wall repair processes, continuing tissue damage, and aortic wall degeneration.

Methods

Tissue Collection

Aortic aneurysm and skin samples were collected from MFS patients (6 males, 5 females; age 8 to 78 years) undergoing elective aortic aneurysm repair surgery. In 9 subjects aneurysm tissue was obtained from the aortic root and there was no dissection (aneurysm diameter 4.3 to 6.5 cm). One of these subjects had neonatal MFS. In 2 other subjects, tissue was obtained from the aortic arch and descending thoracic aorta, respectively. In these 2 subjects, although aortic dissection was present, tissue from the nondissected part of the aortic wall was sampled. The diagnosis of MFS was based on the revised diagnostic criteria of dePaep.17 Normal aortic root tissue and skin samples were collected from organ donors (5 males, 3 females; age 22 to 56 years) through the Queenslanders Donate organization. The study was approved by the Institutional Ethics Committee and subjects or relatives gave informed consent.

Specimens were incubated in Dulbecco’s modified Eagle media (DMEM) (Invitrogen, Calif) containing 10% fetal calf serum (FCS) (Invitrogen), and antibiotics (penicillin, streptomycin and fungizone; Invitrogen) and processed within 24 hours of collection.

Aortic Wall Histopathology

A piece of aorta was fixed in 10% buffered formalin for 24 hours, processed, embedded in paraffin, and cut into 5-μm cross-sections. Sections were dewaxed and rehydrated in ethanol and water. Serial sections then underwent hematoxylin and eosin staining and Alcian blue/Verhoef van Gieson staining for standard histochemical analysis.2 Separate sections were selected for specific immunohistochemistry study. Sections were dewaxed and incubated in 3% H2O2 for 5 minutes to block endogenous peroxidase reaction, washed, and incubated in 10% normal horse serum for 1 hour to block nonspecific binding. Biotinylated hyaluronan binding protein was used for hyaluronan identification (1:500; Seikagaku, Tokyo, Japan) and antibody against CD34 for vascular progenitor cell identification (1:250; HyCult Biotechnology, Uden, the Netherlands). All primary antibodies including CD44 (1:100; Neomarkers, Calif), MT1-MMP (1:200; Neomarkers), β-3 integrin (1:100; Abcam, Cambridge, UK); smooth muscle α-actin (1:500; Immunon, Pa); TGFβ1 to 3 (1:250; Santa Cruz, Calif), a gift from Dr A. Bobik, Melbourne, Australia) were applied and incubated overnight in a humid chamber at room temperature. The sections were then washed and secondary antibody applied (1:100) for 1 hour. After washing, ABC solution (1:100; Vector ABC kit, Calif) was applied for 1 hour followed by 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma, Mich) for 5 minutes. Sections were counterstained with hematoxylin, dehydrated in ethanol and xylene, and mounted in EUKITT (Lomb Scientific, New South Wales, Australia). Positive staining was identified as areas of dark brown color. For immunofluorescence FITC secondary antibody (Chemicon, Calif) was applied for 45 minutes at 37°C then mounted in antifade mounting media (Chemicon). A positive reaction was viewed as green. Negative controls were processed exactly the same but without the primary antibody.

VSMC and Fibroblast Cell Culture

For VSMC culture the tunica adventitia was separated from the tunica media under aseptic conditions in the cell culture hood after methods published previously.2 Cells from passage 1 to 4 were used. Briefly, pieces of tunica media were incubated in collagenase type I ( Worthington Biochemical Corporation, NJ; 5 μg/ml) at 37°C in a shaker water bath for 1 hour. Collagenase was removed and the tissue was incubated in elastase (1 μg/ml) for 30 minutes. Collagenase was added and incubated further until a single cell suspension was obtained. The cell suspension was centrifuged at 1000 rpm for 5 minutes. The pellet was plated on cell culture dishes and incubated in DMEM containing 10% FCS and antibiotics at 37°C with 5% CO2.

