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Proatherogenic Flow Conditions Initiate Endothelial Apoptosis via Thrombospondin-1 and the Integrin-Associated Protein

M. A. Freyberg,* D. Kaiser,* R. Graf,† J. Buttenbender,‡ and P. Friedl†,[1]

†*Institut für Biochemie, Technical University Darmstadt, Petersenstrasse 22, D-64287 Darmstadt, Germany;*
**CytoTools GmbH, Petersenstrasse 22, D-64827 Darmstadt, Germany; and*
‡*Institut für Strömungslehre, Petersenstrasse 30, D-64287 Darmstadt, Germany*

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Recently it has been shown that vascular endothelial cells (EC) are completely devoid of apoptosis if cultivated under a steady laminar flow and that apoptosis is induced by turning off the flow. An autocrine loop of thrombospondin-1 (TSP-1) and the $\alpha_v\beta_3$ integrin/integrin-associated protein (IAP) complex has been identified as the molecular coupling device between flow and apoptosis. Lack of blood flow is a rare and mostly transient phenomenon whereas irregular flow conditions are permanently present at arterial bifurcations and sites of abnormal vessel morphology. Irregular flow conditions are established here either by the action of a cone-and-plate type flow apparatus generating a uniform turbulent flow or in a flow chamber by insertion of a local hindrance creating a zone of unsteady laminar flow with vortex formation and lowered shear stress. In both cases apoptosis is induced either throughout the entire monolayer or restricted to the locally defined area. Flow disturbance and apoptosis are coupled by the described autocrine loop of TSP-1 and the integrin/IAP receptor complex. *In vivo* atherosclerotic lesions occur predominantly at sites of flow irregularities, which are thought to be pro-atherogenic. Thus we propose a key role of the identified mechanosensitive apoptosis induction for the initiation of atherosclerosis.

Key Words: **apoptosis; endothelium; thrombospondin-1; integrin; integrin-associated protein; atherosclerosis; hemodynamics; shear stress.**

The EC of the vascular system constitute the inner lining of all blood vessels. They are involved in the regulation of various physiological processes such as vascular tone or vascular remodeling. A variety of pathological situations are related to endothelial malfunctions as for example focal development of atherosclerosis. The hemodynamic forces resulting from the blood flow *in vivo* play an important role in the growth and structure of blood vessels: flow direction and flow rate not only modulate cell shape and intracellular microfilament alignment (1, 2) but also regulate expression of various genes (3). Furthermore hemodynamic forces protect EC against damaging stimuli (4, 5).

Recently it has been shown that fluiddynamic forces are *per se* essential for endothelial survival: lack of such forces leads to onset of apoptosis in confluent EC monolayers and organ cultures (6, 7). Offset of flow and apoptosis are coupled via an autocrine loop of TSP-1 secreted by EC and its receptor complex of $\alpha_v\beta_3$ integrin and IAP presented on the endothelial membrane (8). Here we investigate the influence of irregular flow conditions leading to severe deviations from steady laminar flow, asking the question whether a disturbance of regular flow will also activate the autocrine loop described for the lack of hemodynamic forces? The results will be discussed taking into consideration the implications for atherosclerosis.

MATERIALS AND METHODS

Material. Rabbit polyclonal antiserum and mouse monoclonal antibodies against TSP-1, purified TSP-1 and mouse monoclonal antibody against IAP are supplied from Dr. Vischer (Münster), mouse monoclonal antibodies against the integrin subunit α_v from Alexis, goat anti-rabbit antibodies from Dianova (Hamburg), FITC-conjugated rabbit anti mouse antibodies and non-immune mouse IgG from Becton-Dickinson. Tissue culture flasks and plates are purchased from Falcon, tissue culture media and supplements from GIBCO except for newborn calf serum from Sebak (Munich).

Abbreviations used: EC, endothelial cells; HUVEC, human umbilical cord vein EC; IAP, integrin-associated protein (CD47); TSP-1, thrombospondin-1.

[1] To whom correspondence should be addressed. Fax: +49-6151-164759. E-mail: di58@pop.tu-darmstadt.de.

