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Shear stress-induced collagen XII expression is associated with atherogenesis

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Abstract

Fluid shear stress has been shown to modulate various endothelial functions. We selected a shear stress-specific clone, identified as collagen XII, from a bovine aortic endothelial cell (BAEC) cDNA library. We confirmed that shear stress induces collagen XII expression at both the mRNA and protein levels in cultured BAECs and human umbilical vein ECs (HUVECs) by stimulating transcription. When HUVECs were exposed to shear stress, they secreted collagen XII protein and it was deposited underneath them. Strong expression of collagen XII was found in the intima of human aortic wall lacking atherosclerotic lesions, whereas weak expression was seen in the intima of atherosclerotic plagues. Furthermore, the downstream portion of atherosclerotic plaques showed apparently weak collagen XII expression compared with the upstream portion. These results suggest that collagen XII expression induced by fluid shear stress may play a role in stabilizing the vascular structure and preventing the formation of atherosclerotic lesions.

Keywords: Collagen XII; Shear stress; Endothelium; Atherosclerosis

Fluid shear stress caused by blood flow modulates the functions of endothelial cells (ECs) by regulating the gene expression of various proteins, including vasoactive substances, growth factors, adhesion and chemoattractant molecules, coagulation factors, and cytokines [1–5]. Shear stress may be involved in the pathogenesis of atherosclerosis because atherosclerotic plaques occur preferentially at the branching sites of the arteries; these sites experience turbulent blood flow and low shear stress [6,7].

Shear stress may also modulate the production of the extracellular matrix (ECM), an essential supporting structural framework of the vessels, by the ECs. For instance, the secretion of laminin by the ECs is up-regulated under shear stress, whereas that of fibronectin is down-regulated [8–10]. Inversely, endothelial functions, including adhesion, protein synthesis, proliferation, and migration, are influenced by the composition and organization of the ECM [11,12]. Recently, we prepared an EC cDNA library to select clones that are specifically expressed in response to laminar shear stress. By screening the cDNA library, we selected a shear stress-specific clone, which we identified as collagen XII.

ECMs are composed of several types of collagens, laminin, fibronectin, and vitronectin. Collagen XII belongs to the group of fibril-associated collagens with interrupted triple helices (FACITs). It may stabilize the fibril spacing by bridging the adjacent collagen fibrils, such as collagen I or III, and mediate fibril interactions with other extracellular and cell-surface molecules [13–15]. Expression of the collagen XII gene in fibroblasts is up-regulated in stretched collagen I gels [16].

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Furthermore, collagen XII is up-regulated by mechanical stimuli in several tissues, including tendons, ligaments, periosteum, arterial smooth muscle, and heart valves [17,18]. These findings suggest that collagen XII expression is regulated by mechanical stress. However, little is known about the role of collagen XII in the arterial walls under shear stress. Therefore, we examined shear stress-induced collagen XII expression using cultured ECs and human aortic tissues.

Materials and methods

Materials. The Oligotex-dT30, cDNA Synthesis Kit, DNA Ligation Kit, Random Primer DNA Labeling Kit Version 2.0, and Taq Cycle Sequencing Core Kit were from Takara Shuzo. The EXlox *Eco*RI/*Hin*dIII Arms Kit, EXlox vector arms, PhageMaker In Vitro Lambda Packaging System, and host cells ER1647 and BM25.8 were obtained from Novagen. The plasmid midikits were from Qiagen. All procedures were performed according to the manufacturers' technical manuals.

Cell cultures and exposure to shear stress. Bovine aortic ECs (BAECs) were obtained from thoracic aortae and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. Human umbilical vein ECs (HUVECs) were harvested from human umbilical vein with 0.05% trypsin with 0.02% EDTA, plated on 0.1% gelatin-coated dishes, and cultured in DMEM containing 20% FCS, 5 ng/mL basic fibroblast growth factor (bFGF), 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. These cells were plated on plastic slides or polyester sheets and flow experiments were performed as described previously [19]. A confluent monolayer of ECs in a parallel-plate flow chamber was subjected to steady unidirectional laminar shear stress. Control or static ECs were grown in the same manner as sheared ECs and placed into fresh medium before being maintained in an incubator.

