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Induction of human inhibitor of apoptosis protein-2 by shear stress in endothelial cells

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Abstract To disclose the anti-atherosclerotic mechanisms of steady laminar shear stress, we analyzed the expression of human inhibitor of apoptosis protein-2 (HIAP-2), whose gene was selected from a cDNA library of sheared endothelial cells (ECs), on ECs. HIAP-2 was dose-independently and time-dependently induced in ECs by shear stress, regulated at the transcriptional level. HIAP-2 expression was also identified in vivo. Shear stress-mediated inhibition of EC apoptosis was associated with the inhibition of caspase-3 activity, suggesting that the shear stress prevents EC apoptosis via negative regulation of caspase-3 by the increment of HIAP-2.

Key words: Human inhibitor of apoptosis protein-2; Caspase-3; Shear stress; Endothelial cell; Atherosclerosis

1. Introduction

From the evidence of the specific localization of lipid deposition on the arterial tree, hemodynamic forces are suggested to be an essential factor in the initiation of atherogenesis [1,2]. Furthermore, the shear stress generated by distinct blood flow (uniform laminar shear stress vs. turbulent shear stress) is suggested to play an antipodal role in the induction or prevention of atherosclerosis by changing the endothelial functions regulating the specific genes which may be involved in atherogenesis [3,4]. Growth regulation of endothelial cells (ECs) by these two different flow patterns has also been shown to be antipodal. Recently, we showed that the unidirectional steady laminar shear stress may play a key regulatory role in the anti-atherosclerotic sequence by maintaining EC stability through the blocking of the cell cycle events of EC proliferation [5]. In addition, EC death was also regulated by shear stress. EC apoptosis preferentially occurs in the downstream part of human plaques, where eddies and separation of the blood stream from the vessel wall occur, resulting in the plaque wall being exposed to low mean shear stress [6]. In contrast, relatively high laminar shear stress inhibited apoptosis of ECs in vitro [7,8]. Since EC death may be involved in plaque disruption, thrombus formation [9] and increasing permeability of the vessel wall, the inhibition of apoptosis by laminar shear stress may also contribute to the development of the anti-atherosclerotic microenvironment in arterial walls, in association with the suppression of EC growth by the same shear stress.

To obtain appropriate probes for elucidating the molecular mechanisms of anti-atherosclerotic functions of laminar shear stress, we have selected specific clones expressed in response to laminar shear stress from an EC cDNA library [10]. Using one of these probes, here we present evidence that the laminar shear stress inhibits EC apoptosis through the induction of a specific apoptosis inhibitor protein.

2. Materials and methods

2.1. Materials

The mouse anti-human inhibitor of apoptosis protein (IAP) monoclonal antibody (B75-1), which identifies c-IAP-1 as an ~72 kDa band on SDS–PAGE, for immunoprecipitation/Western blotting and immunostaining of cultured ECs, and a caspase-3 assay kit were obtained from PharMingen. The goat anti-human c-IAP-1 polyclonal antibody (N-19) for immunostaining of the arteries was from Santa Cruz Biotechnology. Anti-Flag M2 monoclonal antibody was from Sigma.

2.2. Exposure to shear stress

Bovine aortic ECs (BAECs) and human umbilical vein ECs (HUVECs) were obtained and cultured as described previously. These cells were plated on plastic slides or polyester sheets, and flow experiments using a parallel-plate flow chamber, which generates a unidirectional laminar shear stress, were performed [10]. Control ECs were maintained in an incubator.

2.3. Sequence analysis and homology search

The genes whose expressions were upregulated by laminar shear stress were isolated from a library of BAECs which were exposed to 30 dyne/cm^2 shear stress for 4 h. After amplification of these clones by polymerase chain reaction (PCR), the full sequence of one of these clones was detected and the homology of nucleotide and amino acid sequences was investigated as described previously [10].

2.4. Northern blotting

Northern blotting was performed as described previously [10]. Blots were hybridized with either our isolated BAEC cDNA (for BAECs) or a 623 bp reverse transcription PCR (RT-PCR) product (for HUVECs), which was obtained from HUVEC RNA using a primer set encoding the human IAP-2 (HIAP-2, bp 433–1055, GenBank accession number U45879), and GAPDH. Blots were quantified by image analyzer and the data are presented as relative values (target gene/GAPDH).

To examine whether the mRNA expression was caused by the enhancement of transcription or the stabilization by shear stress, 1.5 or 3 μmol/l actinomycin D was added to the medium containing 10% fetal calf serum (FCS) after BAECs were exposed to 20 dyne/cm^2 shear stress for 4 h, and the cells were either incubated in the static

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condition or exposed to the shear stress for 2 or 4 h before RNA extraction. Some BAECs were exposed to shear stress in the presence or absence of actinomycin D for 4 h and then RNA was extracted. The experiment was repeated more than three times.

