Influence of a laminar steady-state fluid-imposed wall shear stress on the binding, internalization, and degradation of low-density lipoproteins by cultured arterial endothelium

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ABSTRACT Fluid mechanical steady-state laminar wall shear stresses of 30 dyne/cm² (high stress) and less than 1 dyne/cm² (low stress) have been applied for varying times to confluent cultures of bovine aortic endothelial cells (BAECs) by means of two parallel plate channel flow chambers in series. BAEC cultures not exposed to shear or flow (no stress) were also studied. A shear stress of 30 dyne/cm² resulted in cellular elongation and alignment, changes that were largely complete by 24 hr. In experiments in which BAECs were incubated with 125I-labeled low-density lipoprotein for 2 or 24 hr in the presence of shear stress levels, 125I-LDL internalization at 24 hr was increased (p < .05) in response to high-stress conditions. This increased uptake of 125I-LDL was observed in BAECs prealigned for 24 hr under high stress and in BAECs undergoing alignment in the presence of circulating 125I-LDL. BAECs were also exposed to shear stress for 24 hr in the presence of a lipoprotein-deficient circulating medium to maximize LDL receptor expression. Receptor-mediated 125I-LDL internalization and degradation measured immediately after shear stress were both significantly enhanced (p < .01) in BAECs exposed to high stress. Furthermore, 125I-LDL binding studies at 4°C revealed a significant increase (p < .01) in specific 125I-LDL binding to BAECs exposed to high stress relative to those exposed to low or no stress. Nonspecific 125I-LDL endocytosis was not influenced by shear stress levels. High stress did not influence the incorporation of 3H-thymidine into BAEC DNA, indicating that the observed increase in LDL endocytosis was not dependent on an increase in cellular replication. The influence of high stress on LDL receptor expression and function suggests that the LDL receptor pathway is influenced by factors other than the cellular availability of exogenous cholesterol, in particular the recognition by BAECs of fluid mechanical signals and their transduction within the cell or on the cell surface. Our findings provide one potential mechanism through which hemodynamic shear stress might participate in cellular cholesterol homeostasis as well as atherogenesis.


RESEARCH INTEREST is focusing increasingly on the roles of various modalities of hemodynamic stress in the etiology and pathogenesis of atherosclerosis. Localized fluid dynamic stresses associated with specific vascular geometries such as arterial curvatures, flow dividers, or branching sites may contribute to the unique focal topography of the atheromatous plaque that characterizes the disease process in both man and experimental animals. Of particular interest is the spectrum of biological mechanisms through which wall shear stress might participate in atherogenesis. A considerable body of evidence now implicates low-density lipoprotein (LDL) transport and receptor-mediated metabolism as key components of atherogenesis. Employing a parallel-plate channel flow viscometric device, we describe in this study the influence of both high (30 dyne/cm²) and low (<1 dyne/cm²) steady-state, laminar, fluid-imposed wall shear stress on the total and receptor-mediated binding, internalization, and catabolism of LDL by confluent cultures of bovine aortic endothelial cells (BAECs).
Methods

**BAEC culture.** BAECs were isolated from aortas obtained from a local slaughterhouse. The cell isolation procedures were based on methods described by Schwartz. Briefly, the excised aortas were rinsed with Dulbecco's phosphate-buffered saline (PBS) containing penicillin (360 U/ml), streptomycin (360 μg/ml), and fungizone (7.5 μg/ml). After dissection under sterile conditions and ligation of all branches, the aortas were rinsed via a cannula with Hank's balanced salt solution (HBSS) with Ca++ and Mg++ and then filled to slight distension with collagenase (1 mg/ml PBS, Type I, Worthington, Bedford, MA) at 37°C. After a 15 min incubation at 37°C, the incubation medium containing the dispersed BAEC was collected. Each aorta was subsequently rinsed with M199 (GIBCO, Grand Island, NY) containing 10% fetal calf serum (FCS, KC Biologicals, Lenexa, KS) to remove residual BAECs, and the resulting media were pooled and centrifuged at 200 g for 5 min; the BAEC pellet was then resuspended in M199-10% FCS for plating at a density of 10⁴ cells/cm². The BAECs were maintained in M199-10% FCS, serially passaged when confluent with 0.25% trypsin, and used between 2 and 4 passages.

