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Laminar Flow Induces Cell Polarity and Leads to Rearrangement of Proteoglycan Metabolism in Endothelial Cells

Johannes Grimm, Ruprecht Keller, and Philip G. de Groot*

From the Department of Clinical Chemistry and Pathobiochemistry of the RWTH Aachen, Aachen, West Germany and the Department of Haematology*, Academic Hospital Utrecht, Utrecht, The Netherlands

Key words

Proteoheparan sulfate – Heparan sulfate – Endothelial cells – Shear rate – Cell polarity

Summary

Bovine aortic endothelial cells in tissue culture were incubated with [^{35}S]-sulfate to examine the pattern of proteoglycan production at various shear rates. It was observed that culturing bovine aortic endothelial cells under laminar flow changes the secretion pattern of proteoglycans to that observed in perfused organ cultures, induces polarity of secretion, down-regulates proteoglycan biosynthesis and decreases uptake of sulfate. These results indicate that surface shear is a major force influencing the endothelial cellular phenotype. The changed pattern of proteoglycan release under static conditions may contribute to the anticoagulant effect of endothelial cells which is much needed when activated coagulation factors are not dispersed by flow.

Introduction

Endothelial cells are polar cells which provide a non-thrombogenic surface on their luminal side and a highly thrombogenic surface on their abluminal side. One group of molecules that are produced by endothelial cells and are present as matrix components and as surface associated molecules are the proteoheparan sulfates (1, 2).

Recently, we described three different proteoheparan sulfate species, HSI, HSII and HSIII, produced by cultured bovine aortic endothelial cells, and their transport kinetics (3, 4).

We showed that the three forms of proteoheparan sulfate are not derived from each other but are unique species with specific transport kinetics. HSI is found associated with the plasma membrane and transported to the culture medium by shedding of plasma membranes. HSII is found in the extracellular matrix and secreted to the culture medium while HSIII is only found in the culture medium.

A very striking observation was that the proteoglycan production by endothelial cells in an aorta organ perfusion culture is entirely different to the proteoglycan pattern observed for endothelial cells in tissue culture (4). HSIII is the only labelled medium product secreted that was observed in the organ culture system.

We postulated that the striking difference between the organ culture and the tissue culture situation is not just due to a tissue culture artefact but reflects a change in phenotype because of which the endothelial cell reacts to the particular conditions of tissue culturing. Two possible mechanisms might induce these changes, first there is no physiological subendothelial matrix, and secondly tissue cultured endothelial cells are not exposed to flow.

We have investigated the effect of laminin, fibronectin, collagen I/III, collagen IV and amnion membrane on the endothelial proteoglycan synthesis and release (4). There are some changes in the HSI/HSIII ratio of endothelial cells, grown on matrix components. However, the pattern of medium proteoglycans was still similar to those cells which were grown on plastic.

In this report we describe the influence of laminar flow on in-vitro endothelial proteoglycan production utilizing a flow chamber (5). Based on these results we suggest that laminar flow has a major influence on the endothelial cell phenotype with regard to cell polarity and proteoglycan production.

Materials and Methods

Bovine aortic endothelial cells were isolated and cultured as described (3), cells of passages 2–5 were used in this study; for radioactive incorporation experiments cells were cultured in sulfate deficient MEM (Flow Laboratories, Meckenheim, W-Germany) plus 10% fetal calf serum; the flow chamber system was set up as described elsewhere (5). The perfusate was recirculated through the perfusion chamber under non pulsatile laminar flow. Total perfusion volume was 15 ml. Flow rates were converted into shear rates (s^{-1}). Shear forces can be calculated from shear rates by: shear rate × viscosity.

This means that in the experimental system described here, a shear rate of 100 s^{-1} is equal to a shear force of 1 dyn/cm^2. [^{35}S]-sulfate was purchased from Amersham (Braunschweig, W-Germany). Sephadex G-25, Sephadex G-50, Sepharose CL-6B were obtained from Pharmacia (Heidelberg, W-Germany).