cDNA library. After BAECs on polyester sheets were exposed to a 30-dyne/cm^2 shear stress for 4 h, the total RNA was extracted by the guanidinium isothiocyanate and cesium chloride gradient procedure of Chirgwin et al. [20]. Messenger RNA was isolated with Oligotex-dt30 and cDNA was synthesized with an oligo(dT)$_{18}$ primer and the cDNA synthesis kit. After methylation by *Eco*RI and *Hin*dIII methylases, 5 μg cDNA in 5 μL of Tris-EDTA buffer was ligated to 4 μg dephosphorylated directional *Eco*RI/*Hin*dIII linkers (GCTTGAATTCAAGC) and digested with *Eco*RI and *Hin*dIII with the DNA ligation kit and the EXlox *Eco*RI/*Hin*dIII arms kit. Linker cDNA (5 μg) was ligated to 0.5 μg EXlox vector arms and the in vitro packaging of the vector into the EXlox phage was performed with the In Vitro Lambda Packaging System.

Library screening by differential hybridization. Specific cDNA clones expressed in response to the laminar shear stress were selected by screening the library using the differential plaque hybridization method. After amplification of the phage library, 250 μL phage libraries (2.27×10^4 pfu/mL) in EXlox vectors were incubated with an equal amount of *Escherichia coli* (strain ER1647, 5×10^8 cells/mL) suspension for 20 min at 37 °C to allow the phage to adsorb to the host; top agar was added and then 5700 phages per plate were plated in the top agar. After the plates were incubated for 9 h, the plaques on each plate were transferred to two positively charged nylon membranes (Hybond-N$^+$, Amersham International PLC). Nucleic acid probes that were labeled with [α-^{32}P]dCTP (110 TBq per mmol/L) by the Random Primer DNA Labeling Kit Version 2.0 were synthesized from the cDNA libraries of control BAECs, and the cDNA on the membranes was hybridized with the probes. Shear stress-specific clones were identified as plaques that did not hybridize with the probes. After isolation and amplification, phages with shear stress-specific clones (positive clones) were converted to plasmid clones automatically by infecting the host strain BM25.5. Positive plasmid clones were amplified by polymerase chain reaction (PCR) and screened twice (second and third screening) by dot-blot hybridization using probes from the cDNA library of both sheared and static control BAECs, which were equally labeled by [α-^{32}P]dCTP. The absorbance of the dot images was evaluated quantitatively using a Bio Image Analyzer (BAS-2000 II, Fujifilm) and positive clones whose absorbances were twice over that of the control were selected.

Sequence analysis and homology search. The shear stress-specific clones were sequenced using the Taq Cycle Sequencing Core Kit and a fluorescence automatic sequencer (SQ3000/32, Hitachi Seisakujyo, Japan), according to the manufacturer's protocol.

Northern blotting. Northern blotting was performed to analyze the mRNA expression of the gene as described previously [19]. Equal amounts of total RNA were subjected to electrophoresis on 1% agarose and transferred to positively charged nylon membranes. Blots were hybridized with our isolated BAEC cDNA and GAPDH, and labeled with [α-^{32}P]dCTP by random priming. After the membranes were washed, the blots were visualized by autoradiography and quantified using the Bio Image Analyzer. The data are presented as relative values (target gene/GAPDH).

Western blot analysis. After being subjected to shear stress, ECs on two polyester sheets were harvested by scraping with PBS and the cells were lysed in lysis buffer (1% Triton X-100, 150 mM NaCl, and 20 mM Tris–HCl, pH 8.0) and mixed, followed by centrifugation. The protein concentration of the supernatant was determined using the Bio-Rad protein assay reagent. Equal amounts of lysate protein were separated on a 5% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane, blocked with 8% nonfat dried milk in TBST (20 mmol/L Tris–HCl, pH 7.5; 150 mmol/L NaCl; and 0.05% Tween 20) at 4 °C overnight. The membrane was incubated with the primary rabbit antibody for collagen XII (LSL, Japan; 1:500 dilutions in TBST) for 1 h at room temperature. Immunoblots were developed with a peroxidase-conjugated secondary antibody (Wako Life Science, Japan; 1:5000 dilution in TBST), visualized with enhanced chemiluminescence reaction reagents (Amersham Life Science, USA), and analyzed with the public domain NIH image program.