2.5. Immunoprecipitation and Western blotting

After subjected to shear stress, HUVECs were lysed and an equal amount of lysate protein (360 μg) was used to perform immunoprecipitation by antiserum using IMMUNOcatcher (CytoSignal), and 5 μl of immunoprecipitate in 40 μl sample buffer was separated by SDS–PAGE, transferred onto a nitrocellulose membrane and then incubated with an antibody. The immunoblots were analyzed as described previously [5]. The same c-IAP-1 antibody or anti-Flag M2 antibody was used for both immunoprecipitation and Western blotting. The experiment was repeated more than four times. For binding assay of HIAP-2 to second mitochondria-derived activator of caspases (Smac), cell extracts were immunoprecipitated with an anti-Flag M2 antibody and then immunoblotted with an anti-HIAP-2 antibody.

2.6. In situ hybridization

Human aortas at the bifurcation of the intercostal artery, obtained within 3 h after death from two cadavers of people who died at 37 and 83 years old, and bovine aortas were fixed with 4% paraformaldehyde and embedded in paraffin. The plasmid carrying HIAP-2 cDNA, which was isolated from bovine ECs subjected to shear stress [10], was linearized with *Hin*dIII and *Acc*65I, and digoxigenin (DIG)-labeled cRNA probes were synthesized using a DIG RNA labeling kit (Boehringer Mannheim). After treatment with proteinase K, the sections were fixed, followed by treatment with triethanolamine-HCl (TEA) and 0.1 mol/l TEA–0.25% acetic anhydride. The hybridization mixture containing denatured DIG-labeled antisense or sense riboprobe was applied over each section and hybridization was carried out for 16 h at 50°C. After non-hybridized transcripts were digested with RNase A, hybridized probe was detected by anti-DIG antibody conjugated to alkaline phosphatase and 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate. The nuclei were counterstained with methyl green.

2.7. Immunohistochemistry

Human aortas, obtained from two cadavers of people who died at 58 and 73 years old, and HUVECs that had been subjected to 20 dyne/cm^2 shear stress for 24 h were reacted with polyclonal and monoclonal antibodies for human c-IAP-1, respectively, and the bound antigens were visualized as described previously [10].

2.8. Analysis of DNA fragmentation and caspase-3 activity

After being exposed to the shear stress in Dulbecco's modified Eagle's medium (DMEM) without serum, sheared and static control BAECs were lysed and treated with RNase A, and with proteinase K, and then the extracted DNA was separated by electrophoresis in a 1.5% agarose gel.

HUVECs were exposed to the shear stress in DMEM containing either 5 ng/ml basic fibroblast growth factor (bFGF) without FCS for 8 h or 20% FCS without bFGF for 18 h. The caspase-3 activity was assayed by use of a kit with a synthetic fluorogenic caspase-3 substrate (Ac-DEVD-AMC) according to the manufacturer's protocol. For confirmation, a caspase-3 aldehyde inhibitor (Ac-DEVD-CHO) was added with the substrate in the medium of some static cells in every experiment.

2.9. RT-PCR

Single-strand cDNA was synthesized from 2 μg of total RNA using primer oligo(dT)$_{12-18}$ and SuperScript Preamplification System (Gibco BRL, Life Technologies) and amplified by PCR using Taq DNA polymerase (Takara). Four primer sets encoding sense and antisense sequences were used to detect the expression of HIAP-1 (550 bp), HIAP-2 (1067 bp), GAPDH (696 bp) [11], and survivin (431 bp) [12] in ECs. Reactions were carried out for 32 cycles consisting of 1 min denaturing at 94°C, 1 min annealing at 55°C, and 1 min elongation at 72°C. The products were separated by electrophoresis in 1.2% agarose and visualized with ethidium bromide.

2.10. Transfection of Smac

HUVECs were transfected with pcDNA 3.1(−) vector carrying full-length Smac, an inhibitor of IAPs, and a Flag tag (a gift from Dr. Xiaodong Wang, Howard Hughes Medical Institute, University of Texas) [13], or pcDNA 3.1(−) vector, using FuGENE 6 transfection reagent (Roche). 7 μg DNA in 352 μl diluted FuGENE 6 reagent was added to the cells/sheet in DMEM containing 20% FCS and 5 ng/ml bFGF and incubated for 20 h. After transfection, cells were exposed to shear stress (20 dyne/cm^2) for 12 h in the medium without bFGF and then the caspase-3 activity of cells and the binding ability of HIAP-2 to Smac were analyzed.