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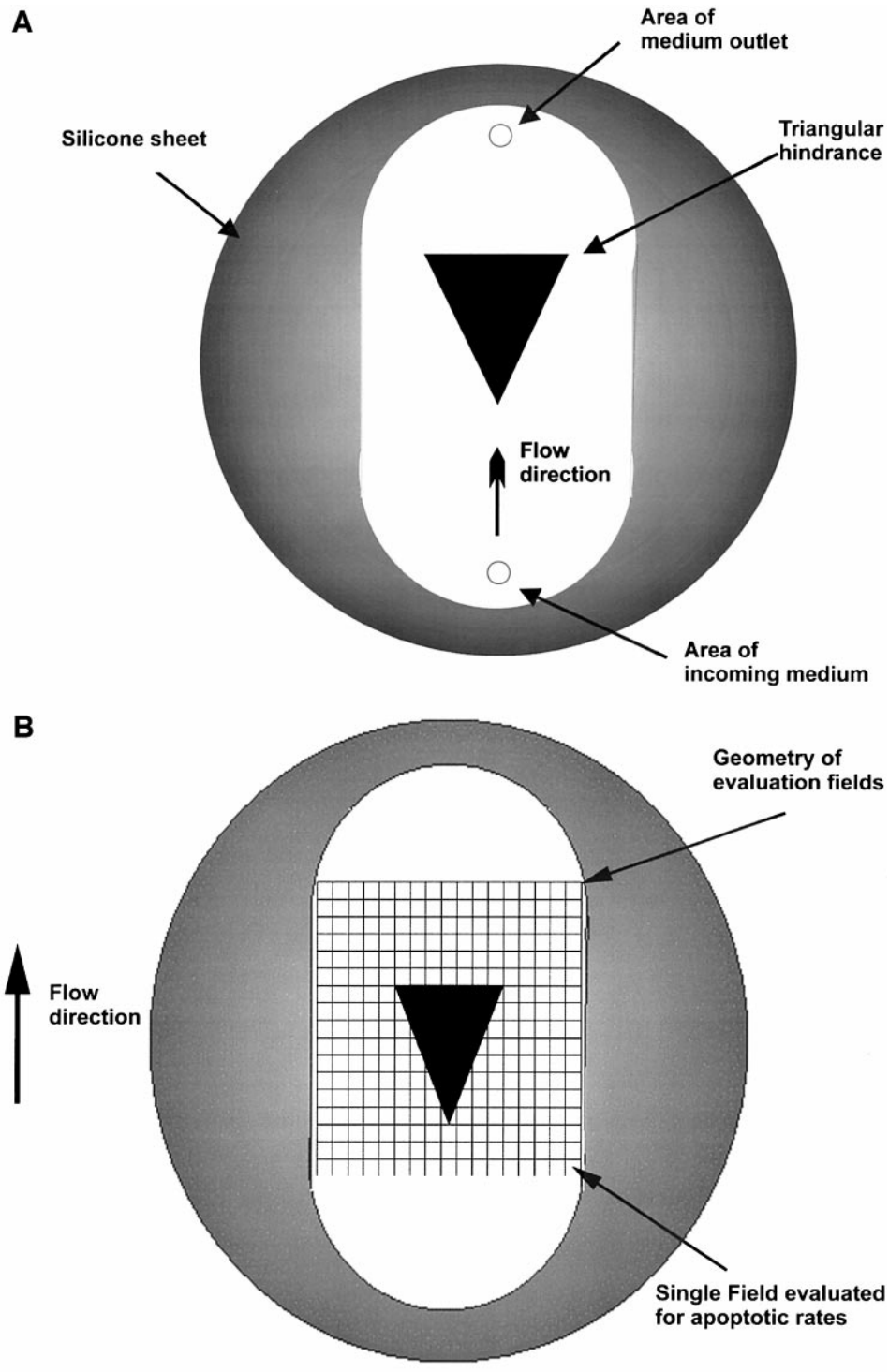


FIG. 1. Geometry of the perfusion area. (A) The geometry of the chamber's flow zone and the placement of the triangular hindrance are shown. (B) The arrangement and size of the single fields evaluated for apoptotic rates are shown.

Cell culture. HUVEC are prepared and maintained in culture as previously described (9–11). For the assays described below cells are seeded in 35 mm cell culture dishes and grown to confluence. For a cultivation under defined flow conditions the plate is converted into a flow chamber as described previously (12) otherwise the cultivation is continued as "static". For the simulation of flow disturbances either a steric hindrance—a triangular sheet of silicon—is inserted into the flow chamber or the cells are exposed to turbulent shear stress in a cone-and-plate-type flow apparatus (13).

