Shear stress inhibits apoptosis of human endothelial cells

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Abstract Physiological levels of shear stress alter the genetic program of cultured endothelial cells and reduce endothelial cell turnover in vivo. To test the hypothesis that shear stress interferes with programmed cell death, apoptosis was induced in human umbilical venous endothelial cells by growth factor withdrawal or incubation with tumor necrosis factor α (TNFα) for 18 h. Apoptosis was quantified by ELISA specific for histone-associated DNA fragments and confirmed by demonstrating the specific pattern of internucleosomal DNA fragmentation detected by electrophoresis and immunohistochemical staining. The TNFα (300 U/ml)-mediated increase in DNA fragmentation was completely abrogated by shear stress. Furthermore, shear stress dose-dependently reduced DNA fragmentation induced by growth factor withdrawal with maximal effect at 45 dyn/cm^2. Inhibition of the CPP32-like proteases with Ac-DEVD-CHO (100 μM) revealed similar anti-apoptotic effects. In contrast, CPP32-independent induction of endothelial cell apoptosis by C_2-ceramide (50 μM) was not prevented by shear stress. Thus, we propose that shear stress interferes with common cell death signal transduction involving the CPP32-like protease family and may contribute to endothelial cell integrity by inhibition of apoptosis.

Key words: DNA fragmentation; Tumor necrosis factor α; Serum depletion; ICE-like protease

1. Introduction

Shear stress has been shown to alter the genetic growth program of cultured endothelial cells [1] and reduces endothelial cell turnover in vivo [2]. Endothelial cell turnover might be due either to increased proliferation or to reduced cell death. Apoptosis or programmed cell death is a preprogrammed death pathway that is constitutively expressed in many cells, albeit in an inactive form [3], and refers to the morphological alterations that include cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation [4,5]. Apoptotic cell death results from transduction of death signals triggered by a wide variety of exogenous stimuli [6]. We and others have previously shown that the cytokine tumor necrosis factor α (TNFα) or other inflammatory stimuli affect endothelial cell viability and induce apoptosis in vitro in bovine, porcine and human endothelial cells [7–9]. Moreover, withdrawal of survival factors has been demonstrated to cause apoptosis of endothelial cells [10]. Apoptosis is mediated by multiple pathways that involve a complex array of biochemical regulators and molecular interactions. Although the upstream signalling of apoptosis is uncertain, the family of cysteine proteases, the interleukin-1β converting enzyme (ICE)-like proteases as well as CPP32-like proteases, have been implicated in apoptosis induced by TNFα and other stimuli [11,12], suggesting an important role in signal transduction of apoptotic signals.

Since shear stress alters diverse physiological responses of endothelial cells [13], we therefore hypothesized that shear stress may affect endothelial cell apoptosis induced by exogenous stimuli or by withdrawal of survival factors.

2. Materials and methods

2.1. Reagents

HUVEC, endothelial basal medium and supplements were purchased from Cell Systems/Clonetics, Solingen, Germany, and FCS and the DNA molecular weight marker were from Gibco, Berlin, Germany. [^{32}P]dCTP was delivered by Amersham, Braunschweig, Germany. Klenow polymerase and DNA fragmentation assay were from Boehringer Mannheim, Mannheim, Germany. Ac-YVAD-CHO and Ac-DEVD-CHO were from Bachem Biochemica GmbH, Heidelberg, Germany and C_2-ceramide was from Biomol, Hamburg, Germany.

2.2. Cell culture and shear stress exposure

HUVEC were cultured in endothelial basal medium supplemented with hydrocortisone (1 μg/ml), bovine brain extract (12 μg/ml), gentamicin (50 μg/ml), amphotericin B (50 ng/ml), epidermal growth factor (10 ng/ml), and 10% foetal calf serum until the third passage. After detachment with trypsin, cells were grown for at least 18 h. Confluent monolayers of HUVEC were grown onto 6-cm wells and exposed to laminar fluid flow in a cone-and-plate apparatus as described [14,15]. A cone of shallow angle (cone angle: 5°) rotates with a controlled frequency of 300 Hz (5 dyn/cm^2), 600 Hz (15 dyn/cm^2) or 900 Hz (45 dyn/cm^2) and thereby applies fluid laminar shear stress of controlled magnitude to the endothelial monolayer grown on the plates.