Reverse transcription PCR. Single-strand cDNA was synthesized from 2 μg of total RNA using primer oligo(dT)$_{12–18}$ and the SuperScipt Preamplification System (Gibco-BRL Life Technologies, USA) and amplified by PCR using Taq DNA polymerase (Takara, Shuzo, Japan). The primer sets encoding sense and antisense sequences were 5′ AGCAAGGGATCAAGAAGCAA 3′ and 5′ ATTCTGAGTCAA CGGCATCC 3′; 5′ CCACCCATGGCAAATTCCATGGGCA 3′ and 5′ TCTAGACGGCAGGTCAGGTCCACC 3′ for GAPDH. The reactions were carried out for 30 cycles consisting of 30-s denaturing at 94 °C, 30-s annealing at 57 °C, and 60-s elongation at 72 °C. The products were separated by electrophoresis in 2% agarose and visualized with ethidium bromide.

Immunofluorescence microscopy. Cells were washed with PBS, fixed for 10 min in 4% paraformaldehyde, and then permeabilized for 20 min with 1% Tween 20 in PBS. Nonspecific protein binding sites were then blocked for 1 h with 1% bovine albumin in PBS. Cells were then incubated overnight at 4 °C with rabbit polyclonal antibodies against collagen XII and a mouse monoclonal antibody against CD34 (Dako, Carpinteria, CA). Goat anti-rabbit IgG conjugated with Alexa Fluor 488 (green) and goat anti-mouse IgG conjugated with Alexa Fluor 568 (red) (Molecular Probes, OR, USA) were used as secondary antibodies, and then the fluorescence was observed with a laser scanning confocal microscope (Fluoview, Olympus, Japan).

Immunohistochemistry. Immunostaining was performed using the labeled streptavidin biotin method (DAKO). After human aortae were obtained at autopsy, blood flow direction was indicated and longitudinal sections of the whole plaque were obtained in a direction parallel to the long axis of the aorta. The samples were fixed with 10% buffered

formalin and embedded in paraffin. They were cut into 3-μm thick sections. Polyclonal antibodies against collagen XII were used at a 1:200 dilution. Immunostaining was detected with the 3-amino-9-ethylcarbazol substrate chromogen system (AEC; Nichirei, Tokyo, Japan), resulting in red staining, then the sections were counterstained with hematoxylin. Double immunostainings were also performed. The first staining for seprase was done as described above. After washing in 0.1 M glycine–HCl buffer (pH 2.2) for 1 h, a second staining for HHF35, a marker for muscle actin (DAKO, diluted 1:600 v/v), using alkaline phosphatase-conjugated streptavidin, was visualized with Fast Blue substrate solution (Nichirei), which resulted in blue staining.

Results

cDNA library screening and homology search

A total of 7356 plaques containing the phage cDNA library were screened and nine clones whose absorbancy ratio of mRNA to GAPDH was >2 times higher than that of the static control were selected. The partial sequences of the nine clones were analyzed. Homology searching revealed that the sequence of one of our isolated genes had 93% homology to the short variant transcript of human collagen XII (COL12A1) (Accession No. NM_080645). One main band of approximately 7 kb was observed for collagen XII mRNA in BAECs; this was consistent with the size of the small variant of collagen XII.

Collagen XII mRNA expression in cultured ECs

To examine the shear-force dependence of collagen XII mRNA expression, BAECs were subjected to shear stress for 4 h at 5, 20, 30, and 50 dyne/cm^2. Shear stress-induced collagen XII mRNA expression and the highest level of collagen XII mRNA expression was obtained by 20 dyne/cm^2 (Fig. 1). The time course of BAEC exposure to 20 dyne/cm^2 of shear stress indicated a rapid and transient increase in the amount of collagen XII mRNA. The collagen XII mRNA levels were first evident after 1 h, peaked after 4 h (to a 6–7-fold increase over the control level), and decreased gradually after 6 h (Figs. 2A and B). Collagen XII mRNA in HUVECs was also induced by shear stress. The time course of collagen XII mRNA expression in HUVECs showed the same pattern as in BAECs (Fig. 2C).