**Examination by electrophoresis.** Type C, well tissue culture cluster dishes (Costar, Cambridge, MA) and were used within 1 day after attaining confluence. The quality of representative BAEC cultures was monitored by their light microscopical appearance, the presence of Factor VIII antigen, and ultrastructural characteristics.

**Isolation and labeling of LDLs.** LDLs were isolated from serum from fasting healthy young male volunteers by preparative ultracentrifugation with a Beckman L8 ultracentrifuge and a Type 60 Ti rotor. Very-low-density lipoproteins (VLDLs) were removed by centrifugation (d = 1.006 g/ml) for 24 hr at 40,000 rpm at 14°C. The solvent density was then adjusted to d = 1.063 g/ml with solid KBr and recentrifuged as above. Since lipoproteins were isolated from fasting normocholesterolemic subjects who typically exhibit low levels of intermediate-density lipoproteins (d = 1.006 to 1.019 g/ml), the d = 1.006 to 1.063 g/ml fraction of the lipoprotein was collected as the source of LDLs. Examination by electrophoresis revealed a single β-migrating band. The isolated LDLs were recentrifuged at d = 1.063 g/ml for an additional 24 hr to remove contaminating proteins. After isolation, the LDLs were dialyzed overnight under nitrogen against PBS containing EDTA. LDLs were iodinated with 125I using Bilheimer's modification of the iodine monochloride method of McFarlane at a molar ratio less than 1. Percent free iodide was determined by precipitating the LDLs with trichloroacetic acid (TCA, final concentration 10% w/v) and measuring 125I activity in the TCA-soluble fraction. Less than 1% of the total activity was present as free iodide. 125I labeling of the lipid moiety of LDL was determined by extraction of the LDLs as described by Folch et al. Lipid labeling represented less than 5% of the total LDL labeling. To assess whether the isolation and labeling of the LDLs had resulted in any chemical modification, agarose electrophoresis of the nascent and iodinated LDLs was performed routinely. LDL fractions showing any altered electrophoretic mobility were not used. Isolated LDL and radiolabeled LDL preparations were stored under nitrogen at 4°C for a maximum of 1 week until use.

**Preparation of lipoprotein-deficient serum.** Lipoprotein-deficient serum (LDS) was prepared by adjusting the density of homologous serum (FCS) to 1.2 g/ml with solid KBr and centrifuging at 105,000 g for 48 hr at 10°C in a Beckman L5-65 ultracentrifuge. The lipoprotein-free serum was then dialyzed against PBS, pH 7.4, for 24 hr at 4°C. Finally, the LDS is sterilized by filtering with a 0.2 μm filter.

**125I-LDL endocytosis measurements.** Details of procedures to quantitate 125I-LDL binding, internalization, and degradation have been reported previously by Goldstein et al. In the first series of experiments 125I-LDL internalization was assessed in BAEC cultures exposed to 10 μg 125I-LDL/ml circulating medium (M199-20% FCS) at 37°C for 2 or 24 hr during shear stress periods. The concentration of LDL in this medium ranged from 20 to 30 μg/ml, which exceeds the concentration required to saturate BAEC receptors and is sufficient to downregulate BAEC LDL receptor expression. Exposure of BAECs to circulating radiolabeled LDL under shear stress conditions was initiated either simultaneously with the induction of shear stress (concurrent shear stress studies) or after 24 hr of prior exposure to shear stress (prestress studies). The BAECs were contained within two parallel-plate channel flow chambers in series so that one set was exposed to high wall shear stress (30 dyne/cm²) and the other set was exposed to low wall shear stress (< 1 dyne/cm²).

After exposure to shear stress, the BAEC-containing coverslips were removed and placed in a 16 mm well of a multowell culture dish containing 2% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis) in PBS at 4°C for rinsing. The BAECs were rinsed a total of five times, the final two rinses of 10 min duration, with 2% BSA, followed by one rinse with ice-cold PBS, and the surface bound 125I-LDL was removed by incubating the BAECs with 0.5 ml of heparin (grade II, Sigma, 10 mg/ml PBS) for 60 min. The heparin incubation fluid was collected and pooled with three 0.5 ml PBS rinses at 4°C and counted for radioactivity in a Beckman Model 310 gamma spectrometer as a measure of 125I-LDL bound to the cell surface. The remaining BAECs were finally solubilized with 0.1N NaOH and the lysate combined with three PBS washes of the coverslip and counted as above as a measure of internalized 125I-LDL. Because 125I-LDL binding to the cell surface does not attain equilibrium at 37°C, only the 125I-LDL internalization measurements were analyzed.