Incorporation of [^{35}S]-Sulfate into Endothelial Proteoglycans at Different Flow Rates

Bovine aortic endothelial cells were seeded on gelatine coated glass cover slips and grown to confluency (approx. 2 × 10^5 cells/coverslip). Cells were exposed to various flow rates in sulfate deficient medium for 60 min after which period [^{35}S]-sulfate was added to a concentration of 200 µCi/ml (0.04 mM sulfate). Incubation was continued for 60 min. The medium was removed, desalted on Sephadex G-50, lyophilized, dissolved in 1 ml 0.1 M Tris/HCl, 1 mM PMSF, 1 mM EDTA pH 7.5 and digested with 1 U Chondroitinase ABC at 37° C for 60 min. Aliquots were chromatographed on Sepharose CL-6B (0.5 × 50 cm) in 0.13 M Tris/HCl, 0.1% SDS, 1 mM PMSF, 1 mM EDTA pH 7.3 in order to separate the different species of proteoheparan sulfate (3). The three different proteoheparan sulfates synthesized by bovine endothelial cells were extensively characterized elsewhere (3). Fractions of 160 µl were collected at 1 ml/h and assayed for radioactivity. The recovery in all chromato-

Abbreviations:
CHAPS: (3-(3-Cholamidopropyl)-dimetylammonio)-1-propane-sulfonate; EDTA: Ethylendiamin-N,N,N,N,-tetraacetate; PMSF: Phenylmethylsulfonylfluoride; SDS: Sodiumdodecylsulfate; Tris: Tris-(hydroxymethyl)-aminomethane

Correspondence to: Dr. Ph. G. de Groot, Department of Haematology, Academic Hospital Utrecht, Catharijnesingel 101, 3500 GV Utrecht, The Netherlands

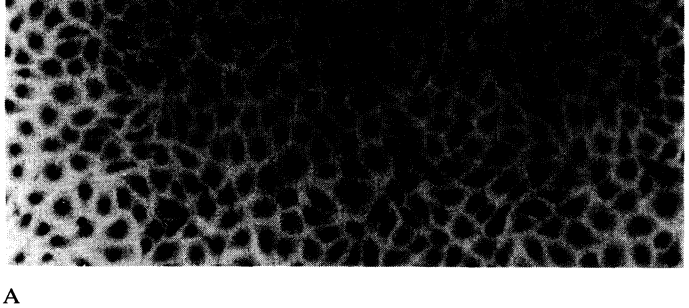
graphies was always higher than 95%. In some cases (desalting on Sephadex G-50) columns were washed after chromatography with 0.1 M Tris/HCl, 4 M guanidine hydrochloride, 0.1% Triton X-100 pH 7.5. In none of the experiments we observed [^{35}S]-labeled material which was still attached to the column.

In another experiment confluent bovine aortic endothelial cells were incubated with 200 µCi [^{35}S]-sulphate/ml at no flow and at a flow rate of 100 s^{-1}. The cells were washed 10 times with DMEM at 4° C, and the cell-layer dissolved by boiling in 7 M urea, 2% SDS, 0.13 M Tris/HCl, 1 mM PMSF, 1 mM EDTA pH 7.5 for 15 min and chromatographed on a Sephadex G-50 column (2 × 25 cm) in 0.5 M NH_4CH_3COO, 5% ethanol. Fractions of 5.2 ml were collected and assayed for radioactivity. The V_o of the G-50 chromatography was lyophilized, digested with Chondroitinase ABC and chromatographed on Sepharose CL-6B as described above. The medium from the same incubation was worked up as described.

Results

To test the effect of laminar flow on endothelial proteoglycan synthesis and release, we utilized a flow chamber, described elsewhere (5). All bovine aortic endothelial tissue cultures used were confluent. Cells were examined for the degree of confluency and damage before and after the shear experiments (Fig. 1). Under the conditions which were applied in the experiments, we did not observe any destruction of cells.

To condition the cells to the laminar flow and to the sulfate deficient medium, cells were first exposed to surface shear in the sulfate deficient medium for 60 min. Then [^{35}S]-sulfate was added and shearing was continued for another 60 min. We have chosen an incubation time of 60 min because all endothelial proteoglycans are present in approx. equal amount in the culture medium after this period under non-flow conditions (3).