Shear stress stimulates transcription of the collagen XII gene

To examine whether the shear stress-induced expression of collagen XII depends on enhanced transcription of the collagen XII gene, actinomycin D, an inhibitor of mRNA synthesis, was added before the application of shear stress. The pretreatment with actinomycin D completely inhibited the collagen XII mRNA expression

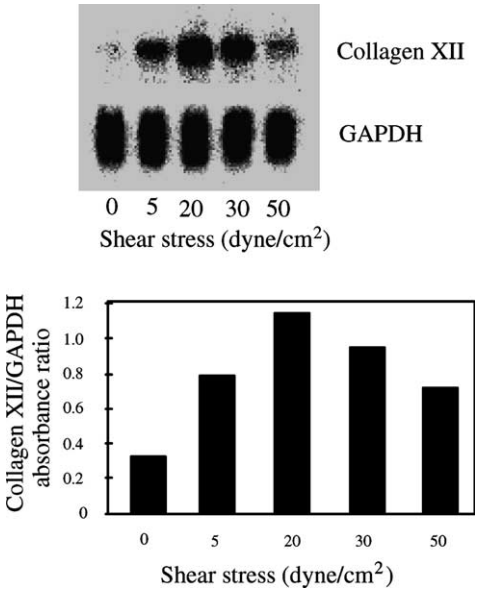

Fig. 1. Collagen XII mRNA expression in BAECs subjected to varying levels of shear stress for 4 h. The same blot was hybridized with GAPDH cDNA. BAECs kept in a static condition served as "time 0." This result is representative of three independent experiments.

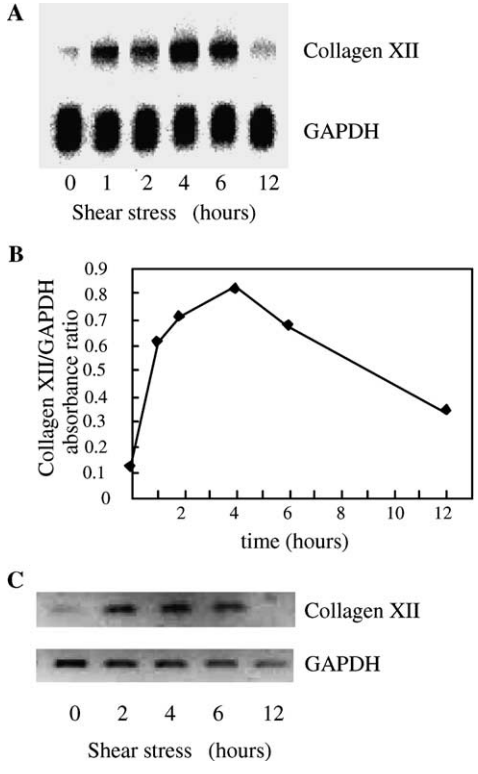

Fig. 2. Time course of collagen XII mRNA expression in BAECs (A and B) and HUVECs (C) induced by 20 dyne/cm^2 of shear stress. The same blot was hybridized with GAPDH cDNA and the relative absorbance ratios of the mRNA levels of collagen XII to GAPDH were quantified (B). This result is representative of three independent experiments.

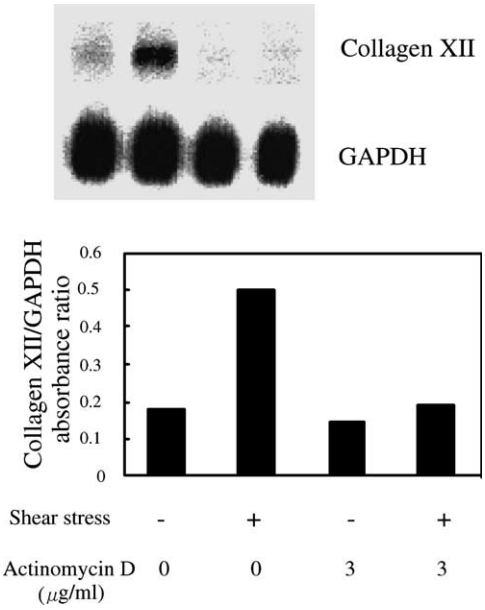

Fig. 3. Effect of actinomycin D on the expression of collagen XII mRNA induced by shear stress (20 dyne/cm^2). ECs were either subjected to shear stress or incubated in a static condition for 4 h, in the absence or presence of actinomycin D (3 μg/mL). The same blot was hybridized with GAPDH cDNA and the relative absorbance ratios of the mRNA levels of collagen XII to GAPDH were quantified. This result is representative of three independent experiments.