The second set of experiments was designed to assess LDL receptor status after BAEC exposure to shear stress. In this case, the circulating medium consisted of M199-20% LDS. In addition to exposure of BAEC to high- and low-stress conditions, a third set of BAECs was incubated under static conditions in a humidified CO₂ incubator at 37°C in the same medium as contained within the circulating shear stress apparatus. The third BAEC set was denoted as no shear stress. To measure receptor-mediated postshear 125I-LDL internalization and degradation, BAEC cultures upregulated under shear conditions were removed at the end of the shear stress period and incubated immediately with 10 μg 125I-LDL/ml 10% LDS in M199 for 2 hr at 37°C in the presence or absence of a 50-fold excess of unlabeled LDL. At completion of the incubations, 0.5 ml of the incubation medium was removed and treated with TCA at a final concentration of 10% (w/v). After centrifugation (Microfuge, 12,000 rpm, 2 min), the supernatant was removed and treated with 5% AgNO₃ (w/v) as described by Henriksen et al. to precipitate free labeled iodide. After a second centrifugation, the 125I activity remaining in the final supernatant was counted as a measure of 125I-LDL degradation (ng LDL/mg cell protein). The BAECs were then rinsed and processed for measurement of 125I internalization as described above. Binding studies were undertaken at 4°C. In these studies, BAEC cultures that were LDL receptor upregulated under shear stress were removed from the flow chambers or incubator (no shear control) at the end of the shear stress period and immediately precooled to 4°C in 0.5 ml 2% BSA in PBS for 30 min before initiation of binding studies. After precooling, the BAECs were incubated with 10 μg 125I-LDL/ml 2% BSA at 4°C for 2 hr. The BAECs were rinsed with 2% BSA and PBS at the end of this incubation, and
the surface-bound 125I-LDL was removed by incubating the cells with heparin for 60 min at 4°C as above. The heparin incubation fluid was collected and pooled with three 1 ml PBS washes at 4°C for counting as a measure of 125I-LDL binding (ng LDL/mg cell protein). 125I-LDL binding studies were performed in the presence or absence of an excess (200 to 500 μg/ml) of unlabeled LDL to allow determination of specific or receptor-mediated binding.

**BAEC replication: 3H-thymidine incorporation.** The influence of both high shear stress (30 dyn/cm²) and low shear stress (<1 dyn/cm²) on total 3H-thymidine incorporation into BAECs was studied. Additional controls consisted of quiescent human skin fibroblast cultures with and without the addition of FCS and static BAEC cultures not exposed to shear or flow. After 24 hr of exposure to high or low shear, or simultaneous maintenance in static culture, the coverslips with BAEC or fibroblasts were placed in 24 well cluster culture dishes containing 0.5 ml of medium (20% LBS in Ham's F-12) with 2 μCi 3H-thymidine (53 Ci/mmol; ICN, Irvine, CA). After a 2 hr incubation at 37°C, the medium was removed, the cultures were rinsed twice with HBSS-0.05% EDTA, and the cells were removed by trypsinization (0.5 ml of 0.25% trypsin with 0.5% EDTA) for 5 min at 37°C. Duplicate aliquots (0.2 ml) were removed for cell counts, and duplicate 0.2 ml aliquots were placed in microfuge tubes, to which was added 0.5 ml of 5% TCA (w/v). After 5 min in an ice bath, the cell suspensions were centrifuged, the supernatants discarded, and the cell pellets resuspended. The TCA treatment was repeated, and the cell pellets were dissolved in 0.2 ml of 1% sodium dodecyl sulfate (SDS) in 0.1N NaOH with mixing for 15 min at 37°C. After transfer to scintillation vials containing 5 ml Ready-Solv Hp, (Beckman), the 3H-thymidine activity (dpm/10⁸ cells or pmoL thymidine/μg cell protein) was counted in a Beckman liquid scintillation spectrometer (Model LS 5801).