3. Results

3.1. IAP mRNA and protein expression in cultured ECs

The full-length sequence of one of our isolated BAEC genes (1572 bp) was 88% homologous to human inhibitor of apoptosis protein 1 mRNA (HIAP-1/C-IAP-2/hMIHC; GenBank accession number U45878), and 83% homologous to human inhibitor of apoptosis protein 2 mRNA (HIAP-2/C-IAP-1/hMIHB; GenBank accession number U45879). One main band was observed for the mRNA of IAP in BAECs, which was approximately 4.5 kb in size, indicating that it represents the BAEC homolog of HIAP-2 mRNA, since the mRNA sizes for HIAP-1 and HIAP-2 have been shown to be 6.5/8.7 kb and 4.5 kb, respectively [14].

When BAECs were subjected to shear stress for 4 h, HIAP-2 mRNA expression was induced by 5–70 dyne/cm^2 shear stress, with a nearly 11-fold increase over that of control by 20 dyne/cm^2, although the levels of expression by 5 and 70 dyne/cm^2 were usually low (Fig. 1). The mRNA expression was first evident at 1 h after the exposure of BAECs, became maximal at 4 h, maintained a high level until 8 h, and then decreased gradually to the control level by 24 h (Fig. 2A,B).

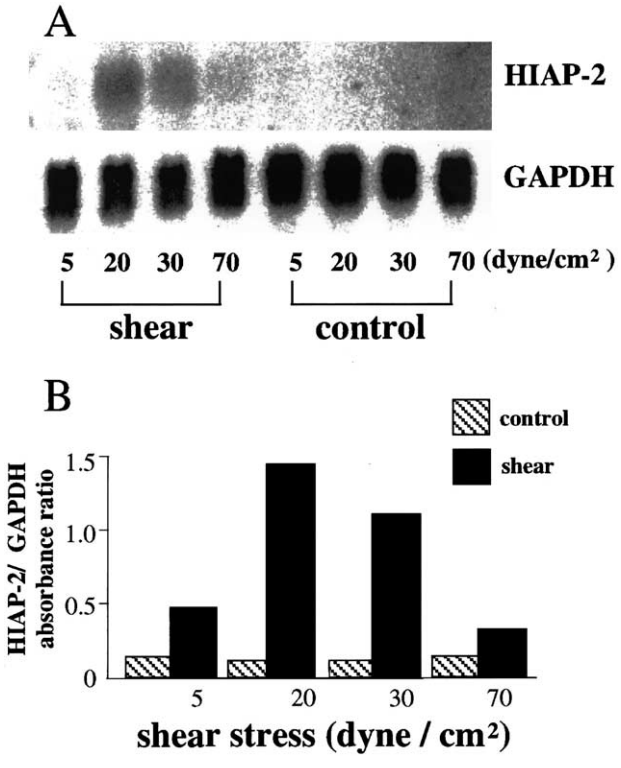

Fig. 1. HIAP-2 mRNA expression in BAECs subjected to shear stress for 4 h (A). The same blot was hybridized with GAPDH cDNA and relative absorbance ratios were quantified by image analyzer (B).

