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## Fluid shear stress stimulates incorporation of hyaluronan into endothelial cell glycocalyx

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**Gouverneur, Mirella, Jos A. E. Spaan, Hans Pannekoek, Ruud D. Fontijn, and Hans Vink.** Fluid shear stress stimulates incorporation of hyaluronan into endothelial cell glycocalyx. *Am J Physiol Heart Circ Physiol* 290: H458–H462, 2006. First published August 26, 2005; doi:10.1152/ajpheart.00592.2005.—Vascular endothelial cells are shielded from direct exposure to flowing blood by the endothelial glycocalyx, a highly hydrated mesh of glycoproteins, sulfated proteoglycans, and associated glycosaminoglycans (GAGs). Recent data indicate that the incorporation of the unsulfated GAG hyaluronan into the endothelial glycocalyx is essential to maintain its permeability barrier properties, and we hypothesized that fluid shear stress is an important stimulus for endothelial hyaluronan synthesis. To evaluate the effect of shear stress on glycocalyx synthesis and the shedding of its GAGs into the supernatant, cultured human umbilical vein endothelial cells (i.e., the stable cell line EC-RF24) were exposed to 10 dyn/cm<sup>2</sup> nonpulsatile shear stress for 24 h, and the incorporation of [<sup>3</sup>H]glucosamine and Na<sub>2</sub>[<sup>35</sup>S]O<sub>4</sub> into GAGs was determined. Furthermore, the amount of hyaluronan in the glycocalyx and in the supernatant was determined by ELISA. Shear stress did not affect the incorporation of <sup>35</sup>S but significantly increased the amount of glucosamine-containing GAGs incorporated in the endothelial glycocalyx [168 (SD 17)% of static levels, *P* < 0.01] and shedded into the supernatant [231 (SD 41)% of static levels, *P* < 0.01]. Correspondingly with this finding, shear stress increased the amount of hyaluronan in the glycocalyx [from 26 (SD 24) × 10<sup>-4</sup> to 46 (SD 29) × 10<sup>-4</sup> ng/cell, static vs. shear stress, *P* < 0.05] and in the supernatant [from 28 (SD 11) × 10<sup>-4</sup> to 55 (SD 16) × 10<sup>-4</sup> ng·cell<sup>-1</sup>·h<sup>-1</sup>, static vs. shear stress, *P* < 0.05]. The increase in the amount of hyaluronan incorporated in the glycocalyx was confirmed by a threefold higher level of hyaluronan binding protein within the glycocalyx of shear stress-stimulated endothelial cells. In conclusion, fluid shear stress stimulates incorporation of hyaluronan in the glycocalyx, which may contribute to its vasculoprotective effects against proinflammatory and pro-atherosclerotic stimuli.

endothelial surface layer; endothelium

THE ENDOTHELIAL GLYCOCALYX is a highly negatively charged, organized mesh on the endothelial cell surface, consisting of membranous glycoproteins, proteoglycans, glycosaminoglycans (GAGs), and associated plasma proteins, and is situated at the luminal side of blood vessels (20). This endothelial layer functions as a protective barrier between endothelial cells and flowing blood by contributing to the endothelial permeability barrier (19), binding anticoagulation factors (13) modulating leukocyte interactions with the endothelium (3, 12), and by limiting myocardial edema (16) and has become in focus for its role as a mechano-shear sensor (22). Recently, we demonstrated that atherogenic stimuli, like oxidative stress (18) and

oxidized LDL (2, 3, 17), perturb the endothelial glycocalyx, resulting in increased glycocalyx permeability and adhesiveness of platelets and leukocytes to the endothelial membrane. Earlier studies, in which sialic acid binding lectins (8) and alcian blue (9) were used, showed that reduced dimensions of the endothelial glycocalyx at arterial sites exposed to disturbed flow patterns associate with increases in endothelial permeability and susceptibility to atherosclerotic lesion formation. Additionally, studies by Woolf (23) and Wang et al. (21) revealed thicker glycocalyces at high shear regions compared with low shear regions and demonstrated that glycocalyx dimension is reduced when rabbits are fed an atherogenic diet. Steady-state glycocalyx dimension is the result of local synthesis and degradation of its constituents, and it is important to know the factors that determine this balance. We hypothesized that fluid shear stress is an important stimulus for glycocalyx synthesis.