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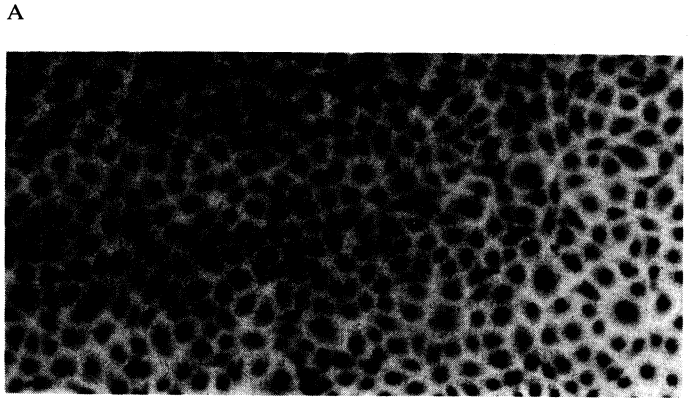

B

Fig. 1 Morphology of endothelial cells before (A) and after (B) exposure for 2 h to flow (shear rate = 100 s^{-1}). After perfusion the cells were fixated with 0.5% glutaraldehyde and stained with May-Grunwald-Giemsa as described previously (3). Original magnification = 100×

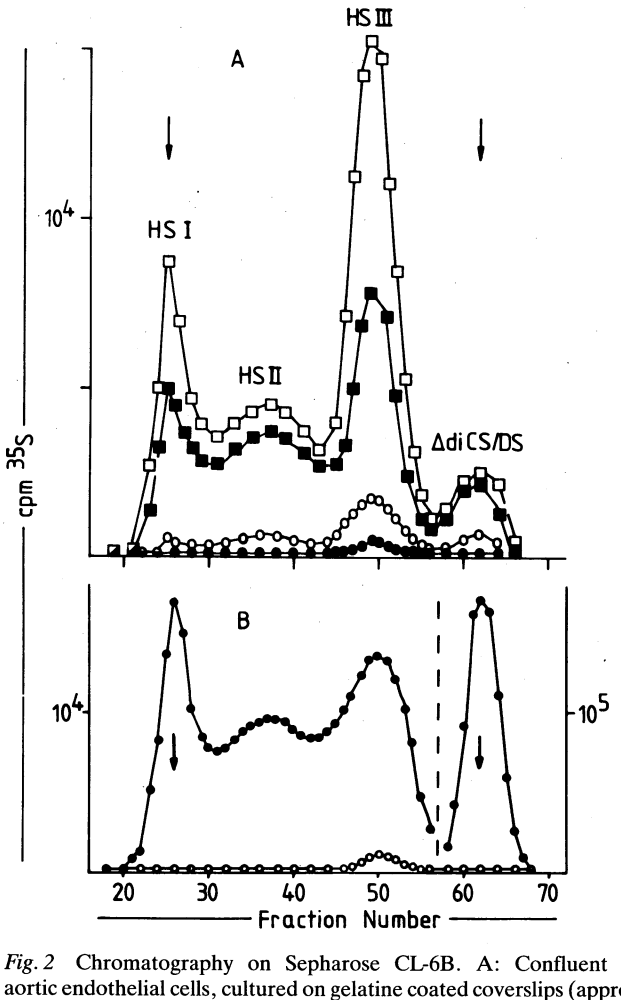

Fig. 2 Chromatography on Sepharose CL-6B. A: Confluent bovine aortic endothelial cells, cultured on gelatine coated coverslips (approx. 2 × 10^5 cells/coverslip) were exposed in sulphate deficient DMEM (Dulbecco's Minimal Essential Medium) + 15% fetal calf serum to the following shear rates: (□–□) = 5 s^{-1}, (■–■) = 10 s^{-1}, (○–○) = 50 s^{-1}, (●–●) = 100 s^{-1}. After 60 min of exposure, carrier-free [^{35}S]-sulphate was added to give a final concentration of 200 µCi/ml (0.04 mM sulphate) and shearing was continued for 60 min. The media were collected, desalted on Sephadex G-50, incubated with 1 U Chondroitinase ABC in 0.05 M Tris/HCl, 0.1 M NaCl, 1 mM PMSF, 10 mM NEM, 1 mM EDTA pH 7.5 at 37° C for 60 min and chromatographed on a Sepharose CL-6B column (0.5 × 50 cm) in 0.13 M Tris/HCl, 1 mM PMSF, 10 mM NEM, 1 mM EDTA pH 7.3 with 1 ml/h. Fractions of 160 µl were collected and assayed for [^{35}S]-activity. Arrows indicate V_o and V_t. The elution positions of plasmamembrane proteoheparan sulphate HSI, matrix proteoheparan sulphate HSII and proteoheparan sulphate HSIII were determined by chromatography of the isolated components. Chondroitinase ABC digestion of proteochondroitin/dermatan [^{35}S]-sulphate leads to unsaturated disaccharides (di CS/DS) which elute at the V_t of the column. B: For comparision the [^{35}S]-elution profile of medium from a 60 min incubation of confluent endothelial cells in tissue culture (●–●) and of a perfusate from a 60 min perfusion with 25 cm of bovine aorta thoracalis (○–○) is given. Data from both experiments are calculated to 2 × 10^5 endothelial cells to make a comparision to Fig. 1A possible

To discriminate between the different endothelial proteoheparan sulfates and endothelial proteochondroitin sulfate, we used the same procedure as described before (3, 4). The medium was desalted, digested with Chondroitinase ABC and chromatographed on Sepharose CL-6B. Under these conditions proteoheparan sulfate HSI [plasma membrane derived (3)], HSII [matrix associated (3)] and HSIII [low molecular weight secretory proteoheparan sulfate (3)] are separated from each other and from