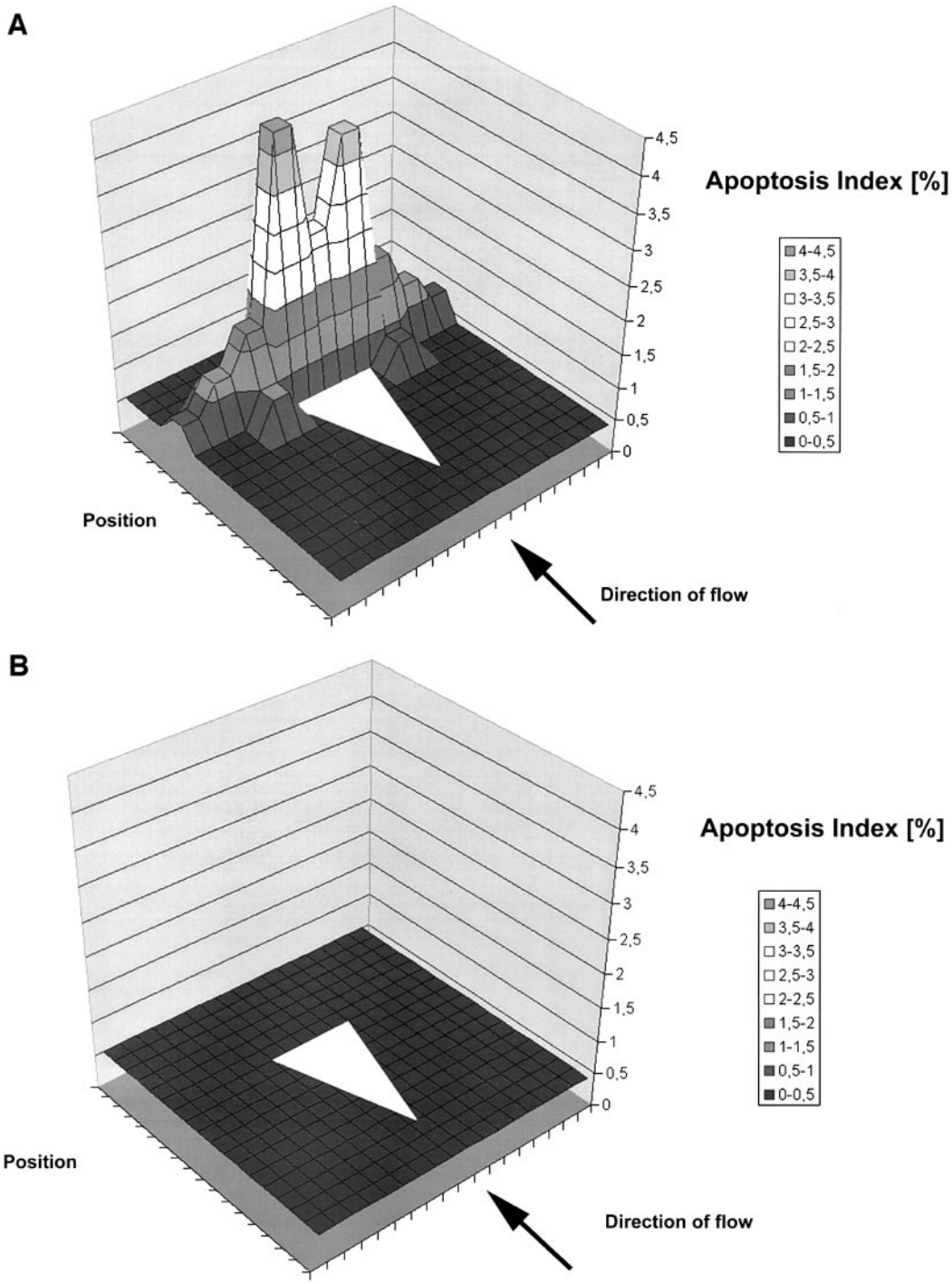


FIG. 2. Apoptosis in areas of disturbed flow. HUVEC are cultivated in 35-mm dishes. Upon reaching confluence, the dish is converted into a perfusion chamber and cultivation continued with a mean shear stress of 5 dyn cm^{-2} (calculated for the chamber without hindrance) for an additional 3 days. Apoptotic rates (expressed as a percentage) for cell populations of individual areas are determined and plotted as bars. A was without and B was with addition of anti-receptor antibodies.

Calculation of flow parameters. Flow parameters in the chamber are calculated by the program Fluent/UNS Release 4.0 (Fluent Incorp.) according to the chamber geometry and the physical medium parameters.

Determination of apoptosis rates. At the end of the test period the cells are fixed, stained with DAPI, morphologically evaluated by fluorescence microscopy and counted with an image analysis system as previously described (14). Apoptotic cells