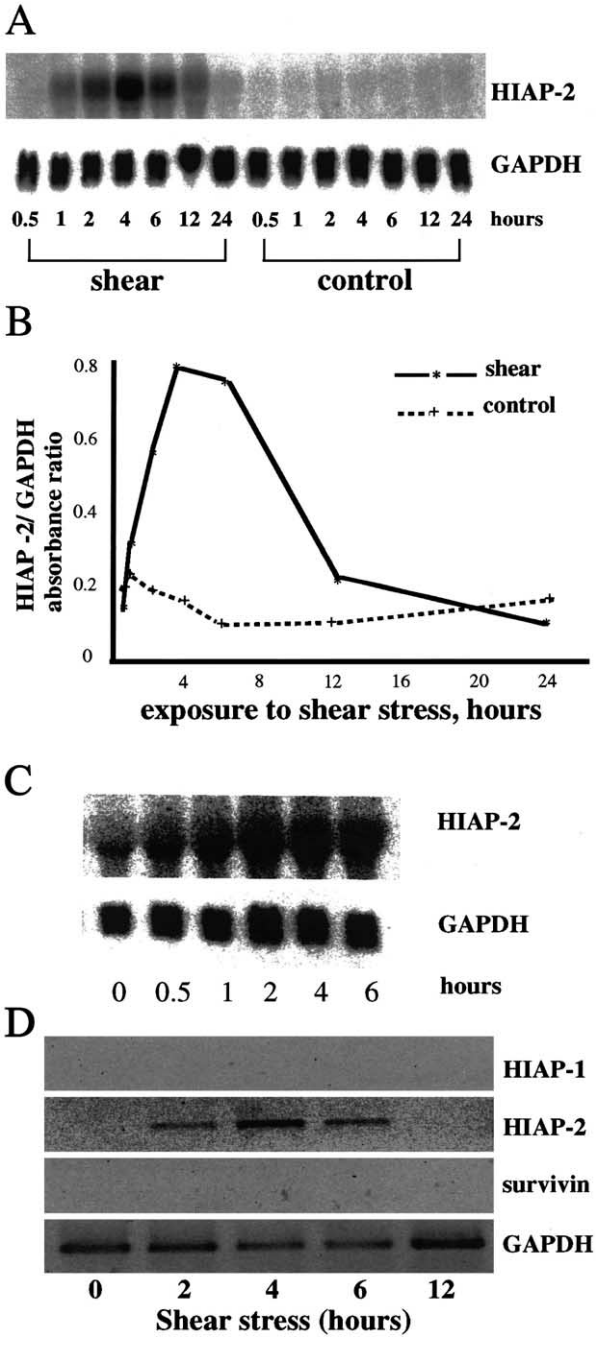

Fig. 2. Time course of mRNA expression of HIAP-2 in BAECs (A,B) and HUVECs (C), and HIAP-1, HIAP-2 and survivin in HUVECs (D) induced by 20 dyne/cm^2 of shear stress, analyzed with Northern blotting and RT-PCR, respectively. The same blot was hybridized with GAPDH cDNA and relative absorbance ratios were quantified (B).

HUVECs also expressed HIAP-2 mRNA after 0.5–6 h exposure to 20 dyne/cm^2 shear stress (Fig. 2C).

The mRNA level induced by the 4 h exposure to shear stress decreased to the control level when the cells were released from the shear stress and incubated in the static condition for 12 h (Fig. 3A). In the presence of actinomycin D during 4 h exposure to shear stress, the mRNA expression was completely abrogated (Fig. 3B). Furthermore, shear stress did not change the time course of mRNA degradation, when the cells were released from the shear stress (20 dyne/cm^2) after a 4 h exposure and either incubated in the static condition or continued to be exposed to the same shear stress in the presence of actinomycin D for 2 and 4 h (Fig. 3C), indicating that this expression of HIAP-2 mRNA is a result of the regulation at the gene transcription level by shear stress.

HUVECs expressed HIAP-2 protein of molecular weight between 65 and 89 kDa after being subjected to shear stress (20 dyne/cm^2) for 4–24 h (Fig. 4). Immunohistochemistry revealed that HUVECs expressed HIAP-2 protein after being subjected to the same shear stress for 24 h (Fig. 5).

The induction of other IAPs, including HIAP-1, and survivin by shear stress was not detected in our HUVECs with RT-PCR (Fig. 2D).

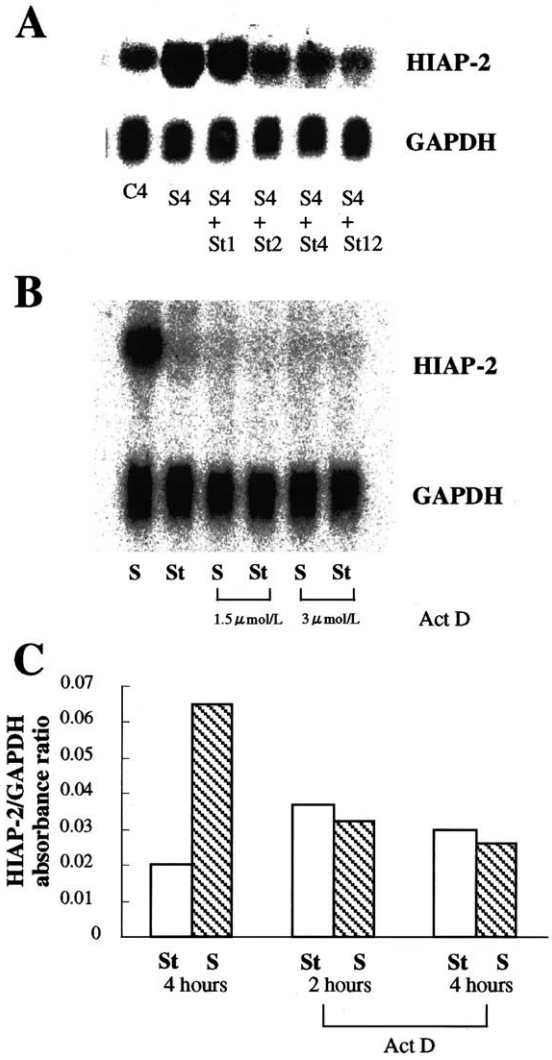

Fig. 3. Regulation of HIAP-2 transcription and mRNA degradation by shear stress (20 dyne/cm^2). Northern blotting. A: ECs were exposed to shear stress for 4 h (S4) and then incubated in the static condition (St) for 1–12 h. C4 indicates a static control incubated for 4 h. B: ECs were either subjected to shear stress (S) or incubated in a static condition (St) for 4 h in either the absence or presence of actinomycin D (Act D). C: After ECs were subjected to shear stress in the absence of actinomycin D for 4 h, ECs were either continued to be exposed to shear stress (S) or cultured in the static condition (St) for 2 or 4 h in the presence of actinomycin D (3 μmol/l).