Cell replication rate was examined further by 3H-thymidine autoradiography. BAECs were initially incubated with 1 Ci 3H-thymidine/ml for 2 hr at 37°C. The BAEC cultures were then cooled to 4°C and rinsed once with PBS and twice with methanol before fixation with methanol for 15 min at 4°C. After fixation, the cells were rinsed five times with water at 4°C and air dried. The fixed cells were then layered with NTB-2 emulsion (Eastman Kodak, Rochester, NY) in a darkroom and sealed in a lightproof container over Drierite for 7 to 10 days. Finally, the emulsion was developed with Kodak Developer D19 for 3 min. Cell replication rate was measured as the percentage of radio-labeled nuclei.

**Shear stress studies: the parallel-plate channel flow chambers.** The parallel-plate device used in this study permits the exposure of cultured endothelial cells to a known fluid-imposed wall shear stress. Each chamber consists of a long channel or rectangular cross section, with adjustable channel height. The design of the channel is based on the parallel-plate device discussed previously. The details of the construction are shown in figure 1. Four ports are located along the channel in which 13 mm plastic or glass coverslips are easily mounted flush with the channel surface. Knowing the flow rate through the channel, which is determined either by a direct measurement of the flow rate or from a measurement of the pressure drop, the wall shear stress is calculated. With this device, a wall shear stress of 60 dyn/cm² can be generated with a flow Reynolds number of 60 and with a channel height of 250 μm. At such a Reynolds number the flow is laminar, and the velocity profile is of a fully developed parabolic form over more than 98% of the channel length.

For the wall shear stress studies described here, two such parallel plate chambers are incorporated into the circuit in series, as shown in figure 2, ensuring that the cells exposed to exper-

**FIGURE 1.** Detailed construction of a four-port, parallel-plate flow chamber used in exposing confluent BAEC monolayers to steady laminar shear stresses.

**FIGURE 2.** Experimental laboratory design illustrating the use of two flow chambers in series, one for high shear stress experimental conditions positioned in an inverted microscope, the other for low shear stress control conditions.
Results

Shape and alignment of BAECs exposed to high stress for up to 24 hr. When confluent monolayers of BAECs are exposed to a fluid shear stress, their response involves a change in shape and orientation. The result is that over a period of 24 hr the cells become more elongated; that is, their shape index decreases and their major axis tends to align with the direction of flow. This is illustrated in figure 3 for a shear stress of 30 dyne/cm². As may be seen, the angle of orientation and the shape index reach a plateau between 12 and 24 hr. Previous studies have demonstrated that these responses of cell shape index and orientation to increased wall shear stress are proportional to the level of shear stress and that these changes are statistically significant (p < .001). No change in BAEC shape or orientation was observed in response to low (< 1 dyne/cm²) or no shear stress after 24 hr. Based on these kinetic observations, experiments were designed to determine the influence of fluid-imposed shear stress on LDL endocytosis both in cells undergoing alignment and in those prealigned by shear stress. The term “prestress” was used to designate experiments in which BAECs were exposed to 24 hr of shear stress to achieve prealignment before introduction of ¹²⁵I-LDL into the circulating medium. “Concurrent stress” designates experiments in which ¹²⁵I-LDL was present in the circulating medium upon initiation of shear stress.

¹²⁵I-LDL internalization, influence of prestress. For both prestress and concurrent stress experimental regimens, ¹²⁵I-LDL internalization studies were performed in the presence of circulating medium containing 20% whole FCS. This was done to determine whether shear stress levels could influence LDL endocytosis without manipulating LDL receptor expression through the use of LDS. In the first series of experiments, the BAEC cultures were incubated with ¹²⁵I-LDL under continu-

ing shear stress after shear stress—induced changes in cell shape and orientation were largely complete. BAEC cultures were exposed to a wall shear stress of 30 and less than 1 dyne/cm² for 24 hr, after which the ¹²⁵I-LDL was added to the perfusate as described above, and the shear stress continued for 2 and 24 hr, respectively, when ¹²⁵I-LDL internalization was measured as described. Eight high- and low-stress experiments were performed in duplicate, and the results are illustrated in figure 4 and detailed in table 1. Although a similar pattern of response to shear stress was observed in the eight studies, the actual amount of ¹²⁵I-LDL internalized between experiments was variable. This variability was primarily associated with the use of BAECs from different aortas. To permit comparison and analyses of the results from the individual experiments, the data were normalized by expressing the ¹²⁵I-LDL internalization observed as a ratio of high to low shear stress (HS:LS) within each experiment. Internalization of ¹²⁵I-LDL was significantly greater in cells exposed to a high wall shear (p = .018) compared with a low shear when the ¹²⁵I-LDL was present in the perfusate for 24 hr. When ¹²⁵I-LDL was present for only 2 hr, there was a trend for the HS:LS ratio to be