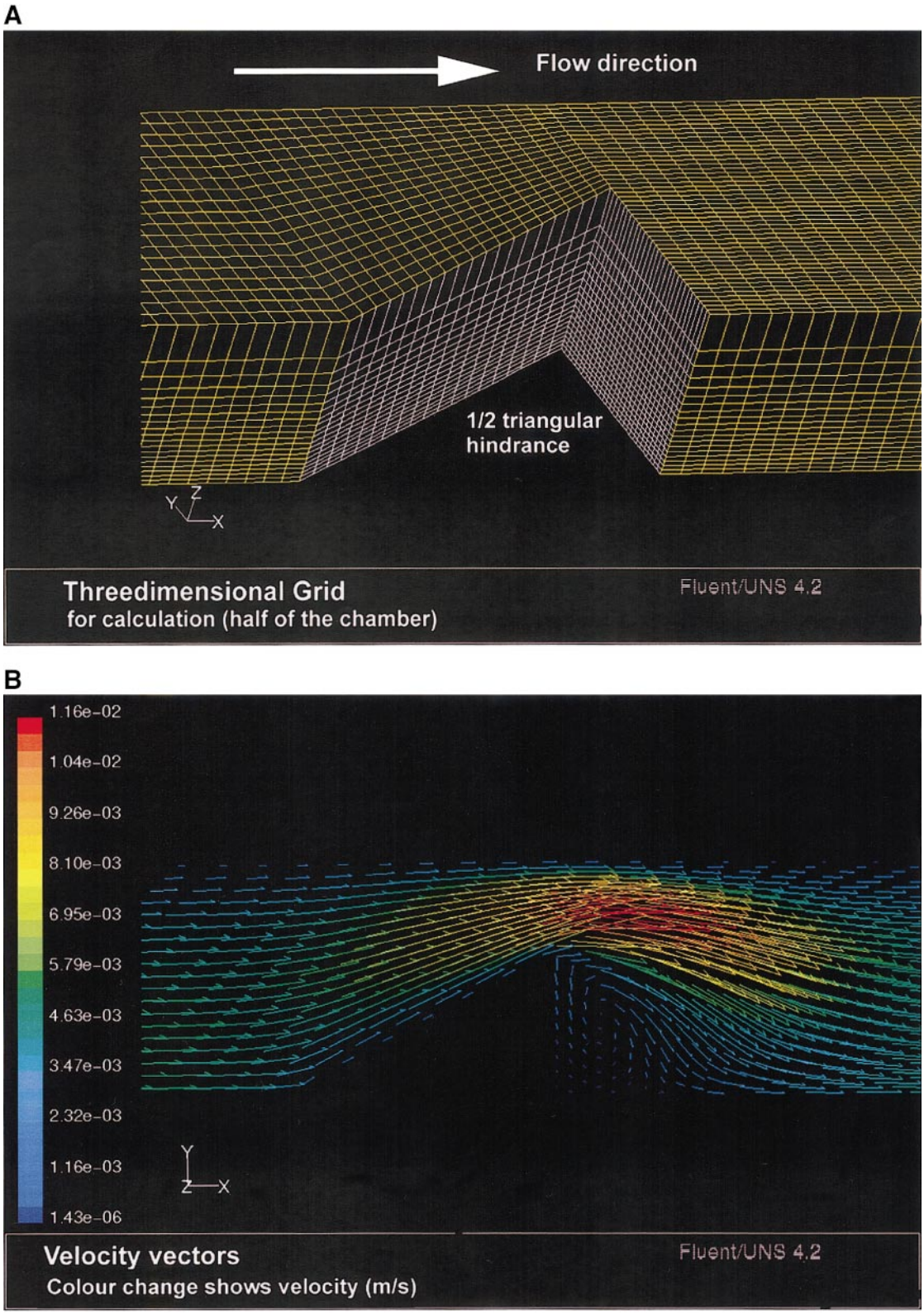
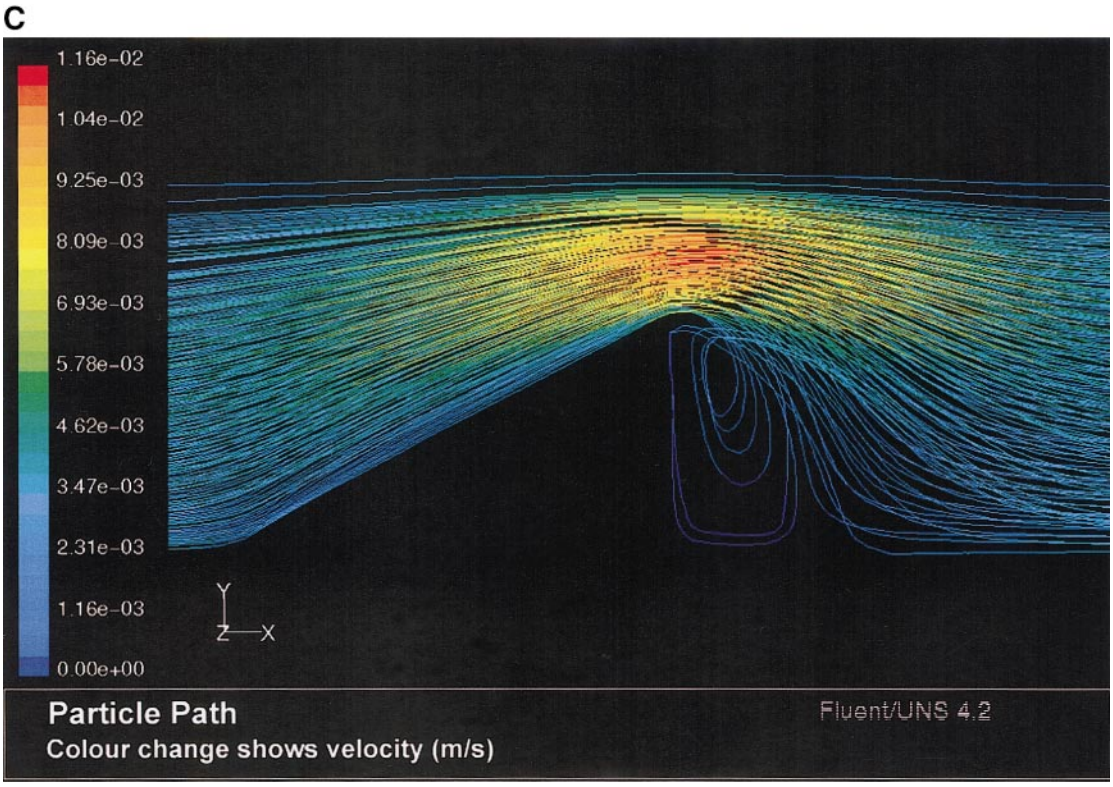


FIG. 3. Fluid dynamic parameters of the perfusion chamber. Several fluid dynamic parameters are calculated for one half of the chamber by the Fluent/UNS program using the exact geometric chamber dimensions and the physical parameters of the flowing medium. (A) The three-dimensional grid in the chamber which is used in the calculations, (B) profiles for velocity vectors, (C) particle path, and (D) wall shear stress. Flow parameters in the chamber were calculated with the Fluent/UNS program, Version 4.0 (Fluent Incorp.) based on chamber geometry and physical medium parameters (Reynolds number 40).