3.2. IAP mRNA and protein expression in the endothelium of aortas

The endothelium of both bovine and human aortas expressed HIAP-2 mRNA, even though not all of the cells reacted (Fig. 6B,C). Some bovine medial smooth muscle cells (SMCs) also expressed HIAP-2 mRNA (Fig. 6B). The many endothelia of human aortas expressed HIAP-2 protein. Some macrophages and intimal SMCs also reacted with anti-HIAP-2 antibodies (Fig. 6D).

3.3. Inhibition of apoptosis by shear stress

Since shear stress induced the gene expression of apoptosis inhibitor, we studied the effect of shear stress on the apoptotic cell death by the induction of apoptosis using a serum withdrawal and bFGF withdrawal method. Exposure of the cells to 20 dyne/cm^2 shear stress for 36 h completely abrogated the apoptotic cell death induced by the serum withdrawal in BAECs, analyzed by DNA fragmentation, similar to previous results in HUVECs [8] (Fig. 7A).

Because the IAP has been shown to inhibit apoptosis through the inhibition of caspase-3 activity (for review, see [14]), we examined whether shear stress-induced IAP expression and death inhibition are concomitant with the decline of caspase-3 activity. In the static condition, a significant increase of caspase-3 activity was observed in the ECs by both serum withdrawal and bFGF withdrawal and this increase was significantly lost in the presence of Ac-DEVD-CHO. Twenty dyne/cm^2 shear stress completely suppressed the activation of caspase-3 induced by both serum withdrawal and bFGF withdrawal (Fig. 7B).

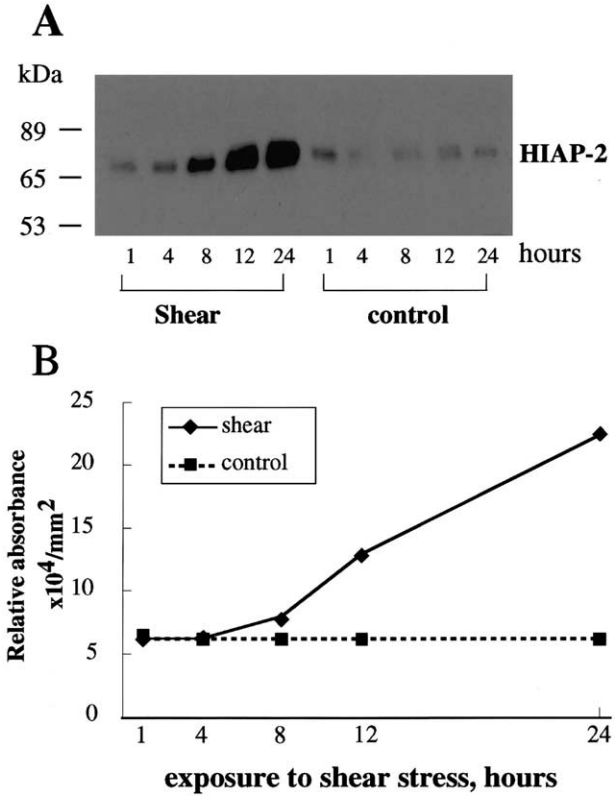

Fig. 4. HIAP-2 protein expression in ECs by shear stress (20 dyne/cm^2), analyzed by immunoprecipitation and Western blotting (A). Immunoblots were analyzed with the public domain NIH image program and data are expressed as relative ratio to background (B).