![FIGURE 4](https://example.com/figure4.png)

**FIGURE 4.** Net intracellular accumulation of ¹²⁵I-LDL (ng/ml) internalized during either a 2 or 24 hr exposure to a shear stress of 30 dyne/cm² (HS) or less than 1 dyne/cm² (LS) at 37°C. For prestress experiments, BAECs were exposed to 24 hr shear stress (HS and LS) before the addition of ¹²⁵I-LDL (100 to 300 dpm/ng LDL) at 10 μg/ml to the circulating medium (20% FCS-M199). Shear stress levels were then maintained for an additional 2 or 24 hr before measuring internalized ¹²⁵I-LDL activity. In concurrent experiments, the ¹²⁵I-LDL (10 μg/ml) was present in the circulating medium at the initiation of the shear stress (HS or LS) exposure, which was maintained for 2 or 24 hr before internalization measurements. At the end of 2 or 24 hr incubation with ¹²⁵I-LDL in the presence of HS or LS, the BAECs on coverslips were removed, rinsed in 2% BSA and PBS, treated with heparin to remove surface-bound ¹²⁵I-LDL, and solubilized with 0.1N NaOH to measure intracellular ¹²⁵I-LDL as detailed in Methods. Data are presented as mean ± SEM, and statistical analyses were performed with Student’s paired t test. Detailed data are presented in table 1.

![FIGURE 3](https://example.com/figure3.png)

**FIGURE 3.** Graphic representation of the time history of the mean shape index and angle of orientation for confluent BAEC monolayers exposed to a steady shear stress of 30 dyne/cm².
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TABLE 1

Influence of a steady-state laminar shear stress of 30 dyne/cm² on 125I-LDL internalization in confluent BAEC cultures (ng/mg cell protein)

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Prestress</th>
<th>Concurrent stress</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>2 hr</td>
<td>24 hr</td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>LS</td>
</tr>
<tr>
<td></td>
<td>HS</td>
<td>LS</td>
</tr>
<tr>
<td>1</td>
<td>54</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>172</td>
<td>235</td>
</tr>
<tr>
<td>3</td>
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<td>8</td>
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<tr>
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<td>263</td>
<td>262</td>
</tr>
<tr>
<td>SEM</td>
<td>± 56</td>
<td>± 51</td>
</tr>
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HS = high shear stress (30 dyne/cm²); LS = low shear stress (<1 dyne/cm²).

greater than 1, but the difference did not attain statistical significance (p > .10).

125I-LDL internalization: influence of a concurrent wall shear stress. The second series of experiments was undertaken to determine the influence of wall shear stress on 125I-LDL internalization during shear-induced changes in cell shape and orientation. BAEC cultures were exposed to both high and low stress for 2 and 24 hr, with 125I-LDL present in the perfusate throughout the application of shear.

Six duplicate experiments were performed. Although the HS:LS ratio was greater than 1 at 2 hr (1.25), this difference did not attain statistical significance (p > .10) (figure 4, table 1). At 24 hr, however, concurrent stress resulted in a statistically significant enhancement of 125I-LDL internalization (p = .028).

When the response of LDL internalization to high shear for 24 hr was compared on the basis of either HS:LS ratios or absolute high shear values, no significant difference was detected between prestressed BAEC and BAEC concurrently stressed upon addition of 125I-LDL.

The above studies establish that the application of laminar steady-state wall shear stress to confluent BAEC cultures enhances the internalization of 125I-LDL. Furthermore, this effect is not dependent on prior shear-induced changes in cell shape and orientation, nor is it limited to the period during which cell geometry is changing. Finally, these studies indicate that this enhanced 125I-LDL uptake occurs even in the presence of lipoprotein-containing FCS.

During the preceding experiments, the percentage of perfusate 125I activity that was not TCA-precipitable, i.e., free 125I, ranged from a mean of 2.85% at 0 and 2 hr to a mean of 3.6% from 24 to 48 hr.