induced by shear stress (Fig. 3). This result revealed that shear stress stimulates transcription of the collagen XII gene.

Collagen XII protein expression in cultured ECs

To test whether shear stress induces collagen XII expression at the protein level, confluent BAECs were subjected to a laminar shear stress of 20 dyne/cm^2 for various periods of time. Elevated levels of small (220 kDa) and large (320 kDa) splice variants of the collagen XII protein were detectable for 4–24 h (Fig. 4A). The expression of collagen XII in HUVECs subjected to shear stress was similar to that seen in BAECs (Fig. 4B).

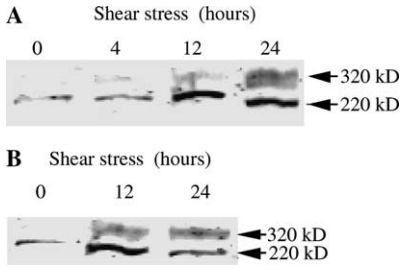

Fig. 4. Collagen XII protein expression in ECs by shear stress. BAECs (A) and HUVECs (B) were exposed to 20 dyne/cm^2 shear stress for the indicated periods, then Western blot analysis was performed to measure the collagen XII protein levels. This result is representative of three independent experiments.

The expression of collagen XII in HUVECs exposed to shear stress was examined using confocal laser scanning microscopy. HUVECs exposed to 20 dyne/cm^2 shear stress for 24 h showed collagen XII grouped into thick fibrils deposited outside the elongated ECs and extending beneath them (Figs. 5B and C), whereas the extracellular collagen XII deposition underneath the static ECs was scant (Fig. 5A).

Immunohistochemistry

The expression patterns of collagen XII in human aortic tissue were investigated using immunohistochemistry. Subendothelial band-like collagen XII expression was observed in the intima that lacked atherosclerotic lesions (Fig. 6A). Some medial smooth muscle cells, not intimal myofibroblasts, showed collagen XII immunoreactivity in this area (Fig. 6D). Together, the major source of collagen XII is derived from endothelial cells. In contrast, collagen XII expression in the intima of atherosclerotic plaques was generally weaker. There was a difference in the collagen XII expression between the different parts of the atherosclerotic plaque: the downstream portion, which was composed of a lot of foam cells and a few myofibroblasts, showed faint staining (Fig. 6C), whereas upstream portion, which was composed of dense fibrous tissue, showed weak and fragmentary staining for seprase (Fig. 6B) in the ECM and some intimal myofibroblasts (Fig. 6E).

Discussion

Our results demonstrated that fluid mechanical stimuli induce collagen XII expression at both the mRNA and protein levels in cultured ECs by stimulating transcription of the gene. The effect of shear stress on collagen XII mRNA expression was transient: it peaked at 4 h then decreased gradually, but collagen XII protein expression was maintained at a high level for at least 24 h. This could be due to an accumulation of collagen XII protein as a consequence of the low degradation rate compared with its mRNA. Intimal collagen XII expression in the human aorta appeared to be modulated according to the strength of the shear stress. Thus, we confirmed that shear stress induces collagen XII production in human ECs.