Table 1 Total cpm of the chromatography fractions HSI, HSII, HSIII and unsaturated disaccharides from degraded proteochondroitin sulfate (PCS) from the medium of 2×10^5 bovine confluent aortic endothelial cells, exposed to various shear rates, as separated on Sepharose CL-6B (see Fig. 1)

Shear (s^{-1})	5	10	50	100
HSI (total cpm)	32,710	14,340	1,010	50
HSII (total cpm)	46,810	31,990	2,850	50
HSIII (total cpm)	96,450	44,820	6,680	1,340
PCS (total cpm)	11,930	7,430	2,210	50

unsaturated chondroitin sulfate disaccharides which elutes at the V_t of the column (Fig. 2).

When endothelial cells were exposed to increasing surface shear, the medium proteoglycan composition gradually changed towards a pattern which was observed for the organ culture situation (Fig. 2) (4). At a shear rate of 100 s^{-1}, the release of HSI and HSII and proteochondroitin sulfate was diminished and HSIII was the only labeled medium product (Fig. 2 and Table 1). This pattern was not further changed at shear rates up to 1,500 s^{-1} (not shown).

In a control experiment we investigated if the changes in proteoglycan composition after shear were due to degradative processes which might be enhanced at higher surface shear. 8×10^6 bovine aortic endothelial cells were incubated with 100 µCi [^{35}S]-sulfate deficient medium for 60 min. The medium was desalted and lyophilized. 75,000 cpm of the [^{35}S]-labeled material was applied to endothelial cells preincubated at no shear or at a shear rate of 100 s^{-1}. Incubation or perfusion was continued for 60 min. Over 99% of the radioactivity applied was recovered from the medium supernatant and the [^{35}S] elution profile of the [^{35}S] labeled material with or without Chondroitinase ABC digestion on Sepharose CL-6B remained unchanged after incubation. In both experiments, incubation at no shear or at 100 s^{-1} for 60 min, the cell layers always contained less than 300 total cpm. We conclude from these control experiments that the observed changes in the proteoglycan composition after shear are due to altered biosynthesis and release of proteoglycans and not to selective adsorption or degradation processes.