Influence of wall shear stress on receptor-mediated 125I-LDL internalization and degradation. Based on our findings that 125I-LDL internalization increased in BAECs exposed to high wall shear stress, a series of experiments was performed to determine whether this enhanced LDL internalization might be mediated via an influence of shear stress on the LDL receptor. Accordingly, confluent BAECs on coverslips were exposed to high, low, or no shear stress for 24 hr in the presence of circulating medium containing 20% LDS to maximize LDL receptor expression. At the end of the shear stress period, the cells were removed and LDL receptor status was assessed by incubating the cells with 125I-LDL at 37°C for 2 hr to measure both receptor- and nonreceptor-mediated 125I-LDL internalization and degradation. Specific or receptor-mediated internalization and degradation were determined as the difference measured in the presence and absence of an excess of unlabeled LDL.

Receptor-mediated internalization of 125I-LDL measured immediately after removal of coverslips from shear stress conditions was significantly enhanced in BAECs exposed to high shear compared with those exposed to no shear (figure 5). Although the overall means of 125I-LDL internalization in cells exposed to low or no shear in nine experiments were identical (56 ng 125I-LDL/mg protein), analysis of the results by Student's t test failed to demonstrate a significant difference (p = .07) between BAECs exposed to high...
FIGURE 5. Specific receptor-mediated and nonspecific $^{125}$I-LDL (ng/mg cell protein) internalization and degradation in confluent BAECs preexposed to shear stress of 30 dyne/cm$^2$ (HS), less than 1 dyne/cm$^2$ (LS), or no shear (NS) for 24 hr at 37° C. After shear stress, BAECs on coverslips were removed and incubated for 2 hr at 37° C with 10 μg $^{125}$I-LDL (100 to 300 dpm/ng LDL)/ml 10% LDS-M199 in the presence or absence of excess (200 to 500 μg/ml) unlabeled LDL. After $^{125}$I-LDL incubations, the medium was collected for measurement of TCA-soluble $^{125}$I activity (degradation, see figure 6) and the cells were rinsed, treated with heparin, and solubilized with NaOH to measure cell internalized $^{125}$I-LDL according to details presented in Methods. Data from nine experiments are presented as mean ± SEM.

compared with those exposed to low shear stress. On the other hand, receptor-mediated degradation of $^{125}$I-LDL was significantly greater (p < .01) in cells exposed to high stress compared with those exposed to low or no shear stress (figure 6). No significant difference in $^{125}$I-LDL internalization or degradation was found between cells exposed to low or no shear when subjected to paired analysis (p > .20). Similarly, nonreceptor-mediated $^{125}$I-LDL uptake and degradation by BAECs was not influenced by shear stress levels (figures 5 and 6).

Influence of high wall shear stress on $^{125}$I-LDL binding.
To determine whether shear stress influenced the availability of cell surface $^{125}$I-LDL–specific binding sites or receptors, $^{125}$I-LDL binding was measured at 4° C over 2 hr immediately after removal of shear stress in six replicate experiments. As illustrated in figure 7, receptor-mediated or specific $^{125}$I-LDL binding was significantly increased in BAECs exposed to high shear stress compared with those exposed to either low or no shear (p < .01). Furthermore, although the overall mean of receptor-mediated $^{125}$I-LDL binding to low shear–treated BAECs was enhanced compared with that of cells exposed to no-stress conditions, this apparent difference was not verified statistically by Student’s paired t test (p > .10). Similar to studies at 37° C, nonspecific binding of $^{125}$I-LDL at 4° C to BAECs measured in the presence of excess unlabeled LDL, was not influenced by exposure to shear stress (figure 7).

Influence of steady-state on endothelial cell replication.
As described in methods, confluent BAEC cultures were exposed to high, low, or no shear stress for 24 hr after which they were incubated for 2 hr with 2 μCi of $^3$H-thymidine. The results are summarized in table 2, where it can be seen that $^3$H-thymidine incorporation into endothelial cell DNA was not significantly different among the three groups of cultures. In particular, exposure to a steady-state high wall shear of 30 dyne/cm$^2$ for 24 hr did not result in a measurable