For adventitial and skin fibroblasts (AF and SF), the tunica adventitia and the dermis layer of the skin were cut into pieces ~1 mm3 and incubated in DMEM containing 10% FCS at 37°C with 5% CO2.

Cell Culture Study

VSMC, AF, and SF were plated on a cover-slip and grown to confluence. They were then fixed in methanol for 15 minutes at −20°C. For migration studies, confluent VSMCs were scratched with a sterile blunt wooden stick to produce a cell-free zone bordered by a straight wound edge. To remove debris, the media was replaced and the cells kept for 24 hours. Cell morphology at the leading edge of migration as well as localization and expression of proteins involved in directional migration (MT1-MMP, CD44, and β-3 integrin6) were assessed using immunohistochemistry as described and Western blot immunoelctrophoresis.

For quantitation of cell loss, triplicate samples of VSMC, AF, and SF were plated in 24-well plates at 105 cells/mL density in DMEM and 10% FCS for 24 hours. The media was removed and cells incubated in DMEM only for 24 hours. Cells were trypsinized and counted in a Coulter counter (Coulter Corp, Fla). The degree of VSMC loss in the absence of FCS was calculated.2

Western Blot

Cell lysates were collected from control and MFS cultures. Total protein were determined using the protein analysis kit (Pierce Biotech, Ill) and equal amounts (10 μg) were electrophoresed in 10% polyacrylamide/SDS gel and electroblotted onto a membrane (Bio-rad, Calif). Rabbit polyclonal antibodies against TGFβ1 to 3 and MT1-MMP and mouse monoclonal antibodies against CD44 and β-3 integrin were used (1:1000).
Quantitative Analysis
An image analysis system (Axiovision version 4.3; Carl Zeiss, Gottingen, Germany) was used to semi-quantitatively score (absent, mild, moderate, and strong staining were given scores of 0, 1, 2, and 3, respectively) areas of TGF\(\beta\)1 to 3 and hyaluronan immunostaining for each subject. Slides were blinded to identity and scored twice by each reviewer (M.N. and M.W.). The average number of microcapillaries in the tunica adventitia per field was determined at magnification of \(\times250\) in all samples.

Statistical Analysis
Data are presented as the mean±SD. N refers to the number of subjects used in a given analysis. The Mann-Whitney nonparametric test was used to compare MFS and control groups with \(P<0.05\) considered significant.

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

Results
Aortic Wall Histopathology
Focal areas of CMD, VSMC loss, and elastic lamellae degeneration in the absence of inflammation were observed in the tunica media of MFS aortic aneurysm (n=11) as previously reported.\(^2\) No CMD was observed in normal aorta (n=8). In MFS, moderate to strong TGF\(\beta\)1 to 3 staining was located in the outer tunica media and tunica adventitia (Figure 1B, 1D, 1F), whereas there was absent to mild staining in control tunica adventitia (Figure 1A, 1C, 1E; TGF\(\beta\)1: mean staining score 1.8±0.4, n=5 versus 0.1±0.5, n=3, \(P<0.05\); TGF\(\beta\)2: 2.0±0.4, n=5 versus 0.8±1.0, n=4, NS; TGF\(\beta\)3: 3.0±0.5, n=5 versus 1.0±1.0, n=2, not significant). In MFS there was strong hyaluronan staining in association with CMD, whereas in the tunica media of control aorta staining was mild (Figure 2A, 2B; MFS 3±0.4, n=5 versus control 1±0.4, n=5, \(P<0.05\)).
MFS compared with control, there was strong expression of CD34 and a 2-fold increase in the number of microcapillaries in the tunica adventitia (5.6±1.8, n=5 versus 2.3±1.5, n=3, P=0.04, per field, respectively), suggesting the presence of angiogenesis. Minimal tissue inflammatory infiltration was present. Microcapillaries in the tunica adventitia of control aorta were mildly stained with CD34 (Figure 3A and 3B). In MFS, outer tunica media VSMCs were positively stained with CD34, whereas in control VSMCs no CD34-positive cells were seen.