2.3. DNA fragmentation

DNA-fragmentation analysis was carried out as described in [16]. Cells (5×10^5 cells) were scraped off the plates and centrifuged at $700\times g$ for 10 min, washed with PBS and resuspended in 500 μl incubation buffer. After centrifugation ($20\,000\times g$, 15 min) the supernatants containing the histone-associated DNA fragments were diluted 1:20 in incubation buffer and were linked to the anti-histone antibody from mouse according to the manufacturer's protocols. Then the DNA part of the nucleosome was bound to anti-DNA-peroxidase. The amount of peroxidase retained in the immunocomplex was determined photometrically with 2,2′-azinodi[3-ethylbenzthiazoline sulfonate] as a substrate. DNA fragmentation was calculated as percent of control values [(optical density of the sample−blank)/(optical density of the control−blank)×100].

2.4. DNA isolation and Klenow labeling

1×10^6 cells were removed from the culture flask, washed with PBS and incubated in lysis buffer (5 mM Tris-HCl, pH 8, 20 mM EDTA and 0.5% Triton X-100) for 15 min at 4°C. After centrifugation for 20 min at $20\,000\times g$ at 4°C the supernatants were treated with RNase A for 1 h at 37°C. A final concentration of 0.5 mg/ml proteinase K and 1% SDS were added and the samples were incubated overnight at 65°C. After isolation of DNA by phenol-chloroform extraction the DNA was precipitated with 70% isopropanol and 0.1 M NaCl. The resulting pellet was resolved in TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA) and the DNA samples were incubated with 5 U of Klenow polymerase, 0.5 μCi [^{32}P]dCTP in the presence of 10 mM Tris-HCl, pH 7.5 and 5 mM $MgCl_2$ for 10 min at room temperature

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according to Rösl et al. [17]. The reaction was terminated by addition of 10 mM EDTA and unincorporated nucleotides were removed by using Sephadex G-50 columns. Labeled DNA fragments were separated on a 1.8% agarose gel, transferred to nitrocellulose membranes and exposed to X-ray film.

2.5. Determination of cell viability and LDH release

Cell viability was assessed as described [7]. HUVEC (1×10^5 cells/ml) were incubated in microtiter plates for 18 h with apoptotic stimuli. Subsequently, cells were treated with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/ml) for 4 h at 37°C, the medium was removed and cells were lysed with 2-isopropanol containing 0.04 M HCl. The metabolized MTT was determined photometrically (595 nm).

For measurement of lactate dehydrogenase (LDH) levels a kit was used (Boehringer Mannheim, Germany). 1×10^5 cells were seeded into 12-well plates. The cell culture supernatant was incubated with pyruvate and NADH and the LDH activity was determined photometrically according to the manufacturer's protocols.

2.6. Immunohistochemistry

Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) was performed as outlined [16] using an in situ cell death detection kit (Boehringer Mannheim, Germany). Cells were washed with PBS and fixed in 4% formaldehyde dissolved in PBS for 30 min at room temperature. After permeabilization (0.1% Triton X-100 in 0.1% sodium citrate) the cells were stained with TUNEL reaction mixture containing fluorescein-labeled nucleotides and visualized using alkaline phosphatase conjugated anti-fluorescein antibodies. After substrate reaction with fast red, apoptotic cells were stained and analysed by light microscopy.

3. Results

3.1. Apoptosis of endothelial cells

Apoptosis is characterized by specific cleavage of the DNA by endonucleases resulting in histone-associated DNA fragments with a length of 180 bp and its multiples. These characteristic DNA fragments were detected using three different techniques such as ELISA specific for histone-associated DNA fragments, separation of DNA fragments in agarose gels demonstrating the typical 180 bp DNA ladder, and immunohistochemical staining of single-stranded DNA fragments by labeling with deoxynucleotidyltransferase as recommended by Sgonc and Wick [18].

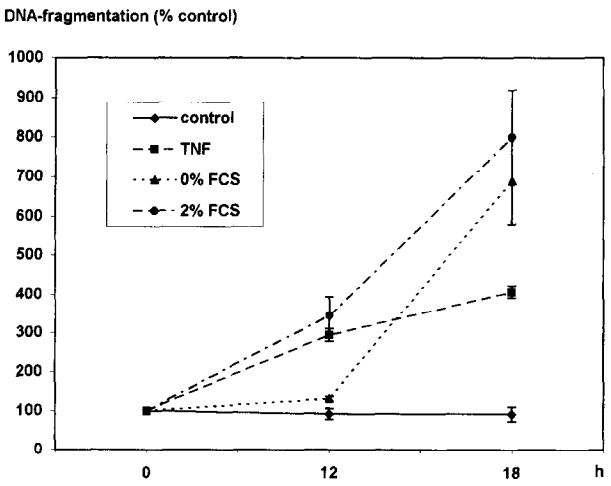

Fig. 1. Time-dependent effect of TNFα or serum depletion on apoptosis in HUVEC. HUVEC were incubated without FCS (0%), in the presence of 2% FCS or with 300 U/ml TNFα for the times indicated. Then DNA fragmentation was determined by ELISA (see Section 2). Data are means ± S.E.M., $n = 3$.