FIGURE 7. Receptor-mediated binding at 4° C to BAECs preexposed to shear stress of 30 dyne/cm$^2$ (HS), less than 1 dyne/cm$^2$ (LS), or no shear (NS) for 24 hr in the presence of a circulating medium containing M199-10% LDS at 37° C. After shear stress, BAECs coverslips were removed, precooled at 4° C for 30 min, and incubated for 2 hr at 4° C with 10 μg $^{125}$I-LDL (100 to 300 dpm/ng LDL)/ml 2% BSA in the presence or absence of an excess (200 to 500 μg/ml) unlabeled LDL. BAECs were next rinsed and the cell surface $^{125}$I-LDL removed with heparin and counted for radioactivity. Data from six experiments are presented as mean ± SEM.
increase in BAEC replication, making it unlikely that the enhanced 125I-LDL endocytosis is the result of shear-induced BAEC replication. As a positive control for the mitogenic assay, cultured human fibroblasts rendered quiescent by a 72 hr preincubation in culture medium M199 containing 1% platelet-poor plasma-derived serum were stimulated with 10% FCS-M199 for 24 hr before incubation with 3H-thymidine as above. A 13-fold increase in 3H-thymidine uptake was observed after stimulation in these fibroblasts compared with the quiescent state, indicating that the assay sensitivity was adequate (data not shown). Cell replication rate as measured by 3H-thymidine autoradiography confirmed these results.

Discussion

The studies described in this report have established for the first time that a laminar steady-state fluid mechanical wall shear stress within the biologically relevant range (i.e., 30 dyne/cm²), enhances significantly the binding, internalization, and degradation of LDL by confluent BAEC cultures. Furthermore, this high wall shear effect reflects an enhancement of the receptor-mediated component of LDL-binding, internalization, and degradation. Quite apart from the potential significance of this wall shear stress effect in the atherogenic process, our findings raise some intriguing biological questions. In particular, how is a fluid mechanical signal such as wall shear stress recognized by the endothelial cell? Furthermore, how is such a signal transduced within the cell or on its surface to regulate changes not only in cell shape and orientation but also in LDL endocytosis, including LDL receptor expression?

In the present series of experiments, we have observed that confluent BAEC cultures exposed to a wall shear stress of 30 dyne/cm² exhibit changes both in cellular shape and orientation that reflect the direction of shear stress and that are complete within 24 hr (figure 3). This influence of high shear stress with laminar flow on the geometry and orientation of confluent BAEC cultures is consistent with the alignment noted by others. It is clear from figure 4 and table 1 that the enhancement of 125I-LDL internalization by shear stress is not dependent on prior shear-induced changes in cell shape and orientation, nor is it limited to the time during which BAEC cell geometry is actively changing. Even though the influence of high wall shear stress on LDL internalization is not limited to the time during active changes of cell shape and orientation, these results do not rule out the possibility that the alterations in cell biology underlying this response, such as enhanced LDL receptor activity, may develop during the time the BAECs are changing their geometry.

Results of the endocytosis experiments conducted in the presence of circulating 125I-LDL also indicate that the elevated levels of the LDL internalization observed in response to high shear may be at least partially independent of exogenous cholesterol concentrations. As described above, the enhanced 125I-LDL internalization was recorded in BAECs incubated with 20% whole FCS, which would downregulate LDL receptor activity in cultured BAEC maintained under static conditions. These data again indicate that shear stress may be influencing LDL receptor activity.

125I-LDL internalization measurements recorded after incubation with 125I-LDL in the presence of shear stress conditions represent the net accumulation of intracellular 125I-activity at the end of the 2 or 24 hr experimental period. The total amount of 125I-LDL internalization within each experiment would be expected to be higher as a result of the loss of internalized radiolabel resulting from degradation and/or exocytosis of the internalized 125I-LDL. The large circulating volume of media (75 ml) relative to the number of BAECs prevented detection of these components of the total 125I-LDL internalization. The recent development of residualizing, high-specific activity 125I-glycoconjugates for labeling proteins, including LDL, makes it possible to account for the amount of LDL degraded by measuring the lysosomal accumulation of nondegradable glycoconjugate.

Studies are in progress to employ this technique with 125I-dilactitol-tyramine to label LDL for internalization studies under shear stress conditions.