### MATERIALS AND METHODS

**Chemicals.** Medium 199 (M199), L-glutamine, antibiotic-antimycotic, and trypsin were obtained from GIBCO-BRL, PBS (pH 7.4) from Fresenius Kabi, and FBS from Biowhittaker. Heparin and endothelial cell growth supplement were obtained from Sigma. The radiochemicals 6-[<sup>3</sup>H]glucosamine (specific activity, ~25 to 40 Ci/mmol) and carrier-free Na<sub>2</sub>[<sup>35</sup>S]O<sub>4</sub> (specific activity, ~43 Ci/mgS) were purchased from ICN, and Molecular Probes provided anti-heparan sulfate, conjugated anti-mouse IgM, custom-made fluorescent (Alexa-555) hyaluronan binding protein (AABP) (no. 400762–1, Seikagaku America), and nuclear stain Syto. The Hyaluronan Enzyme-linked Immunosorbent Assay kit was obtained from Echelon Biosciences (Salt Lake City, UT). Fibronectin was a kind gift from the Sanquin Research Foundation (Amsterdam, The Netherlands).

**Cell culture.** The human EC-RF24 cell line, representing human vascular umbilical endothelial cells that have been immortalized with an amphotrophic replication-deficient retrovirus, contained human papilloma virus 16 E6/E7 DNA (4). EC-RF24 cells have been shown to have retained a diploid karyotype, display no abnormalities, and are able to grow in a polar fashion compared with primary human umbilical vein endothelial cells after being cultured for up to 1 yr. Furthermore, the presence of von Willebrand factor, endoglin, and PCAM-1 was established as well as expression of surface adhesion molecules E-selectin, VCAM-1, and ICAM-1, and the results were comparable to those obtained from primary endothelial cells. The cells were grown on 10 µg/ml fibronectin-coated cell culture flasks in M199 media, supplemented with 20% (vol/vol) heat-inactivated FBS, 50 µg/ml heparin, 12.5 µg/ml endothelial cell growth supplement, 0.2 mmol/l L-glutamine, 100 U/ml penicillin-G, 100 U/ml streptomycin sulfate, and 25 µg/ml amphotericin-B at 37°C in 5% CO<sub>2</sub>.

**Parallel flow chamber.** The parallel flow perfusion chamber used is described in detail by Sakariassen et al. (14). In short, the chamber

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consists of two 1-cm-thick polymethyl methacrylate rectangulars. The top part has a 1-cm-wide depression of 0.6 mm, which represents the chamber width and height when the parts are joined together. Two corks can be placed at the bottom part of the chamber, which each fit the endothelial cell containing Thermanox coverslips, matching the flow path of the top part. Watson-Marlow pump model 323S/D was used to obtain flow and to eliminate the pulse generated by the pump; two windkessels and resistance tubing were placed in the flow setup, and the windkessels were placed before and after the flow chamber and the resistance tubing between the first windkessel and the flow chamber. The final flow that was exposed to the cells was 49.9 (SD 2.73) ml/min, which translates into a shear stress of 9.7 (SD 0.5) dyn/cm<sup>2</sup>. The parallel flow perfusion chamber was generously provided by Dr. P. G. de Groot, Department of Hematology, University Medical Center, University of Utrecht (Utrecht, The Netherlands).