**Cell Culture Study**

There was strong intensity of TGFβ1 to 3 staining throughout the cellular cytoplasm in MFS VSMCs, whereas in controls staining was mild to moderate (Figure 4A, 4B; TGFβ1: MFS mean staining score 1.8±0.5, n=5 versus control 0.1±0.1, n=3, P=0.04; TGFβ2: 2.2±0.5, n=5 versus 0.4±0.5, n=3, P=0.04; TGFβ3: 2.6±0.6, n=5 versus 1.1±1.3, n=2, NS). MFS SF showed moderate to strong intensity of staining of TGFβ1, β2, and β3 in contrast to control, in which there was absent to mild staining (Figure 4C, 4D; TGFβ1: MFS mean staining score 1.3±0.6, n=3 versus control 0.1±0.1, n=2, NS; TGFβ2: 1.7±0.6, n=3 versus 0.1±0.1, n=2, not significant; TGFβ3: 2.7±0.6, n=3 versus 0.6±0.6, n=2, not significant). Distribution of staining of TGFβ1, β2, and β3 was similar. Increased expression of TGFβ1 to 3 in SF and AF in MFS was associated with the presence of strong SMα-actin staining (Figure 5). In MFS VSMCs hyaluronan was distributed diffusely in the cytoplasm, whereas in control VSMCs hyaluronan was located close to the cellular plasma membrane (Figure 6A, 6B; mean staining score MFS: 3±0.6, n=3 versus control: 2±0.5, n=4, not significant).

After incubation for 24 hours in serum-free media there was a 3- to 5-fold increase in cell loss in MFS VSMC, AF, and SF in comparison to control cell cultures (VSMC: 29±4%, n=5 versus 9±5%, n=3, P<0.05; AF: 26±4%, n=5 versus 7±4%, n=5, P<0.05; SF: 26±9%, n=3 versus 5±3%, n=3, not significant). Western blot studies showed a 3-fold increase in the expression of TGFβ1, β2, and β3 in SF (n=2) and VSMC (n=2) derived from MFS in comparison to controls (n=2; Figure 7A), consistent with the immunohistochemical findings in aortic tissue and cultured cells.

The 24-hour scratch wound assay revealed altered distribution of CD44 marker in MFS VSMCs (n=3) compared with control VSMCs (n=3). In control VSMCs, the leading edge of migration was characterized by long cytoplasmic projections strongly stained with CD44 (Figure 8A). In MFS VSMCs the projections were shorter and blunt and CD44 staining was not concentrated at the leading edge of migration (Figure 8B). There were similar changes in the distribution of MT1-MMP and β3 integrin staining in MFS (n=2) compared with control VSMCs (n=3; data not shown). Western blot studies suggested there was no change in level of expression of CD44, MT1-MMP, or β3 integrin in MFS (n=3) compared with control VSMCs (n=3; Figure 7B).
Discussion

Histopathology of MFS aneurysm tissue was consistent with our previous report showing elastic lamellar disruption, focal CMD with minimal inflammatory infiltrate, and loss of VSMCs associated with apoptosis.² There was increased expression of each of the 3 human TGF-β isoforms in aneurysm tissue and in cultured VSMCs and fibroblasts derived from aorta and skin (Figures 1, 4) compared with controls, although this did not reach significance for TGF-β2 and β3 in aneurysm tissue or for TGF-β3 in cultured VSMC. To our knowledge, this is the first report of increased TGF-β expression in MFS vascular tissue. It supports the proposal that in MFS, fibrillin-1 gene mutation associated with microfibril deficiency leads to dysregulation of TGF-β and subsequent pathogenetic effects.³