Results of experiments in which BAEC LDL receptor activity was allowed to develop maximally under shear stress in the presence of lipoprotein-deficient circulating medium before assessment of LDL receptor activity more directly address the question as to the influence to high shear stress on LDL receptor expression. These results indicate that exposure of BAECs to

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TABLE 2

<table>
<thead>
<tr>
<th>3H-Thymidine incorporation (dpm/10³ cells)</th>
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<th>LS</th>
<th>NS</th>
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<tbody>
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<td>No. of experiments</td>
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</tr>
<tr>
<td>Mean ± SEM</td>
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<td>13.5 ± 1.40</td>
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</table>

Abbreviations as in table 1.
high wall shear stress increases the receptor-mediated components of 125I-LDL internalization and degradation compared with BAECs exposed to no shear, as well as degradation compared with BAECs exposed to low shear. The lack of a significant increase in 125I-LDL internalization in BAECs exposed to high compared with those exposed to low stress (p = .07) may be caused by the somewhat higher variability encountered in internalization measurements. Interestingly, results of binding studies performed at 4°C C indicate that this increased endocytosis of 125I-LDL in BAECs exposed to high stress may be caused by an increased availability of specific LDL receptor sites.

Previous reports24, 31 have demonstrated that the number of LDL receptors declines dramatically as cultured endothelial cells attain confluence. Whether high-shear conditions diminish the cell-to-cell communication responsible for this receptor down regulation or more directly influence LDL receptor synthesis, recycling, or expression remains to be investigated.

One of the cellular mechanisms that could potentially contribute to the enhanced uptake of 125I-LDL by BAECs exposed to high shear is an increased rate of pinocytosis. Based on our observations that the non-specific LDL endocytic pathways are not influenced significantly by shear stress levels, it seems unlikely that pinocytosis is the primary mechanism involved in the LDL endocytosis response. Although Davies et al.,9 using horseradish peroxidase as a marker, have demonstrated that cellular pinocytosis increases in BAECs exposed to shear stress, these authors have reported that changes in shear stress amplitude (3 to 13 dyne/cm²) was the determining factor in producing an elevation in fluid-phase endocytosis.

Our findings have established that a steady-state laminar shear stress has not induced a measurable increase in BAEC division, confirming the findings of others9, 10 and making it unlikely that the increased receptor-mediated LDL endocytosis described can be attributed to an increased rate of endothelial cell replication. In sharp contrast to the absence of any effect of laminar shear stress on BAEC replication, it has recently been established that turbulent fluid shear stresses as low as 1.5 dyne/cm² stimulate endothelial DNA synthesis, in the absence of cell alignment, loss of confluence, or cellular loss.10 What influence turbulent or even unsteady laminar shear stress might have on LDL endocytosis remains to be determined.

The influence of shear on endothelial LDL receptor expression and function could result from an increase in receptor number, enhanced exposure of existing receptor domains (thus facilitating ligand-receptor coupling), enhanced receptor affinity, or an accelerated recycling of receptors to the plasmalemmal membrane. These possibilities are presently being explored. It is clearly of importance to understand how a fluid mechanical shear stress signal is translated to the heightened LDL receptor expression and function we have observed. In particular, it would appear that factors other than the availability of exogenous cholesterol16, 17 may regulate cellular LDL receptor expression and function.

The increased LDL receptor expression observed in response to an elevated wall shear stress in vitro may provide insights into the role of hemodynamic forces in the regulation of endothelial function. Since most aortic endothelial cells exist in an environment of elevated wall shear stress in vivo, the increased LDL receptor activity observed in vitro in response to a fluid-imposed shear stress may represent a restoration toward natural endothelial function. Vasile et al.,32 for example, have demonstrated a significant receptor-mediated component of LDL endocytosis in rat aorta in situ. Thus aortic endothelial cells may express LDL receptors in response to shear stress as part of a metabolic adaption to their environment to maintain cholesterol homeostasis. Alternatively, if elevated hemodynamic shear stress enhances transport or transcytosis of LDL to underlying arterial cells and tissue either via receptor or nonreceptor-mediated processes, then hemodynamic forces may also influence the excessive accumulation of arterial wall cholesterol observed in atherosclerosis.

Finally, the response of LDL receptor-mediated endocytosis to hemodynamic forces provides a basis for future investigations not only into the regulation of LDL endocytosis but also into regulation of other receptor-mediated processes involved in endothelial cell biology.

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Influence of a laminar steady-state fluid-imposed wall shear stress on the binding, internalization, and degradation of low-density lipoproteins by cultured arterial endothelium.

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