**Radioactive incorporation studies.** The cells were seeded on fibronectin-coated Thermanox coverslips and attached to confluency for 2 h in M199 media without antibiotics ( $\sim 2 \times 10^5$  cells/coverslip). The cells were placed in a parallel flow chamber, and nonpulsatile flow at shear stress of 9.7 (SD 0.5) dyn/cm<sup>2</sup> was applied for 24 h in complete M199 media without antibiotics, supplemented with 40  $\mu$ Ci/ml 6-[<sup>3</sup>H]glucosamine and 50  $\mu$ Ci/ml Na<sub>2</sub>[<sup>35</sup>S]O<sub>4</sub>. After 24 h of exposure, either under static or flow condition, the cells were placed under static condition, and after 1 h, the supernatant was collected. After 1 h, the cells were treated for 30 min at room temperature with 0.05% (wt/vol) trypsin and for an additional 10 min at 37°C to separate membranous glycoocalyx cell fraction from the cellular fraction.

**Validation of trypsin fraction purity.** To test whether elevated levels of glucosamine over sulfate (G/S) ratio in the glycoocalyx were due to specific incorporation of substrate into GAGs, four additional experiments were performed from which the trypsin-derived glycoocalyx fraction was further purified by DEAE chromatography (15). Briefly, the cells were incubated with 0.05% (wt/vol) trypsin for 30 min at room temperature and for an additional 10 min at 37°C and then centrifuged at 1,500 g for 5 min to separate cells from the trypsinated glycoocalyx proteoglycan fraction. The columns were prepared as described previously (15). The glycoocalyx fraction was applied to the column, and the column was washed to remove nonproteoglycan proteins. Column-retained proteoglycans were eluted with a high salt (1 M NaCl) solution and counted in a scintillation counter. Additionally, the trypsin fraction of GAG-deficient cells [Chinese hamster ovary (CHO)-pgsA745] (6) were also purified by column chromatography and compared with glucosamine and sulfate incorporation of wild-type GAG-containing CHO cells (CHO-K1).

**Fluorescent imaging.** Cells were cultured as stated in *Cell culture* and exposed for 24 h to nonpulsatile flow. Afterward, cells were rinsed with 0.1% BSA/HEPES and subsequently incubated for 30 min at room temperature with 10  $\mu$ g/ml anti-heparan sulfate antibody, 30 min with 10  $\mu$ g/ml Alexa fluor 488 conjugated anti-mouse IgM, and 30 min with 50  $\mu$ g/ml hyaluronan binding protein (HABP), labeled with Alexa fluor 555. The Thermanox coverslips were placed between microscopic object glass and glass coverslip, immersed in  $2 \times 10^{-3}$  mmol/l nucleus stain 44 blue fluorescent nucleic acid (Syto).

The cells were imaged with the use of a fluorescence microscope (Olympus B151; objective, UplanApo  $\times 60/1.2$  water immersion), and 16-bit image stacks of  $50 \times 50 \times 20$   $\mu$ m (XYZ coordinates) with pixel sizes of  $144 \times 144 \times 200$  nm were recorded with the use of a cascade 650 digital camera (Photometrics). The recorded image stacks were deconvolved by using Huygens2 software (Huygens Professional version 2.4.1; Scientific Volume Imaging BV; Hilversum, The Netherlands) and the intensity per plane was calculated, from which cytoplasmic and glycoocalyx intensities were determined.

**Hyaluronan content determination.** Hyaluronan mass was determined by using an enzyme-linked immunosorbent assay kit, commercially available from Echelon Biosciences. The principle is based on

competitive ELISA assay in which the colorimetric signal is inversely proportional to the amount of hyaluronan present in the sample.

**Statistical analysis.** For statistical analysis, two-way paired [<sup>3</sup>H]-glucosamine and Na<sub>2</sub>[<sup>35</sup>S]O<sub>4</sub> incorporation data or unpaired HABP intensity data (Hyaluronan ELISA data) *t*-tests were used when appropriate. A value of *P* < 0.05 was considered statistically significant. Values are means (SD).