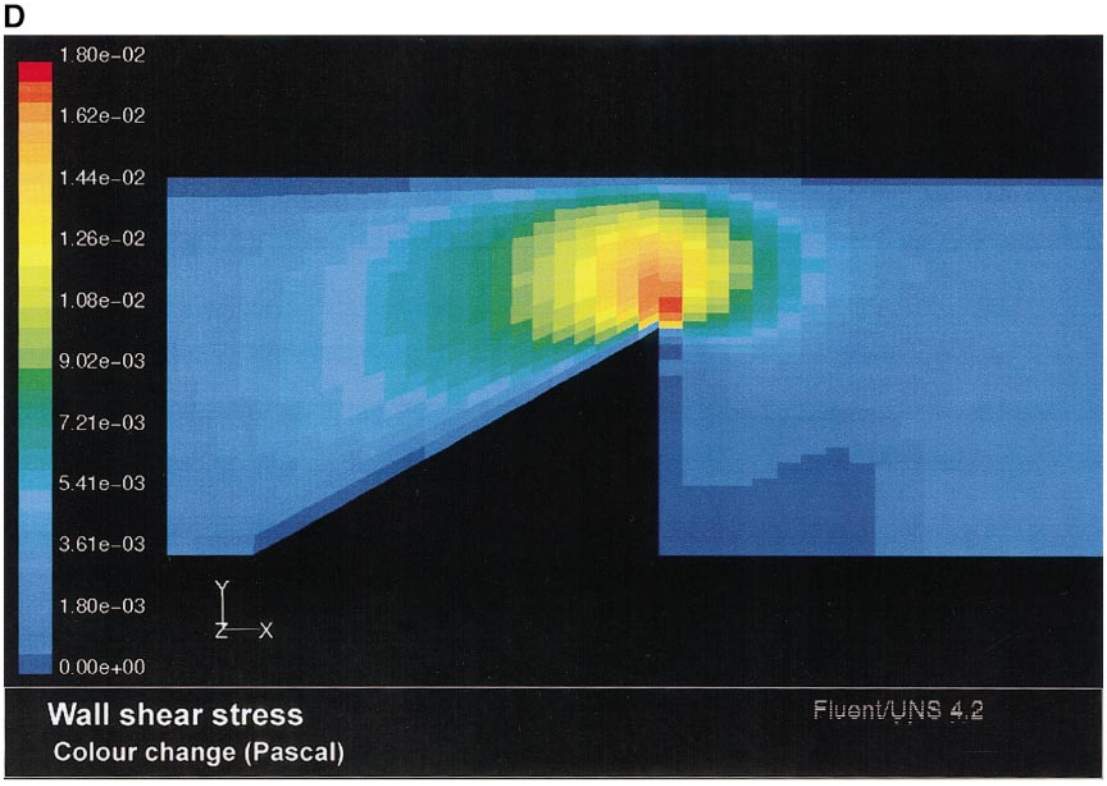


FIG. 3—*Continued*

were counted and the result expressed as percentage of apoptotic cells.

Blocking with antibodies. Blocking is achieved by medium supplementation with antibodies in the following concentrations: a 1:20 dilution of rabbit anti-TSP-1 antiserum or 50 μg ml^{-1} monoclonal anti-TSP-1 antibody for blocking of TSP-1, 50 μg ml^{-1} monoclonal anti-IAP antibody for blocking of IAP, 50 μg ml^{-1} monoclonal anti-α_v antibody for blocking of the integrin subunit α_v.

Immunostaining of IAP (CD47). Medium is aspirated, and the cells are washed twice with PBS, fixed with 2% (w/v) formalin solution on ice for 30 min, washed twice with PBS, incubated for 60 min with PBT [0,5% (v/v) Tween 20 and 0,5% (w/v) BSA in PBS], washed twice with 0,5% (v/v) Tween-20/PBS, and incubated for 120 min with mouse anti-human CD47 antibody (Cymbus Biotechnology) diluted 1:100 in 0,5% (v/v) Tween-20/PBS. The negative control is done with mouse gamma globulin (Dianova, Hamburg) at the same concentration as the CD 47 antibody. After washing three times with 0,5% (v/v) Tween-20/PBS the 1:200 diluted Cy™3-conjugated AffiniPure F(ab)$_2$ fragment Rabbit-anti-mouse IgG (Jackson ImmunoResearch) is given to the monolayer for 90 min followed by further three washes with 0,5% (v/v) Tween-20/PBS. The CD47 positive cells are detected under a fluorescence microscope (Nikon).

Sandwich ELISA against thrombospondin-1. Sandwich ELISA is performed as described previously (8).

RESULTS

The offset of flow constitutes an extreme deviation from regular laminar shear stress. Alternatively the flow itself may be altered either by establishment of turbulence as indicated by an appropriate Reynolds number or by creating an irregular laminar flow, i.e., a flow with continuous changes of flow direction or vortex formation.