The functional roles of collagen XII within the vasculature have not been fully elucidated. However, several reports indicate that the expression of collagen XII is induced in part by mechanical stress. For instance, this phenomenon is observed during periodontal ligament remodeling, in bladder stretch injury, in wounded skin, and in the endomysial fibroblasts of overloaded chicken muscle in vivo [21–23]. These findings are consistent with a study showing that tenascin-C and

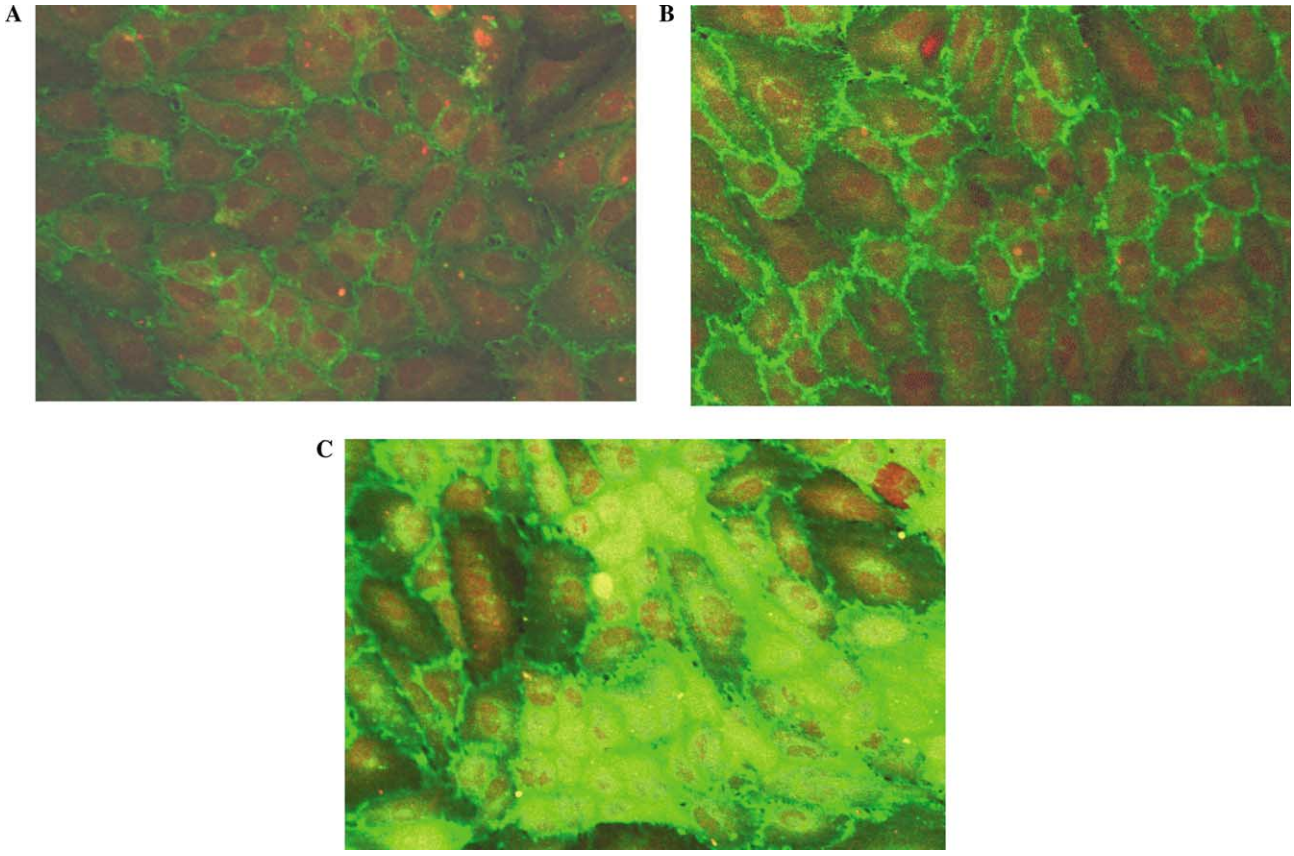

Fig. 5. Confocal images from HUVECs exposed to shear stress. Cells were immunofluorescently labeled for collagen XII (green) and CD34 (red), a marker of endothelial cells. Static cultures are shown in (A). ECs exposed to a shear stress of 20 dyne/cm^2 for 24 h are shown in (B) and (C). (C) shows an image obtained from a horizontal section close to the plastic slide.

collagen XII mRNA and protein are reversibly induced by tensile stress in vitro in dermal fibroblasts cultured on a collagen I matrix [16]. Together, these data suggest that collagen XII may be closely associated with the functional adaptation, remodeling, and regeneration of connective tissue in response to mechanical stress.