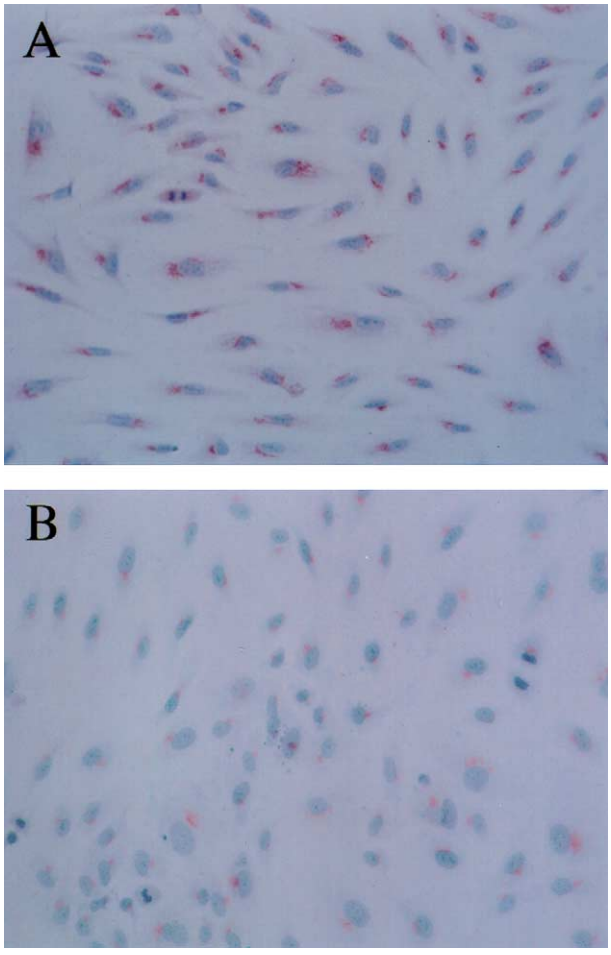

Fig. 5. HIAP-2 protein expression in cultured ECs by shear stress. After HUVECs were exposed to 20 dyne/cm^2 of shear stress (A) or maintained in the static condition (B) for 24 h, the cells were stained with anti-HIAP-2 antibody by the avidin–biotin–immunoperoxidase method.

To obtain direct evidence that shear stress-induced HIAP-2 inhibits caspase-3 activity, we transfected Smac, a caspase activator, by binding IAPs including HIAP-1, HIAP-2, XIAP, and survivin and removing their inhibitory activity [13]. After confirmation of Smac expression in transfected ECs (Fig. 8A), ECs were exposed to shear stress. Increased activity of caspase-3 in apoptotic ECs with pcDNA 3.1(−) was suppressed by shear stress about 75%. However, this suppression effect of shear stress was decreased to 51% in the cells with pcDNA-Smac (Fig. 8C). Simultaneously, the amount of HIAP-2 protein associated with Smac in ECs with pcDNA-Smac increased as a result of shear stress (Fig. 8B).

4. Discussion

The data in this paper demonstrate that laminar shear stress inhibits EC apoptosis by the suppression of caspase-3 activity, in agreement with other studies [7,8]. This shear stress-induced suppression of apoptosis seems to be mediated by the down-regulation of FAS receptor expression, up-regulation of anti-apoptotic Bcl-X_L [7], and activation of Akt kinase [15]. The

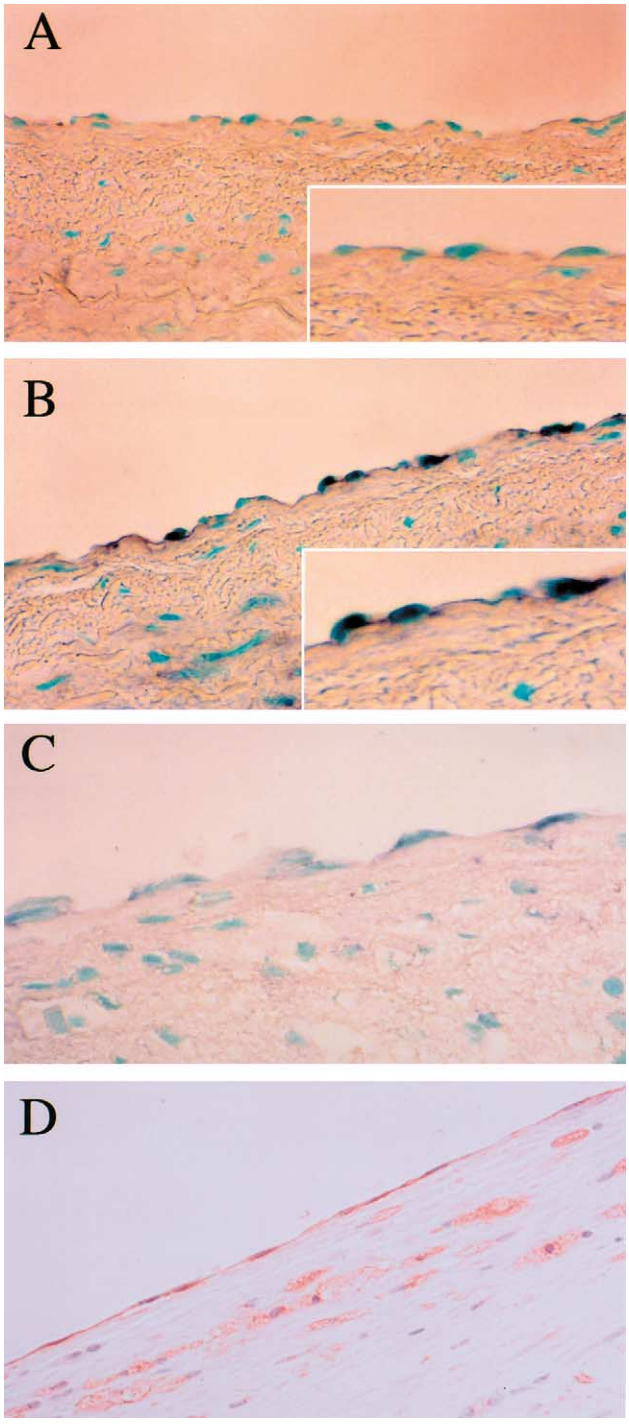

Fig. 6. HIAP-2 mRNA and protein expression in endothelium of bovine (A,B) and human aortas (C,D). No signal was detected by hybridization with sense probe (A), while ECs hybridized with antisense probe show a positive signal (B,C). Abdominal aorta of a 73 year old man was stained with antibody (D). The insets show higher magnification views.