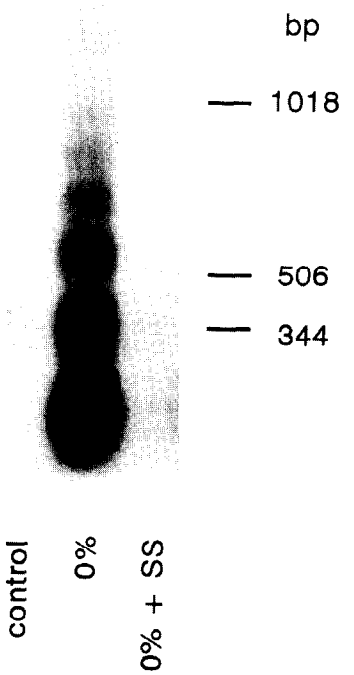

Fig. 2. Agarose gel electrophoresis of DNA fragments. HUVEC were incubated in the absence of serum (0%) with or without additional shear stress (45 dyn/cm^2) for 18 h. Then, DNA fragments were extracted and radiolabeled as described in Section 2 and loaded on 1.8% agarose gels. A representative autoradiograph of three independent experiments is shown.

Incubation with TNFα (300 U/ml) for 18 h increased DNA fragmentation as a measure of induced apoptosis about 4-fold when compared to controls as detected by ELISA (Fig. 1). Elimination of FCS resulted in a 7-fold higher level of DNA fragmentation compared to control cells, whereas up to 8-fold increase in DNA fragmentation was detectable when HUVEC were grown in the presence of 2% FCS (Fig. 1).

Induced DNA fragmentation increased in a time-dependent fashion with maximal effects after 18 h incubation in the presence of 0%, 2% FCS or 300 U/ml TNFα (Fig. 1) and correlated with a decrease of cell viability by 29 ± 2.6, 35 ± 4.2 and 22 ± 6%, respectively, compared to controls. In order to exclude necrosis as a potential side effect of treatment, the release of lactate dehydrogenase (LDH) was measured. No significant increase in LDH release was observed after 18 h incubation with apoptotic stimuli excluding the induction of cell necrosis. However, prolonged incubation for more than 24 h led to significant LDH release (data not shown).

As shown in Fig. 2, these results were confirmed by direct visualization of [^{32}P]dCTP-labeled DNA fragments separated by gel electrophoresis. A typical DNA ladder was detected after incubation of HUVEC with 0% FCS (Fig. 2) as well as with TNFα (data not shown).

3.2. Effect of shear stress on endothelial cell apoptosis

The effect of shear stress was studied by exposure of endothelial cells to laminar steady shear stress in a cone-and-plate apparatus in the presence or absence of apoptosis-inducing stimuli. Laminar shear stress for 18 h dose-dependently decreased apoptosis induced by FCS depletion (Fig. 3). Induc-

tion of apoptosis with TNFα was similarly affected by shear stress. Exposure of HUVEC to laminar flow (15 dyn/cm^2) completely abrogated apoptosis induced by TNFα and reduced DNA fragmentation even below baseline levels (Fig. 4), whereas 5 dyn/cm^2 was less effective (data not shown). Moreover, laminar shear stress significantly reduced apoptosis induced by reduction to 2% FCS (from 830 ± 128% to 386 ± 114%, $p = 0.02$) as measured by ELISA (control cells are set as 100%). The appearance of the typical DNA ladder of 180 base pairs induced by FCS depletion or TNFα was dramatically reduced following 18 h exposure to shear stress (45 dyn/cm^2) (Fig. 2 and data not shown, respectively) confirming the results obtained by ELISA. Furthermore, shear stress-mediated reduction of apoptosis was also demonstrated by immunohistochemical detection of DNA fragments with terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (Fig. 4).