In culture, ECs synthesize and deposit ECM components basally, including several types of collagens

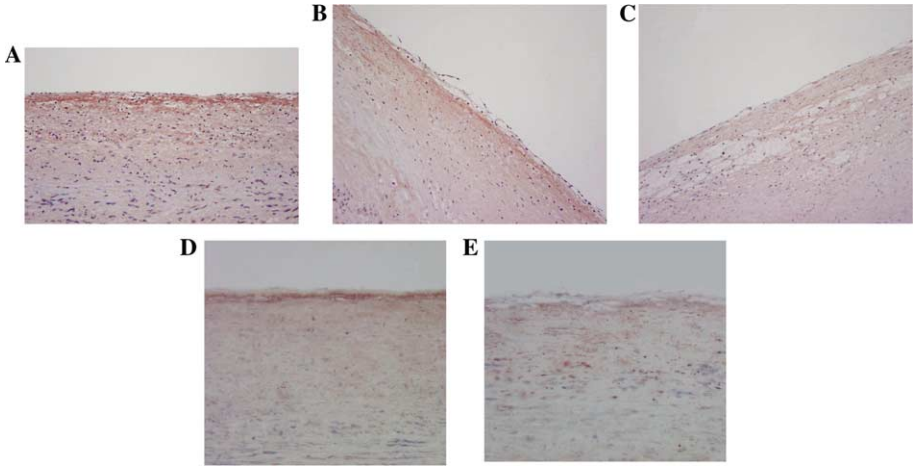

Fig. 6. Immunohistochemical labeling of collagen XII protein in the intima of the human aorta. (A) shows immunostaining of the intima of an aorta that lacks an atherosclerotic lesion. (B) and (C) show the immunostaining of the upstream and downstream portions of an atherosclerotic plaque, respectively. (D) and (E) are double immunostaining (collagen XII; red, HHF35; blue) of an aorta that lacks an atherosclerotic lesion and of the upstream portion of an atherosclerotic plaque, respectively.

[24–26], laminin [27], fibronectin [28,29], and vitronectin [30]. Furthermore, laminin secretion under shear stress is up-regulated, whereas fibronectin is down-regulated [8–10]. Our confocal microscopic study showed that shear stress-induced collagen XII protein was secreted extracellularly and deposited underneath the HUVECs. Collagen XII may mediate the interaction between collagen fibrils and other extracellular matrix proteins, or with the cell surface [14–16]. In the intima of the artery, collagen I is the major collagen [31–33] and collagen XII may be associated with collagen I for stabilizing the vascular structure.

Atherosclerosis develops preferential sites along the vasculature, in regions experiencing oscillatory flow with low mean shear stress that predispose to endothelial dysfunction. On the contrary, unidirectional flow, typical of plaque-free areas, exerts a protective effect on ECs [34,35]. In addition, the upstream sites of atheromatous plaques are under a flow with high shear stress, whereas downstream sites are exposed to relatively low shear stress [34,35]. We found the strong seprase expression in the intima lacking atherosclerotic lesion, suggesting plaque-free unidirectional flows may induce collagen XII secretion from ECs. Furthermore, within an atheromatous plaque, intimal collagen XII expression is weaker in the downstream portion of the atherosclerotic plaque than in the upstream portion. Apoptosis within the plaque was significantly higher in the downstream portion than in the upstream portion [36]. This notion is supported by the observation that the downstream portion of atherosclerotic plaques is prone to disruption [37]. In the upstream part, some intimal myofibroblasts expressed collagen XII protein, which may compensate for a decreased production in ECs. Therefore, shear stress-induced collagen XII expression in the intima may play a role in stabilizing the vascular structure and preventing the development of atherosclerosis.

In conclusion, the present study provides evidence that collagen XII is produced and secreted from human vascular endothelium in a manner regulated by the strength of shear stress. Although further study is needed to clarify the function of collagen XII in the aortic wall, our findings support the idea that the modulated expression of collagen XII may play an important role in the formation of atherosclerotic lesions.

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