Effect of Flow Disturbances on Endothelial Apoptosis

Our first step was to demonstrate *in vitro* that the change from laminar flow to flow disturbance was able to induce of apoptosis in an endothelial monolayer. For this purpose a steric hindrance was inserted into a flow chamber with a confluent monolayer of HUVEC. A triangular silicone sheet was placed in the middle of the laminar flow area (Fig. 1A) creating a zone of disturbed flow downstream from its base. The endothelial monolayer was cultivated for 3 days in this device with medium flow rates exerting an average shear stress of 1 and 5 dyn cm^{-2} (calculated for the chamber without hindrance) (12). The cell population was then evaluated for apoptosis as depicted in Fig. 1B. The whole surface was divided into the depicted areas, where apoptotic rates were determined by counting the apoptotic cells in randomly chosen microscopic fields and the percentage of apoptotic cells per area was calculated on the basis of at least 1000 evaluated nuclei. Figure 2A shows the results for cultivation with 5 dyn cm^{-2}. Behind the base of the triangular hindrance the apoptotic rates per field increased up to 4–4.5%. In the residual chamber the apoptotic rate per field was at the detection limit of 0–0.5%. Similar data was also obtained in experiments with lower shear stress (data not shown).

Calculated Fluid Dynamics

The primary effect of the inserted hindrance was a change in the profile of medium flow. Several fluid dynamic parameters were calculated using the Fluent/UNS program. The exact geometric dimensions, the chosen volume flow and the physical medium parameters were used for velocity calculations by the Navier–Stoke's equation for incompressible fluids and by calculation of local shear stress and pressure gradients with the Caucy–Poisson equation for Newton fluids. To minimize the number of plots the calculations were generated for, the Reynolds number 40 was used for only one half of the chamber. Figure 3A shows the three-dimensional grid in the chamber which was used in the calculations. The calculated patterns of shear stress (Fig. 3B) and flow streamlines (Fig. 3C) reveal a region of greatly lowered wall shear stress (Fig. 3D) as well as separation and reversal of axial flow directly behind the base of the triangular hindrance. Comparison with Fig. 2 shows that this region is identical with the area of increased apoptosis in the endothelial monolayer.

Molecular Coupling of Flow Disturbance and Apoptosis

Recently, thrombospondin-1 and the $\alpha_v\beta_3$ integrin/integrin-associated protein complex have been identified as mechanosensitive death mediators coupling the lack of hemodynamic forces with the triggering of endothelial apoptosis (8). The same molecular mechanism might also be applied to the induction of apoptosis by disturbance of laminar flow. Accordingly the effect of flow disturbances on endothelial apoptosis was investigated in the flow chamber system using the same experimental setup as described above with the addition of antibodies against TSP-1 or IAP respectively α_v or β_3 (antibodies specific to the complete $\alpha_v\beta_3$ integrin complex not only bound to its antigen but also activated the receptor and induced apoptosis, data not shown). Comparison of Fig. 2A with Fig. 2B shows that the presence of anti-IAP antibodies prevents the appearance of apoptosis in the region of disturbed laminar flow beyond the base of the triangular hindrance. The same holds true for either anti-TSP-1 antibodies or anti-α_v antibodies respectively β_3 antibodies (data not shown). Further evidence was produced in an experiment in which postconfluent HUVEC monolayers were cultivated in a cone-and-plate-type flow apparatus for 24 h either under steady laminar shear stress or under turbulent conditions with or without antibodies against

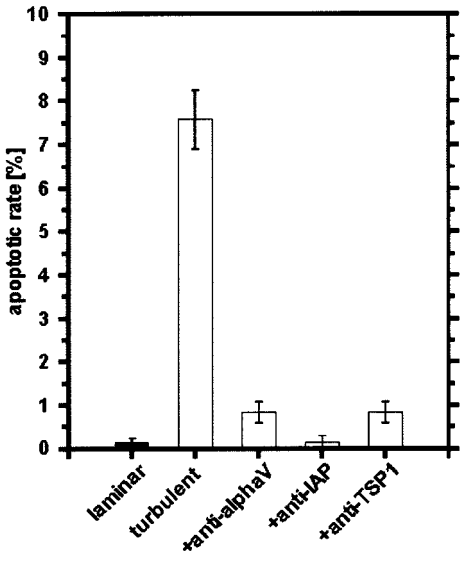


FIG. 4. Effect of turbulence on flow regulated apoptosis. HUVEC are cultivated in 35-mm dishes. Upon reaching confluence, defined flow conditions are established through conversion into a cone-and-plate-type flow apparatus. Cells were cultivated for an additional 24 h at a shear stress of 1–5 dyn cm^{-2} either under laminar flow (solid bar) or under turbulent conditions (open bars) with the indicated antibodies at a concentration of 50 $\mu g\ ml^{-1}$.

the mediators. Figure 4 shows that turbulent conditions increased the rate of apoptotic cells in the monolayer up to 7.6% compared to <0.1% under steady laminar flow. However, under turbulent conditions antibodies against TSP-1 decreased the apoptotic rate to 0.8% and antibodies against IAP respectively α_v to 0.1% as well as 0.8%.