3.3. *Potential mechanisms of shear stress-induced inhibition of apoptosis*

Shear stress transcriptionally increases growth factors such as basic fibroblast growth factor (bFGF) [19] which may prevent FCS-depletion induced apoptosis [10]. Neutralizing antibodies against basic fibroblast growth factor (1 μg/ml), however, did not prevent the protective effect of shear stress (331 ± 60% with 0% FCS and shear stress vs. 292 ± 75% with additional anti-bFGF), suggesting the interaction of shear stress with a common pathway of TNFα- and FCS-depletion-induced apoptosis. The inhibitory effect of shear stress on TNFα- and FCS-depletion-induced apoptosis was mimicked by the inhibition of the interleukin-1β converting enzyme (ICE)-like and CPP32-like cysteine proteases, an important common pathway of apoptosis signal transduction [11]. The CPP32-like inhibitor Ac-DEVD-CHO [20] inhibited TNFα- as well as FCS-depletion-induced apoptosis (Fig. 5). Similar results were achieved with the ICE-like protease inhibitor Ac-YVAD-CHO (100 μM) [12] (data not shown). Interestingly, induction of ICE-independent apoptosis by exogenous C_2-ceramide [21] was inhibited by neither CPP32 inhibitors nor shear stress (Fig. 5).

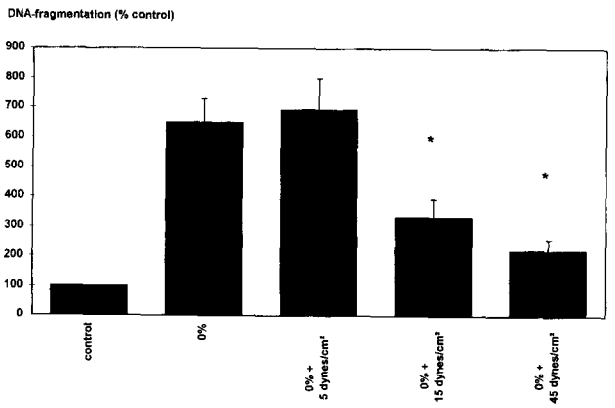

Fig. 3. Dose-dependent effect of shear stress on DNA fragmentation induced by FCS depletion. HUVEC were incubated for 18 h without FCS (0%) and with additional shear stress (SS) of the indicated magnitude and DNA fragmentation was detected by ELISA (see Section 2). Results are means ± S.E.M., $n = 4$. *$p < 0.05$ vs. 0%.

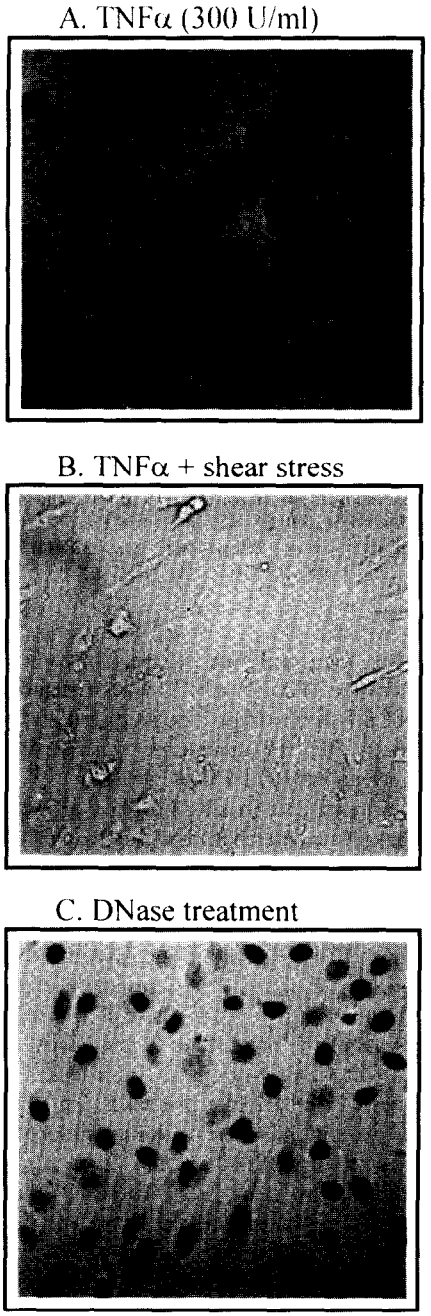

Fig. 4. Immunohistochemical detection of apoptosis induced by TNFα and protective effect of shear stress. Endothelial cells were stained as outlined in Section 2 after treatment with TNFα (300 U/ml) (A), with TNFα and shear stress of 15 dyn/cm^2 (B) for 18 h. DNase 1 treated control cells served as positive control (C). Original magnification ×10 (A,B) and ×40 (C).

4. Discussion

The results of the present study demonstrate that shear stress abrogates apoptosis of endothelial cells induced by TNFα as well as by serum withdrawal. These findings suggest that shear stress significantly contributes to endothelial cell integrity by inhibition of programmed cell death.