The amount and the composition of medium [^{35}S] proteoglycans was essentially the same whether or not the cells were incubated with endothelial cell conditioned sulfate deficient medium or with fresh sulfate deficient medium indicating that there is also no feedback regulation of medium proteoglycans on the release of these substances, at least not in the incubation periods that we investigated (not shown).

There is also a decrease of total [^{35}S]-sulfate incorporation into sulfated polymers. This may be due to down-regulation of biosynthesis. But there may be less trafficing of radioactive sulfate from the medium into the intracellular pool. In another experiment, therefore, we estimated the amount of cellular low molecular weight and high molecular weight [^{35}S] label after incorporating it for 60 min in endothelial cells at non-flow and at a shear rate of 100 s^{-1} (Fig. 3).

In a typical experiment, cells exposed to surface shear contained approx. 50% (47%) less [^{35}S]-labeled material in the low molecular weight fraction (51,320 cpm compared to 96,830 cpm at no shear), appearing at the V_t position of a Sephadex G-50 chromatography. However, the amount of labeled high molecular weight material was decreased by 93% in the shear experiment compared to non-flow conditions. In a control experiment, endothelial cells were incubated with the same amount of radioactivity as in the experiment, described above, at 4° C for

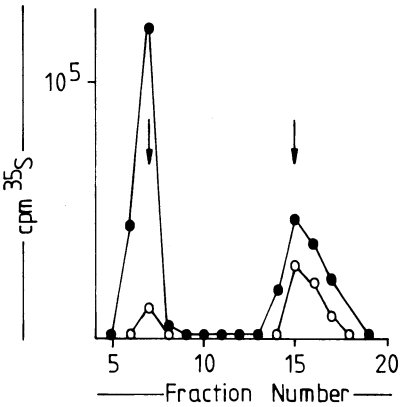

Fig. 3 Chromatography on Sephadex G-50. Confluent bovine aortic endothelial cells were incubated with [^{35}S]-sulphate at no shear (●–●) and a shear rate of 100 s^{-1} (○–○) as described under Fig. 2. The cells were washed 10 × with DMEM at 4° C to remove extracellular radioactivity, dissolved by boiling in 7 M urea, 2% SDS (sodium dodecylsulphate), 0.13 M Tris/HCl, 1 mM PMSF, 10 mM NEM, 1 mM EDTA pH 7.5 for 15 min and chromatographed on a Sephadex G-50 column (2 × 25 cm) in 0.5 M NH_4CH_3COOH, 5% ethanol. Fractions of 5.2 ml were collected and assayed for radioactivity. Arrows indicate V_o and V_t