Flow-Dependent Expression of TSP-1 and IAP

Postconfluent cultures of HUVEC were analyzed for the presence of the mediators after cultivation under steady laminar flow and with turbulence in the cone-and-plate type flow apparatus. TSP-1 was assayed in the culture supernatant by a quantitative ELISA (the SEM of 3 independent assays in brackets). While under steady laminar flow the level of TSP-1 was 15 (±3) ng ml^{-1} 10^6 cells, under turbulence the TSP-1 concentration increased up to 115 (±7) ng ml^{-1} 10^6 cells—a level comparable to static conditions, i.e., 112 (±12) ng ml^{-1} 10^6 cells. These experiments were performed with endothelial monolayers at a cell density of 80,000 cells cm^{-2} and a medium feed of 0.1 ml cm^{-2} thus the active TSP-1 concentrations were under steady laminar flow 12 (±1.2) ng ml^{-1} and under turbulence 92 ng ml^{-1}. Figure 5 shows the effect of TSP-1 concentration on the apoptosis level in static EC cultures: the threshold level of TSP-1 provoking a statistically significant ($p > 0.02$) increase of apoptosis above background was around 50 ng ml^{-1}, saturation was achieved at about 1 $\mu g\ ml^{-1}$ and the half maximal rate is obtained at about 190 ng ml^{-1}. So the observed TSP-1 accumulation under laminar flow was clearly below the threshold level whereas under turbulence the TSP-1 level was in the range of greatest sensitivity of response to dose.

The expression of IAP specifically the $\alpha_v\beta_3$ integrin/integrin-associated protein complex was analyzed by immunostaining of the cell monolayer. Figure 6A2 shows that IAP was not detectable under steady laminar flow but was expressed under turbulence (Fig. 6B2), whereas the $\alpha_v\beta_3$-integrin complex was present under turbulence (Fig. 6B1) as well as under steady laminar flow (Fig. 6A1). These results clearly identified TSP-1 and the IAP as essential switch factors.

DISCUSSION

The importance of a hemodynamic stimulus for the survival of vascular endothelium has been previously reported (6, 15). Recently a mechanosensitive autocrine loop of thrombospondin-1 (TSP-1) and the $\alpha_v\beta_3$ integrin/integrin-associated protein (IAP) complex has been identified as coupling device between flow and apoptosis (8). However lack of blood flow is a rare and mostly transient phenomenon; therefore the physiological role of this mechanism is thought to be the degression of vessels without blood flow for a longer period of time (i.e. needless vessels) thus providing an essential mechanism for vascular remodelling. The question evolves whether this mechanism is involved into a pathophysiological process leading to an endothelial dysfunction.

It has been shown that a variety of defined risk factors lead to a physiological situation conducive to atherosclerosis (16). Although the described risk conditions such as elevated blood lipids and blood pres-

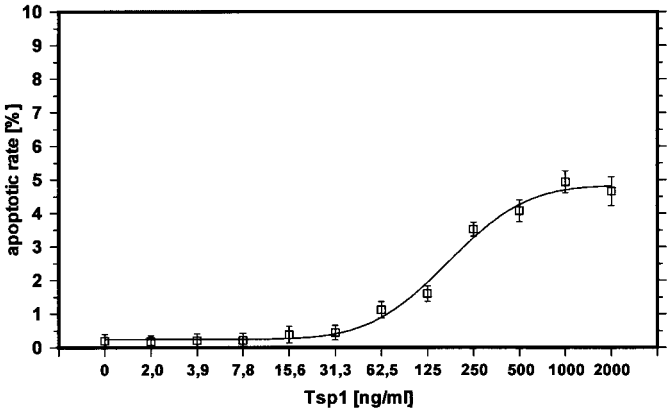


FIG. 5. Dose response of thrombospondin-1 to apoptosis in static cultures. Two days postconfluent monolayers of HUVEC were incubated for further 24 h with fresh medium supplemented with the indicated amounts of TSP-1 and apoptotic rates were determined.