## RESULTS

**Effect of flow on [<sup>3</sup>H]glucosamine and Na<sub>2</sub>[<sup>35</sup>S]O<sub>4</sub> incorporation.** EC-RF24 cells were seeded on fibronectin-coated coverslips and grown to confluency ( $10^5$  cells/coverslip). After the supernatant was replaced with medium without radiolabeled tracers, radiolabeled glucosamine and sulfate-containing GAGs that shedded into the supernatant were measured after 1 h. Subsequently, the cells were counted, and all radioactive counts per minute were normalized to cell number. Exposure to shear stress reduced the number of cells per coverslip by a relatively large amount [from 0.7 (SD 0.3)  $\times 10^5$  to 0.4 (SD 0.3)  $\times 10^5$  cells/coverslip, static vs. flow, respectively, *n* = 7 experiments, *P* < 0.05], but shear stress did not affect sulfate incorporation [sulfate counts  $\cdot$  min<sup>-1</sup>  $\cdot$  cell<sup>-1</sup> in flow cells = 104 (SD 60)% of static cells, *n* = 7 experiments, not significant (NS)]. In contrast, exposure of the cells to shear stress tended to increase total glucosamine incorporation [glucosamine counts  $\cdot$  min<sup>-1</sup>  $\cdot$  cell<sup>-1</sup> in flow cells = 181 (SD 161)% of static cells, *n* = 7 experiments, NS]. Absolute counts of glucosamine and sulfate incorporation per  $10^4$  cells in the various compartments are depicted in Table 1.

To eliminate the confounding interexperimental variation of substrate-label intensity, G/S ratios were determined for each individual experiment, revealing a highly significant increased level of G/S incorporation [160 (SD 42)% of static cells, *n* = 7 experiments, *P* < 0.01]. This increase in G/S ratio was due to increased incorporation of glucosamine containing glycosaminoglycans in the glycoocalyx of flow cells [*n* = 4 experiments, 168 (SD 17)% of static cells, *P* < 0.01; Fig. 1, middle], but no changes were detected in the intracellular pool of incorporated substrate [*n* = 4 experiments, 117 (SD 23)% of static cells; Fig. 1, left]. Furthermore, the increased levels of glucosamine substrate in the glycoocalyx were also reflected by elevated levels of glucosamine containing GAGs shedded into the supernatant [*n* = 4 experiments, 231 (SD 41)% of static cells, *P* < 0.01; Fig. 1, right].

**Specificity of substrate incorporation in glycoocalyx GAGs.** To test whether the glucosamine and sulfate moieties, present in the glycoocalyx, were specifically incorporated into GAGs, glycoocalyx fractions of shear stress-exposed and static cells were passed through DEAE columns, and retained glycoocalyx

Table 1. Absolute counts of [<sup>3</sup>H]glucosamine and [<sup>35</sup>S]sulfate incorporation per  $10^4$  cells in various compartments

	Intracellular	Glycoocalyx	Shedded	Total
<i>Flow</i>				
[ <sup>3</sup> H]glucosamine	764 (SD 665)	353 (SD 333)	384 (SD 307)	1501 (SD 911)
[ <sup>35</sup> S]sulfate	13 (SD 12)	8 (SD 10)	10 (SD 4)	30 (SD 25)
<i>Static</i>				
[ <sup>3</sup> H]glucosamine	626 (SD 411)	174 (SD 93)	159 (SD 84)	959 (SD 403)
[ <sup>35</sup> S]sulfate	10 (SD 7)	5 (SD 3)	14 (SD 7)	29 (SD 13)

Values are means (SD) with units of measure in counts per minute per  $10^4$  cells.