present study demonstrates a new mediator, HIAP-2, of anti-apoptotic pathways induced by shear stress. Laminar shear stress promoted the transcription of HIAP-2 in ECs and maintained the HIAP-2 protein expression at a high level, even after the mRNA expression was diminished. Accumulation of HIAP-2 protein could have occurred as a consequence of the low degradation rate.

Two human IAPs, the human homolog of murine c-IAP-1, were first identified as the proteins associated with tumor necrosis factor receptor 2 (TNFR2), which mediates TNF-induced cell survival [16]. So far, six IAPs have been found in humans: HIAP-1, HIAP-2, XIAP (hILP), NAIP, survivin and BRUCE. HIAP-2 and HIAP-1 have been shown to bind and inactivate activated caspase-3, -7 and -9, as well as procaspase-9, for inhibition of both mitochondria-mediated and receptor-mediated apoptosis (for review, see [14,17]). IAPs are

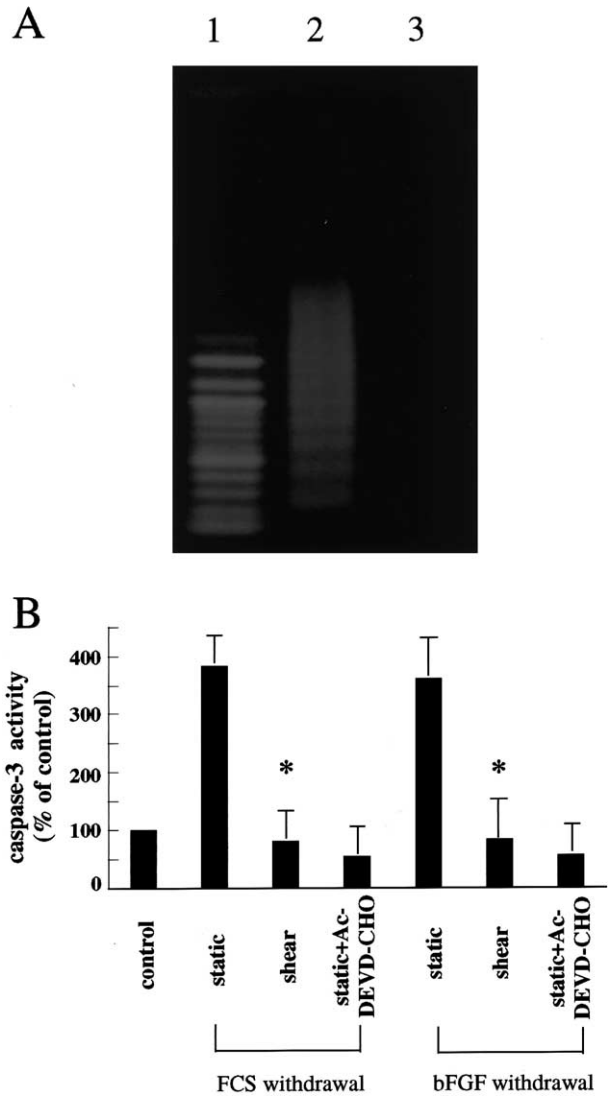

Fig. 7. Inhibition of EC apoptosis and caspase-3 activity by shear stress (20 dyne/cm^2). A: After being exposed to the shear stress in DMEM without serum for 36 h (lane 3), DNA fragmentation of BAECs was analyzed by electrophoresis. Lanes 1 and 2 show a molecular marker and the static control ECs, respectively. B: Apoptosis of HUVECs was induced by either FCS or bFGF depletion. Caspase-3 activity was assayed by measuring the liberated fluorescent AMC from the substrate. The relative value to the static control in DMEM containing both 20% FCS and 5 ng/ml bFGF, which was set at 100%, was calculated in each experiment and the results are expressed as mean ± S.D. of the number of observations ($n = 3$). *$P < 0.01$ vs. static ECs. Ac-DEVD-CHO was used as a positive control.