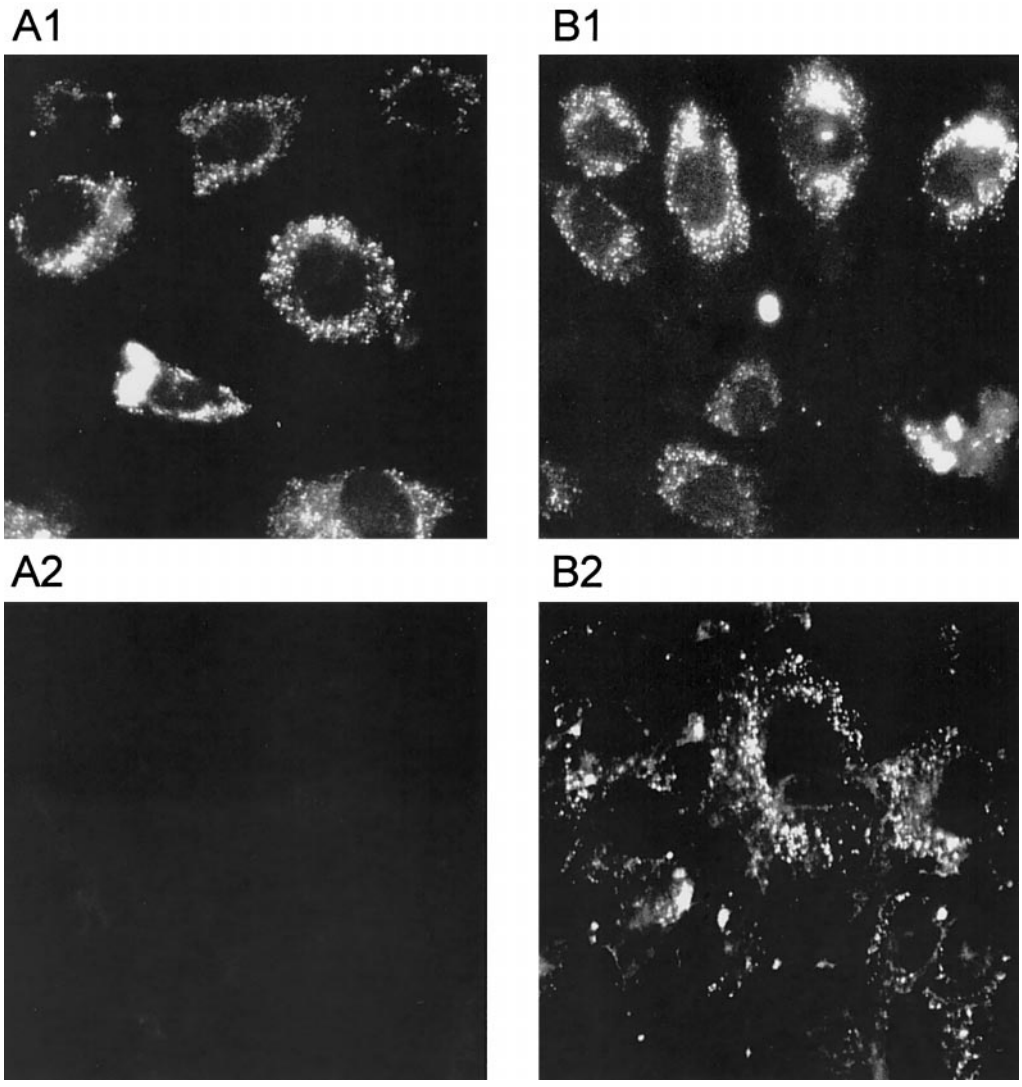


FIG. 6. Flow-dependent expression of IAP. HUVEC are cultivated in 35-mm dishes. Upon reaching confluence, defined flow conditions are established through conversion into a cone-and-plate-type flow apparatus. Cells are cultivated for an additional 24 h at a shear stress of 1–5 dyn cm^{-2} either under laminar flow (A) or under turbulent conditions (B). Monolayers are immunostained for either IAP (2) or the $\alpha_v\beta_3$ integrin (1).

sure, smoking, and stress burden the whole vasculature, atheromas are selectively localized in areas subjected to any exceptional hemodynamic situation. It is usually areas of irregular vessel form or bifurcations that are particularly prone to development of atherosclerotic lesions (17, 18). This selective localization suggests that hemodynamic forces are the initiating stimuli in atherosclerosis. The onset of apoptosis due to flow disturbances may constitute the leading stimulus in initiation of atherosclerosis. It has also been shown that the nature of hemodynamic flow influences the proliferative status of vascular endothelium (19). There are numerous descriptions of atherosclerotic lesions in areas of disturbed flow (17, 18, 20–24, 24–28). The proliferative state in existing endothelium can be interpreted as repair activity due to lethal damage to endothelial cells. Results from *in vitro* cultures of various types of endothelial cells have shown that a lack of hemodynamic forces leads to an equilibrium of apoptosis and proliferation finally resulting in senescence of the cell population (7).

In this work we have shown that endothelial apoptosis is also induced by flow conditions known to be pro-atherogenic. This indicates that *in vivo* the disturbance of laminar flow induces apoptosis, too. So we propose the following model for the initiation of atherosclerosis. In a first step apoptosis is hemodynamically induced leading to an onset of proliferation in the damaged endothelium. If the endothelium is further burdened by the above mentioned risk factors the ex-

haustion of the endothelial proliferative capacity finally leads to an irreversibly dysfunctional endothelium and thus to the initiation of atherosclerosis. The importance of the endothelial proliferative capacity is underlined by the recent finding that vascular EC in contrast to other somatic cells express a residual telomerase activity and that pro-atherogenic factors induce telomerase inactivation (29).

So far, it has remained unclear what molecules are preferentially expressed and active at these sites with the resulting onset of atherosclerosis. Now we can clearly provide evidence that apoptosis regulated via a mechanically induced autocrine loop of Thrombospondin-1 and the $\alpha_v\beta_3$ integrin/integrin-associated protein (CD47) complex might burden the endothelium at the hemodynamically marked spots and in this way initiates atherosclerosis. Further evidence for the involvement of Thrombospondin-1 in the early pathogenesis of atherosclerosis and restenosis has been recently provided by experiments showing that antibodies against thrombospondin-1 reduced neointimal thickening in balloon-injured rat carotid artery (30).

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