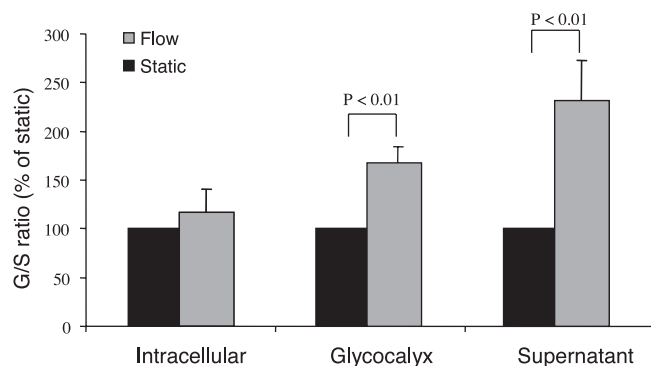


Fig. 1. Effect of flow on cellular glucosamine/sulfate (G/S) distribution. Flow stimulates cellular incorporation of glucosamine relative to sulfate substrate (G/S ratio), as reflected by increases in supernatant G/S ratio ( $P < 0.01$ , right) and in glycocalyx G/S ratios ( $P < 0.01$ , middle), but no changes occurred in intracellular G/S ratios [not significant (NS), left].

proteoglycan-rich fractions were analyzed for relative glucosamine and sulfate content. The shear stress-induced increase in G/S ratio was not affected by DEAE chromatography, being 168 (SD 17)% ( $n = 4$  experiments,  $P < 0.01$ ) and 149 (SD 13)% ( $n = 4$  experiments,  $P < 0.01$ ) before and after the chromatography purification procedure.

The specificity of the column purification method for GAG-incorporated substrate was confirmed by comparing glucosamine and sulfate incorporation levels in the glycocalyx fractions obtained from GAG-containing CHO cells (CHO-K1) and GAG-deficient CHO-cells (CHO-pgsA745). DEAE chromatography lowered CHO-pgsA745 substrate incorporation levels to 6 (SD 1)% and 2 (SD 1)% of wild-type controls, indicating that  $<10\%$  of glycocalyx-incorporated substrate levels can be accounted for by nonspecific cell-surface binding of substrate (data not shown).

**HABP and anti-heparan sulfate binding to static and flow exposed EC-RF24 cells.** Shear stress did not significantly affect intracellular uptake of HABP compared with static cells [65 (SD 65) vs. 53 (SD 25) arbitrary units/ $\mu\text{m}^2$  endothelial surface area in flow ( $n = 6$  experiments) and static ( $n = 4$  experiments) cells, respectively;  $P = \text{NS}$ ]. In contrast, a significant threefold increase was found in glycocalyx-HABP levels in flow-exposed cells compared with static cells [104 (SD 6)% vs. 32 (SD 8)% of cytoplasmic HABP intensity levels,  $P < 0.0001$ ; Fig. 2, left]. The anti-heparan sulfate binding showed no differences in glycocalyx heparan sulfate levels of flow exposed cells compared with static cells [70 (SD 30)% vs. 110 (SD 18)% of cytoplasmic anti-HABP intensity levels;  $P = \text{NS}$ ; Fig. 2, right].

**Effect of flow on hyaluronan content of glycocalyx and supernatant.** The hyaluronan content of the glycocalyx of cells cultured under static condition and flow-exposed cells was determined with hyaluronan ELISA kit. The glycocalyx of flow-exposed cells contained more hyaluronan [46.3 (SD 28.7) ng/cell  $\times 10^4$ ] than the glycocalyx of static-cultured cells [26.3 (SD 24.1) ng/cell  $\times 10^4$  ( $P < 0.05$ );  $n = 6$  experiments; Fig. 3, left]. The hyaluronan content in the supernatant collected under static condition 1 h after 24 h static condition or flow exposure was 27.8 (SD 10.7) versus 55.3 (SD 15.7) ng/cell  $\times 10^4$  ( $P < 0.05$ ;  $n = 7$  experiments; Fig. 3, right).