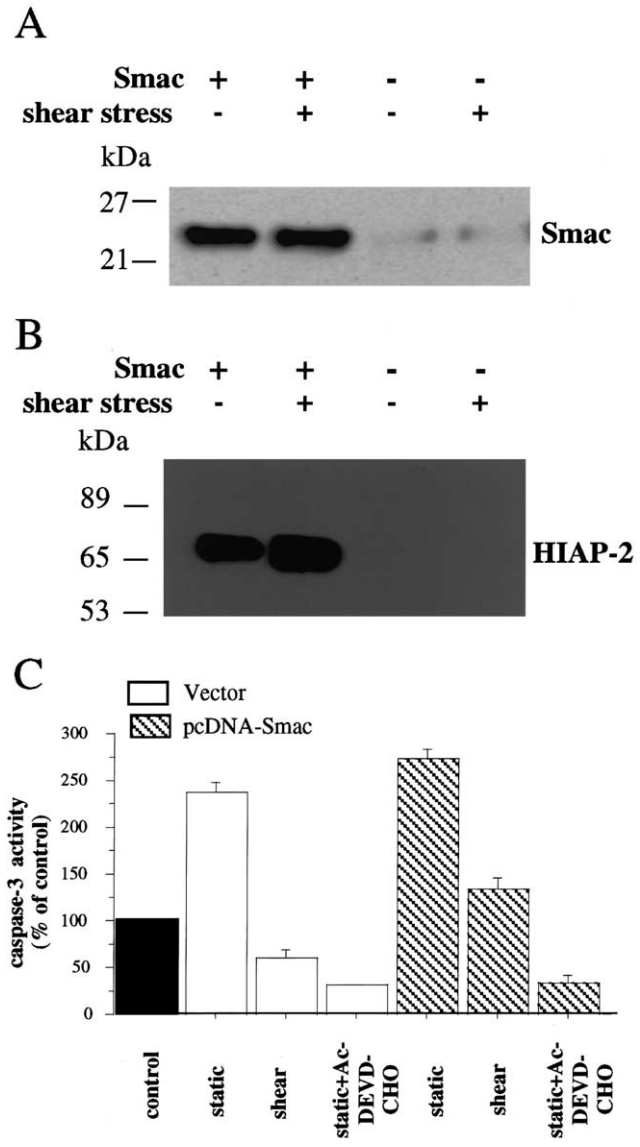

Fig. 8. Down-regulation of caspase-3 by shear stress was interfered with by Smac. HUVECs were transfected with either pcDNA 3.1(−) vector carrying full-length Smac and Flag tag or vector alone. After confirmation of Smac induction with immunoprecipitation and Western blotting using anti-Flag M2 antibody (A), transfected ECs were exposed to the shear stress (20 dyne/cm^2) for 12 h in the medium without bFGF. The cell extracts were first immunoprecipitated with anti-Flag M2 antibody, and then immunoblotted with anti-HIAP-2 antibody (B). Caspase-3 activity was measured and the results are expressed as described in Fig. 7 (C).

negatively regulated by at least two regulatory proteins, Smac and Omi/HtrA2, a serine protease. The two are released from the mitochondria in response to apoptotic stimuli including serum deprivation or UV irradiation and can induce apoptosis in a caspase-dependent manner via its ability to disrupt caspase–IAP interaction [18]. In this study, shear stress significantly suppressed caspase-3 activation and apoptotic cell death induced by the serum or growth factor withdrawal, while this suppression effect of shear stress was inhibited by the complex formation of HIAP-2 with Smac, suggesting that the inhibition of apoptosis by laminar shear stress may result from the binding of shear stress-induced HIAP-2 to activated caspase-3, and that this shear stress-induced HIAP–caspase interaction is negatively regulated by Smac, and possibly by other regulatory proteins, like Omi/HtrA2.

Mediators other than HIAP-2, or the cooperation of other proteins with HIAP-2, cannot be ruled out in the mechanisms of shear stress-induced inhibition of apoptosis. Recently, evidence has shown that p21$^{Sdi1/Cip1/waf1}$, a cell cycle regulator, binds procaspase-3 to inhibit epithelial cell apoptosis [19], and that p21$^{Sdi1/Cip1/waf1}$ prevents the down-regulation of c-IAP in apoptotic peripheral blood cells [20]. Together with our data that p21$^{Sdi1/Cip1/waf1}$ was significantly up-regulated by laminar shear stress in ECs [5], these data suggest that p21$^{Sdi1/Cip1/waf1}$/procaspase-3 complex formation, or collaboration of HIAP-2 with p21$^{Sdi1/Cip1/waf1}$ may also contribute to the shear stress-mediated apoptosis inhibition.

HIAP-2 was expressed in various human organs [16,21]. The present study first showed that the endothelium of the human aorta not only expresses HIAP-1, as shown by another study [22], but also HIAP-2.

Impairment of the regulation of apoptotic cell death and cell growth may contribute to many diseases including restenosis and heart failure [23,24]. Stabilization of ECs through the regulation of cell proliferation and death by laminar shear stress may contribute to the formation of the anti-atherosclerotic microenvironment in vessels.

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