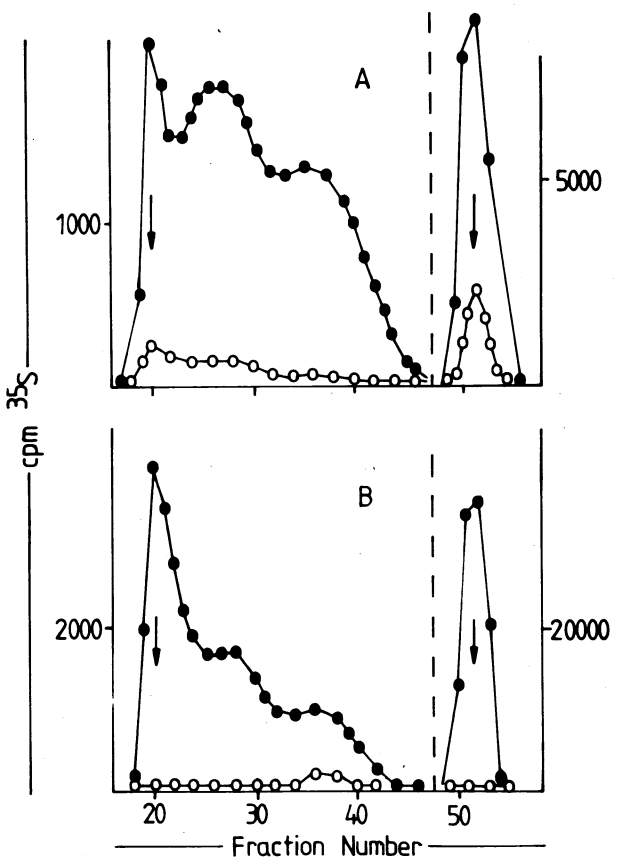

Fig. 4 Chromatography on Sepharose CL-6B. Confluent bovine aortic endothelial cells were incubated with [^{35}S]-sulphate at no shear (○–○) and a shear rate of 100 s^{-1} (●–●) as described under Fig. 2. The endothelial cell layers (A) were dissolved, desalted as described under Fig. 2 and digested with Chondroitinase ABC (see Fig. 2). The media (B) were desalted and incubated with Chondroitinase ABC as described under Fig. 2. All preparations were chromatographed on a Sepharose CL-6B column (0.5 × 50 cm) with 0.13 M Tris/HCl, 1 mM PMSF, 10 mM NEM, 1 mM EDTA pH 7.3 as eluant. Fraction of 200 µl were collected at 1 ml/h and assayed for radioactivity. Arrows indicate V_o and V_t. To characterize the elution pattern, isolated HSI, HSII and HSIII were chromatographed under the same conditions

1 min. Cells were washed as in the above experiment. The cell layers always contained less than 5,000 cpm.

We suggest that apart from less influx of radioactive sulfate into the endothelial cell at higher shear rates there is also a down-regulation in biosynthesis. Since the biosynthesis of the coreprotein is the rate-limiting step in proteoglycan assembly, this down-regulation probably takes place at this early level of proteoglycan biosynthesis. But as we do not known the K_m-values of all the enzymes involved in the intracellular [^{35}S] metabolism, the data, presented in Fig. 3, do not give conclusive evidence for our suggestion.

We have compared the proteoglycan composition in the endothelial cell layer and in the medium from cells exposed to non-flow conditions and to a shear rate of 100 s^{-1} (Fig. 4). In cells, exposed to surface shear, there is less incorporation of [^{35}S]-sulfate into proteoglycans and a change in the proteoheparan sulfate composition in both compartments, cell layer and medium. Proteochondroitin sulfate does not appear in the medium of cells at higher surface shear but it is the main labeled proteoglycan in the cell layer besides HSI and HSII. We conclude from these data that the cells at a surface shear higher than 10 s^{-1} still produce all proteoglycans as determined under non-shear conditions but release these proteoglycans in a polar fashion: HSII and proteochondroitin sulfate remain associated with the cell layer, HSIII is released into the medium supernatant. HSI, the plasma membrane component, is not released into the medium at higher surface shear but is still present in the cell layer. We conclude from this, that the plasma membrane shedding which leads to the release of HSI with a high transportation rate under non-shear conditions is also diminished once the cells are exposed to a surface shear higher than 100 s^{-1}.

Discussion

In a recent report (3) we have described the proteoheparan sulfate production by bovine aortic endothelial cells in tissue culture and showed that bovine aortic endothelial cells produce at least three different species of proteoheparan sulfate. The main product was a proteoheparan sulfate of approx. 195,000 which is probably transported into the medium via plasma membrane shedding. If this shedding of plasma membrane proteoheparan sulfate reflected a physiological process one should be able to find it in plasma or serum. This proteoheparan sulfate, however, has never been described as a component of the blood circulation. Furthermore, the appearance of a matrix associated proteoheparan sulfate and a proteochondroitin sulfate in the culture medium of endothelial cells ought not to be there according to the physiological state of an endothelial cell in vivo. These cells construct the non-thrombogenic surface of the vessel wall. Therefore, matrix associated substances, some of which are highly thrombogenic, need to be released in a polar fashion only to the subendothelial side of the cell.

For these reasons we have analyzed the proteoheparan sulfate production and organ culture system in which endothelial cells are still attached to their physiological subendothelial matrix and are exposed to surface shear (4). Utilizing the aorta perfusion system we have shown that there is a striking difference between the proteoheparan sulfate production by endothelial cells from tissue culture compared to organ culture perfusion system.