**Time-dependent effect of endothelial cell flow exposure on hyaluronan in culture media.** Measuring the hyaluronan content in the media of shear stress-stimulated endothelial cells

during different times of flow exposure revealed that the hyaluronan content in the media was already significantly increased after 1 h of flow stimulus [static vs. flow-exposed cells, 23.5 (SD 10.9) vs. 58.6 (SD 36.5) ng/cell  $\times 10^4$ ;  $P < 0.05$ ;  $n = 6$  experiments; Fig. 4, left bar]. The amount of hyaluronan produced by shear stress-stimulated cells increased further to 83.3 (SD 74.7) ng/cell  $\times 10^4$  and 540.7 (SD 327.7) after 6 and 24 h of shear stress stimulation ( $n = 6$  experiments;  $P < 0.05$ , Fig. 4, middle and right bars). No significant changes in the amount of hyaluronan produced by static cells were observed over time.

## DISCUSSION

The present study demonstrates that exposure of cultured endothelial cells for 24 h to a shear stress of 10 dyn/cm<sup>2</sup> stimulates incorporation of glucosamine-containing GAGs in the glycocalyx, which is accompanied by elevated levels of glucosamine-containing GAGs in the supernatant. These increases were confirmed by direct demonstration of increased hyaluronan concentrations in the glycocalyx and in the supernatant, as well as by a threefold increase in the incorporation of HABP in the glycocalyx. The fact that shear stress did not affect net sulfate levels in the glycocalyx does not rule out an effect of shear stress on the incorporation of sulfated GAGs in the glycocalyx. In addition to its incorporation in hyaluronan, glucosamine is also incorporated in sulfated sugars like heparan sulfate and chondroitin sulfate. The lack of changes in sulfate incorporation could be due to altered activities of sulfotransferase enzymes or a modification of sulfated GAG chain length. Therefore, more detailed studies are required to determine whether shear stress also affects sulfated GAGs in addition to the clear increases in hyaluronan incorporated in the glycocalyx.

Previous studies by others have focused predominantly on the effects of shear stress on the endothelial metabolism of sulfated GAGs. Arisaka et al. (1) used pig aortic endothelial cells exposed to shear-stress levels of 15 and 40 dyn/cm<sup>2</sup> in a parallel flow chamber for periods of 3, 6, 12, and 24 h. The cells were incubated for 12 h with Na<sup>35</sup>S]O<sub>4</sub> after shear-stress

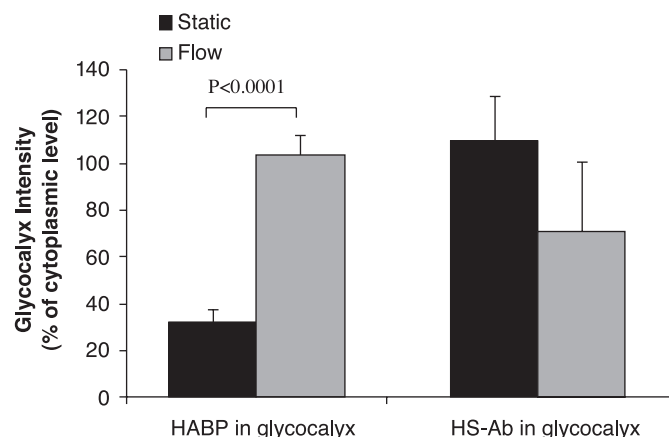


Fig. 2. Glycocalyx hyaluronan binding protein (HABP) and anti-heparan sulfate (HS) staining on flow and static cells. Shear stress induced threefold increase in glycocalyx HABP label intensity ( $P < 0.0001$ , left) compared with glycocalyx of static cultured cells. Anti-HS label binding showed no differences in glycocalyx HS levels of flow-exposed cells compared with static cells (NS, right). HS-Ab, HS antibody.