The presence of apoptosis was confirmed by three different techniques for detecting the characteristic DNA fragments. An ELISA technique, specific for oligosomal histone-bound

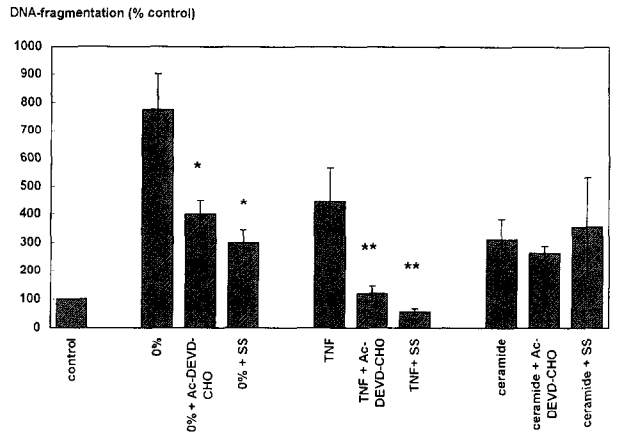

Fig. 5. Effect of shear stress and inhibition of CPP32. HUVEC were incubated with TNFα (300 U/ml), C_2-ceramide (50 μM) or with serum-free medium (0%) for 18 h with or without additional shear stress (SS) or the CPP32 inhibitor Ac-DEVD-CHO (100 μM). DNA fragmentation was determined by ELISA. Results are means ± S.E.M., $n = 3$ with $^{*}p < 0.05$ vs. 0%, $^{**}p < 0.05$ vs. TNFα.

DNA fragments, agarose-DNA gel electrophoresis, ensuring the specific cleavage of DNA [3,17], and immunohistochemical detection of DNA strand breaks. Additionally, the appearance of necrosis after the incubation time investigated was excluded by measurement of LDH release.

Enhanced expression of basic fibroblast growth factor by shear stress has been described and might contribute to the protective effect of shear stress on apoptosis by serum depletion, because this growth factor may reduce apoptosis due to FCS depletion [10]. However, neutralizing antibodies against bFGF were not effective in inhibiting apoptosis induced by FCS depletion, suggesting that additional mechanisms must be operative in abrogating endothelial cell apoptosis by shear stress. The results of the present study demonstrate that shear stress interferes with apoptotic signal transduction above or at the level of the ICE/CPP32-like proteases. This hypothesis is supported by the finding that ceramide-induced apoptosis which does not require CPP32- or ICE-like protease activity [21] was not affected by shear stress. The CPP32-like protease activity, but not its transcriptional levels, was reduced by shear stress (data not shown), suggesting posttranslational modification or involvement of shear stress in the signal transduction upstream of ICE/CPP32-like proteases or directly on the ICE/CPP32-like protease family.

Injury of the vascular endothelium is a critical event early in the pathogenesis of atherosclerosis [22]. Importantly, atherosclerotic lesions preferentially develop in regions with low shear stress or even turbulent blood flow [23] suggesting a protective role of physiological levels of shear stress for the functional integrity of endothelial cells [24]. Several studies have demonstrated that physiological levels of shear stress induce significant alterations in endothelial cell structure and function, including increased production of tPA [25], nitric oxide [14] and prostacyclin [26], decreased synthesis of endothelin-1 [1], and increased levels of TGFβ [1], all of which are recognized to exert potent anti-atherosclerotic functions. Indeed, it has been shown that increased blood flow inhibits neointimal hyperplasia in endothelialized vascular grafts [27]. The results of the present study considerably extend these previous findings by demonstrating that shear stress protects endothelial cells from being driven into apoptosis upon exogenous stimulation with TNFα as well as upon withdrawal of survival factors. Thus, shear stress might importantly contribute to the integrity of the endothelial cell layer and thereby inhibit damage to the arterial vascular wall. This conclusion is also strongly supported by experimental in vivo findings showing that endothelial cells of lesion-prone regions, which are associated with low shear stress or even turbulent blood flow, are characterized by an increased endothelial cell turnover rate [2] suggesting a link between low or absent shear stress and endothelial cell death.

In summary, the results of the present study demonstrate that shear stress abrogates endothelial cell apoptosis induced by different stimuli. Shear stress-mediated inhibition of endothelial cell apoptosis might importantly contribute to the functional integrity of the endothelial cell layer and thereby inhibit damage to the arterial wall, which is a key event for initiating atherosclerotic lesion development.

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