In this report we show that the pattern of proteoglycans released by tissue cultured endothelial cells can be transformed into the pattern of endothelial cells in organ culture by exposing cultured cells to flow. There is a continuous shift of the proteoglycan pattern from the tissue culture to the organ culture situation with increasing flow. These changes take place at shear forces which are lower than observed physiologically in arteries or veins (6).

There are mainly three changes induced by laminar flow:

1. Polar secretion.
   Only HSIII is found in the medium supernatant of endothelial cells in organ culture and in the perfusion chamber. The matrix associated proteoglycans HSII and proteochondroitin sulfate are part of the cell layer in both systems.
2. Down regulation of proteoglycan production.
   The production of the endothelial proteoglycan is decreased by a factor 100–1,000 with increasing surface shear. Whether this down-regulation is due to decreased synthesis of the core protein or due to a change in the processing of the glycosaminoglycan chains is not known.
3. No shedding of plasma membrane proteoheparan sulfate HSI.
   The reason for plasma membrane proteoheparan sulfate shedding under non-flow condition is not clear. Some authors favour a model in which proteoheparan sulfate derived from the extracellular plasmamembrane is liberated by limited proteolysis (7). On the other hand there may be vesiculation of endothelial plasma membranes at low shear. Such vesiculation processes have been described in detail for erythrocytes (8). To induce such vesiculation processes changes in the cytoskeletal system have to occur (9, 10), which have been determined for endothelial cells under different surface shear conditions.

Recently it was shown (11) that endothelial cells, after wounding the cell layer, show some differences in the proteoglycan release compared to those from confluent cultures. By these results, which are still related to the tissue culture system, it has already been indicated that the medium proteoglycan pattern from endothelial cells is highly dependent on the physiological state of the endothelial cell. However, we do not have any indication that the laminar flow initiates the cells to enter the cell cycle, which was in agreement with findings by Davies et al. (12). One can, therefore, imagine that there are many more drastic differences between the in-vivo and the in-vitro situation. Not only proteoheparan sulphate synthesis and release by endothelial cells is influenced by flow. Also other endothelial cell functions, such as arachidonic acid metabolism, are modulated by flow.

The production of prostacyclin by vascular endothelial cells under physiologic flow conditions is significantly higher than that of cells under stationary conditions (13, 14).

From the stand-point of a cellular physiologist an endothelial cell in tissue culture may be compared to an endothelial cell in-vivo in a closed artery or vein (non-flow conditions) with little or no communication to the subendothelial tissue, growing on a totally non-physiological matrix. Such a situation, which probably occurs very rarely in-vivo, leads to the expression of a cellular phenotype which may not have very much to do with the phenotype of an endothelial cell f.i. in the aorta of a living animal.

Obviously the aorta perfusion system does not exactly represent the true in-vivo situation of endothelial cells. There are still differences between the perfused organ in-vitro and the aorta of a living animal with regard to blood components, wall compliance, pulsatile wave contoures etc. Therefore, the observed difference between the proteoglycan production by endothelial cells in organ culture and tissue culture have to be interpreted with caution and our conclusions are still hypothetical.

Nevertheless, our finding that flow is the major force influencing the cellular phenotype with regard to cell polarity and proteoglycan metabolism of cultured endothelial cells may have some important physiological implications. Although only a little subfraction of the endothelial proteoheparan sulfates bind to antithrombin III (14, 15), the dramatic increase of the proteoheparan sulfate release into the culture medium supernatant

may indicate that endothelial cells actively interfere with the blood coagulation system if they are not exposed to flow. These findings provide further evidence that the qualitative nature of blood flow may have a controlling role in endothelial cell function.

It has been shown by in-vivo experiments that the velocity of thrombus formation is low at very low flow rates, reaches a maximum at increased flow and decreases at higher rates of mean blood velocity (16). One reason for the decrease in thrombus formation velocity at very low flow rates may be that endothelial cells under non-flow and low flow conditions participate in preventing blood coagulation in a closed artery or vein by releasing antithrombogenic heparan sulfates.

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