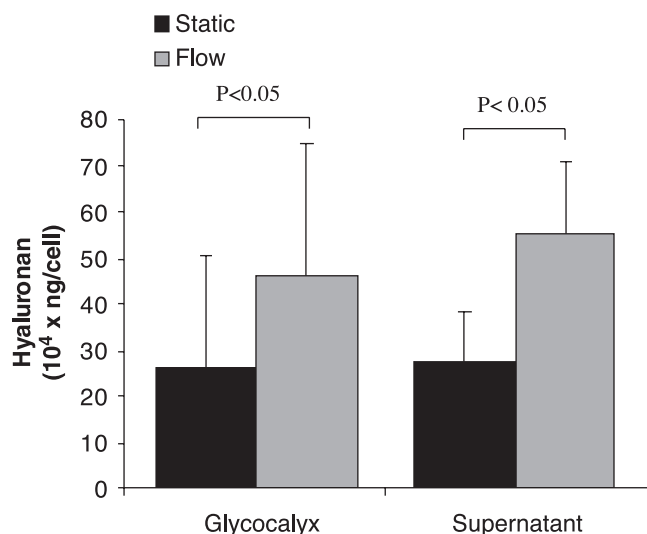


Fig. 3. Effect of flow on hyaluronan content of glyocalyx and supernatant. Flow-exposed cells contained more hyaluronan in glyocalyx compared with glyocalyx of cells cultured under static condition (left). Similar differences were observed for hyaluronan content in supernatant collected 1 h after 24-h static versus flow exposure ( $P < 0.05$ ; right).

exposure. These authors demonstrated increased synthesis of sulfated GAGs after high shear stress of 40 dyn/cm<sup>2</sup> and also a small, but significant, increase at 15 dyn/cm<sup>2</sup>. Our lack of shear stress-induced increases in the incorporation of sulfate at a relatively low shear-stress level of 10 dyn/cm<sup>2</sup> is therefore consistent with their conclusion that moderate-to-high shear-stress levels are required to stimulate synthesis of sulfated GAGs. Elhadj et al. (5) exposed bovine aortic endothelial cells for 7 days to <0.5 dyn/cm<sup>2</sup> before increasing shear rates for 3 days to 5 and 23 dyn/cm<sup>2</sup>. Na[<sup>35</sup>S]O<sub>4</sub> was added to the sheared cells during the final 24 h. The focus of that study was on soluble-released material, which was purified by Sepharose CL-6B and DEAE chromatography. No significant increase in the net sulfated GAG synthesis was detected, but a shift in its size distribution was reported, indicating that a modulation of specific sulfation patterns may occur despite limited effects of low shear-stress levels on sulfated GAG synthesis. Florian et al. (7) exposed bovine aortic endothelial cells to a short-term (3 h) oscillatory shear stress of 10 (SD 15) dyn/cm<sup>2</sup>, as well as steady shear stress of 10 dyn/cm<sup>2</sup>. Shear stress-induced endothelial nitric oxide production was found to be abolished when cells were treated with heparitinase, indicating that endothelial-sulfated GAGs may contribute to the mechanotransduction of shear forces from the extracellular to the intracellular compartment.

In summary, these experiments demonstrate 1) that sulfated GAGs in the endothelial glyocalyx may function as a mechanotransducers as reported before for hyaluronic acid (11), 2) that shear-stress exposure alters the size distribution of endothelial-sulfated GAGs, and 3) that high levels of shear stress may also increase sulfated GAG synthesis. The current study, however, demonstrates for the first time that shear stress also increases hyaluronan content in the endothelial glyocalyx. However, it must be noted that the loss of a significant number of cells in the shear stress experiments may have selected for an endothelial phenotype that is most responsive to shear stress in terms of extracellular hyaluronan incorporation and attach-

ment. The current experiments were performed on an immortalized human umbilical vein endothelial cell line exposed to a laminar, nonpulsatile flow profile. Future studies are required that incorporate different flow profiles on arterial as well as microvascular types of endothelium to test whether shear-stress stimulation of endothelial hyaluronan incorporation is a general response or possibly limited to specific conditions and certain vascular sites.