Hyaluronan content in MFS tunica media was increased and occurred in close proximity to elastic lamellae and areas of CMD. Hyaluronan was co-localized with TGF-β distribution (Figures 1, 2). In control aorta hyaluronan was mainly distributed in the tunica intima and adventitia. In cell culture there were similar findings with increased hyaluronan content in VSMCs derived from MFS compared with control VSMCs and in aortic and skin fibroblasts (data not shown) using immunostaining, but the differences were not significant (Figures 6). These findings are consistent with previous work showing increased hyaluronan content in MFS cultured SF.¹³ Because increased hyaluronan expression has been observed in association with differentiation of normal human lung fibroblasts into myofibroblasts, hyaluronan content may be important in the determination of myofibroblast phenotype.¹⁸ In the present study increased expression of TGFβ in SF and AF was associated with myofibroblast differentiation (Figures 4 and 5). The findings support the proposal that TGFβ regulates hyaluronan levels and that hyaluronan content is important in the determination of myofibroblast phenotype.

The scratch migration assay in this study suggested the presence of altered directional migration in MFS VSMC (Figure 8) and in aortic and skin fibroblasts. The disorganized migration front was associated with absence of CD44 localization at the leading edge of cytoplasmic projection despite normal cell expression of CD44 in Western blot analysis. These were similar findings for two other proteins involved in cell migration (MT1-MMP and β-3 integrin). The normal level of hyaluronan CD44 receptor expression in MFS despite increased cell culture hyaluronan content supports the finding that increased hyaluronan content rather than CD44 expression was likely to be the primary factor underlying cell migration characteristics.¹⁸ The abnormal distribution of intracellular proteins as well as disorganization of extracellular matrix distribution² in cultured MFS SF, AF, and VSMC is consistent with excess production of increased hyaluronan. Accumulation of hyaluronan and associated differentiated myofibroblasts may lead to reduced levels of free CD44 and impaired cellular migration.
Previous reports of increased VSMC apoptosis in tissue of MFS aneurysm tissue is consistent with findings in the present study, in which MFS cell loss during culture was also abnormal. Signals arising from aortic wall apoptotic cells may be responsible for the significant increase in number of CD34-positive microvessels in MFS. However, the response was incomplete with absence of inflammatory infiltrate in tissues surrounding the injury. The high hyaluronan environment might be one factor inhibiting extravasation of inflammatory cells and CPCs. The reason for the presence of CD34-positive VSMCs in the outer media of MFS aneurysm is not clear; however, the presence of excess hyaluronan might stimulate dedifferentiation of these cells.

The diagnosis of MFS in this study was determined using the Ghent criteria, although in some subjects the diagnosis remains uncertain. Fibrillin-1 gene mutation has been identified in 2 subjects in this MFS cohort, and in these subjects abnormal fibrillin-1 expression has been previously reported. Molecular diagnosis of MFS is difficult and not widely available. In this cohort, absence of molecular diagnosis limits the conclusions that might be made regarding an association between MFS and altered expression of TGFβ. The study is further limited by the small number of subjects in each subgroup. Although immunostaining scores were higher in MFS compared with controls, the results did not always reach significance. The anti-inflammatory agent aprotinin was used during surgery. However, it seems unlikely that the short time frame between aprotinin use and removal of tissue for investigation would influence the histological findings.

In summary, the study has shown abnormal regulation of TGFβ with increased expression of TGFβ isoforms in aortic aneurysm tissue and that this is associated with an increase in tissue hyaluronan content. The findings are consistent with excess activity of TGFβ in MFS having an important role in regulation of hyaluronan metabolism, reduction of VSMC cell survival and impairment of tissue repair, factors that may all contribute to the development of aortic aneurysm.

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### Figure 7
A, Western immunoblot electrophoresis shows increased expression of TGFβ1, β2, and β3 in MFS SF and VSMC compared with controls. B, Western immunoblot electrophoresis shows expression of CD44, MT1-MMP, and β3 integrin are similar in control and MFS VSMC.

### Figure 8
Localization of CD44 (green) and long cytoplasmic protrusions at the leading edge of migration in control VSMC (A) in the wound healing assay. In contrast, MFS VSMC show an absence of CD44 localization and blunt cytoplasmic border at the leading edge (B) (250×).
Disclosures

None.

References


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