*Functions of hyaluronan in endothelial glyocalyx.* The contribution of hyaluronan to the endothelial glyocalyx and its functional implications are only recently recognized. Mochizuki et al. (11) demonstrated that hyaluronidase treatment of the endothelial glyocalyx in isolated canine femoral arteries attenuates flow-induced production of nitric oxide to 19 (SD 9)% of control arteries. This reduced ability of the vascular endothelium to transduce fluid shear stresses into biochemical synthesis of nitric oxide did not affect agonist-stimulated endothelial nitric oxide production, indicating that hyaluronan GAGs play a specific role in the mechanostimulation of the endothelium. Electron microscopic observation of discrete glyocalyx structures in rat myocardial capillaries confirmed that hyaluronidase treatment eliminates most of these structures from the luminal endothelial surface (16). Loss of glyocalyx hyaluronan subsequently resulted in significant swelling of the pericapillary space and anatomic compression of myocardial capillaries, indicating that removal of hyaluronan GAGs from the endothelial glyocalyx increases transcapillary fluid loss and probably leakage of plasma macromolecules. This concept is supported by previous in vivo studies (10) revealing that elevating plasma levels of hyaluronidase increases the permeation of dextrans of  $\leq 150$  kDa mol mass into the endothelial glyocalyx of hamster cremaster muscle capillaries. In the latter study, it was elegantly demonstrated that glyocalyx permeability barrier properties could be restored by reconstitution of the hyaluronidase-degraded hyaluronan GAGs by a mixture of chondroitin sulfate and hyaluronan, demonstrating that association of hyaluronan with endothelial proteoglycans is essential to exert its permeability barrier properties. In line with these recent reports on the contribution of hyaluronan GAGs to glyocalyx vasculoprotective properties, our current

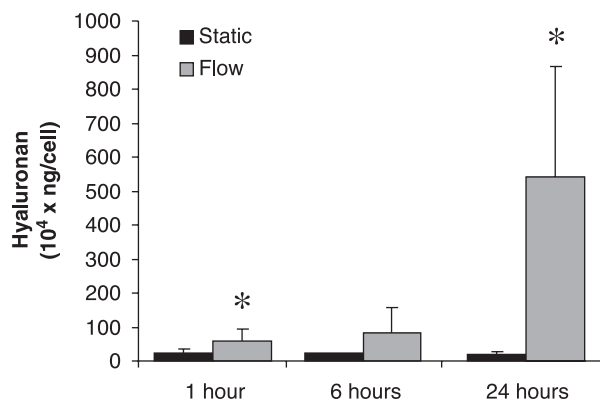


Fig. 4. Time-dependent effect of endothelial cell flow exposure on hyaluronan content in cell-culture media. With hyaluronan content of endothelial cells in collected media being measured during different times of flow exposure, hyaluronan content in media was already significantly increased after 1 h of flow stimulus ( $*P < 0.05$ ; left). After cells were exposed for 24 h of flow, hyaluronan content in 24-h collected media was about 20 times higher in flow-exposed cells compared with 24-h static cultured cells ( $*P < 0.05$ ; right).

finding that shear stress is an important stimulus for the incorporation of hyaluronan into the endothelial glycocalyx might increase our understanding of the relation between spatial shear-stress distributions and the localization of vulnerable vascular sites.

In conclusion, we demonstrate in the current study for the first time that fluid shear stress stimulates the incorporation of hyaluronan in the endothelial glycocalyx. Enhanced incorporation of hyaluronan in the endothelial glycocalyx might contribute to optimal endothelial function and protect against pathogenic vascular perturbation and warrants future studies on the relation between hyaluronan synthesis and endothelial function at lesion-prone vascular sites exposed to complex flow patterns.

#### GRANTS

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