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Turbulent fluid shear stress induces vascular endothelial cell turnover *in vitro*

(hemodynamic forces/endothelial growth control/atherosclerosis)

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ABSTRACT The effects of hemodynamic forces upon vascular endothelial cell turnover were studied by exposing contact-inhibited confluent cell monolayers to shear stresses of varying amplitude in either laminar or turbulent flow. Laminar shear stresses (range, 8–15 dynes/cm^2; 24 hr) induced cell alignment in the direction of flow without initiating the cell cycle. In contrast, turbulent shear stresses as low as 1.5 dynes/cm^2 for as short a period as 3 hr stimulated substantial endothelial DNA synthesis in the absence of cell alignment, discernible cell retraction, or cell loss. The results of these *in vitro* experiments suggest that in atherosclerotic lesion-prone regions of the vascular system, unsteady blood flow characteristics, rather than the magnitude of wall shear stress *per se*, may be the major determinant of hemodynamically induced endothelial cell turnover.

Hemodynamic forces have been implicated in the initiation, localization, and development of atherosclerotic vascular disease (1, 2). Little is known, however, about the effects of such forces upon the endothelial cell lining of blood vessels, the integrity of which is essential for normal vascular function. In certain areas of the aorta and its main branches, blood flow characteristics are both variable and complex. In locations such as the descending thoracic aorta and distal carotid arteries, pulsatile laminar flow is prevalent (3), whereas in other regions, such as coronary arteries and the carotid bifurcation, secondary flows, vortices, and intermittently changing flow directions are encountered (4). The distribution of atherosclerotic lesions in susceptible species, including humans, is closely correlated with the location of disturbed flow in the major vessels (5). Time-dependent flow separation and unsteady secondary flow typically occur in localized regions that are usually well defined and of limited size. Furthermore, turbulence will occur in the largest arteries under conditions of increased flow velocity and cardiac output (4). Thus, shear stresses, which are the direct tractive forces acting on the endothelial cell surface as a result of blood flow, are highly variable in magnitude, frequency, and direction in such regions.

Autoradiographic studies *in vivo* have demonstrated increased endothelial DNA synthesis in localized areas of the aorta and its major branches, suggesting that locally increased endothelial cell turnover, perhaps as a result of injury, may occur near branches and bifurcations (6, 7). Increased cell turnover need not imply denudation of the endothelium and indeed during the initiation and early development of atherosclerotic lesions the endothelium remains a confluent monolayer of cells (8).

The role of fluid shear stress in promoting endothelial cell injury and/or turnover is uncertain: both high and low shear stresses have been implicated. High shear stress has been linked to alignment of endothelial cells (9), cell loss (10), increased arterial permeability (11), and enhanced endothelial biosynthetic capabilities (12). Atherosclerotic lesions often occur in areas of predicted high shear stress, such as distal to flow dividers (13). Paradoxically, however, there are regions of predicted high laminar shear stress *in vivo* that are spared atherosclerosis, and more recent experiments suggest that the occurrence of low shear stresses in regions of laminar flow separation correlates well with lesion development (14–16). The periodicity, direction, and frequency of shear stresses are extraordinarily complex in regions of disturbed flow whether laminar or turbulent shear stresses prevail (17–19). It has been suggested, therefore, that unsteady flow patterns or fluctuating shear stress amplitude and direction may be more directly correlated with endothelial biology and atherogenesis than mean shear stress amplitude alone (17–19). In contrast to laminar flow, turbulent flow at an equivalent mean shear stress level includes a wide range of shear stress frequencies and flow directions. In this report, therefore, we assess independently the effects of shear stress amplitude and two types of flow, turbulent versus laminar, upon endothelial cell turnover *in vitro*.

MATERIALS AND METHODS

The structure and function of endothelial cells were studied *in vitro* under carefully controlled conditions of flow in a modified cone-plate viscometer, the construction of which has been described in detail (20). Bovine aortic endothelial cells were isolated and cultured on glass coverslips as described (21). Confluent monolayers were exposed to laminar or turbulent flow in the cone-plate apparatus for various time periods ranging from 3 to 24 hr. Laminar flow was generated using a cone angle of ½° (21, 22). Turbulent flow was induced by increasing the flow velocity and using a cone angle of 5°. Time-mean shear stress levels were extrapolated from previous measurements and theory (20–23) and were estimated to be accurate to ±20%. Estimates of shear stress fluctuation in turbulent flow were made by a combination of flow visualization and hot-film gauge measurements as described (24). No sharp peaks were observed in the spectrum of fluctuation in turbulence, and stress excursions were limited to approximately 25% of the mean value. Up to 12 endothelial monolayers on coverslips (1 cm^2 area, 1.5 × 10^5 endothelial cells per coverslip) were subjected to flow in the apparatus for varying periods and the fraction of cells committed to the cell cycle was assessed by autoradiography. Immediately following exposure to flow, coverslips of cells were removed to Petri dishes for incubation with 0.2 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine per ml for 24 hr. The fraction of the cell

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population that synthesized DNA during this period was determined by autoradiography according to procedures described in ref. 25. Cell density in the monolayer was measured in phase-contrast photomicrographs taken immediately upon removal of each monolayer from the flow apparatus and cells were examined by both phase- and Nomarski interference-contrast microscopy for morphological change. Replicate coverslips of endothelial cells were trypsinized immediately following exposure to shear stress and DNA content was measured by fluorescence cytometry using propridium iodide. DNA content was plotted against cell frequency and the area beneath peaks corresponding to G_0/G_1 and G_2/M phases (n and $2n$ relative amounts of DNA, respectively) was measured as an estimate of cell cycle progression (26). The area under the curve between these peaks was considered to represent cells traversing S phase.

RESULTS

The shape of confluent cultured endothelial cells normally observed under static culture conditions (Fig. 1*A*) was altered by exposure to unidirectional shear stresses of 8 dynes/cm^2 in laminar flow within 24 hr (Fig. 1*B*). Individual cells became ellipsoidal and the cell population assumed an axial alignment in the direction of flow. In contrast, application of a mean shear stress as low as 1.5 dynes/cm^2 for 16 hr in turbulent flow resulted in random orientation of cells in the monolayer. Furthermore, many cells rounded up out of the plane of the

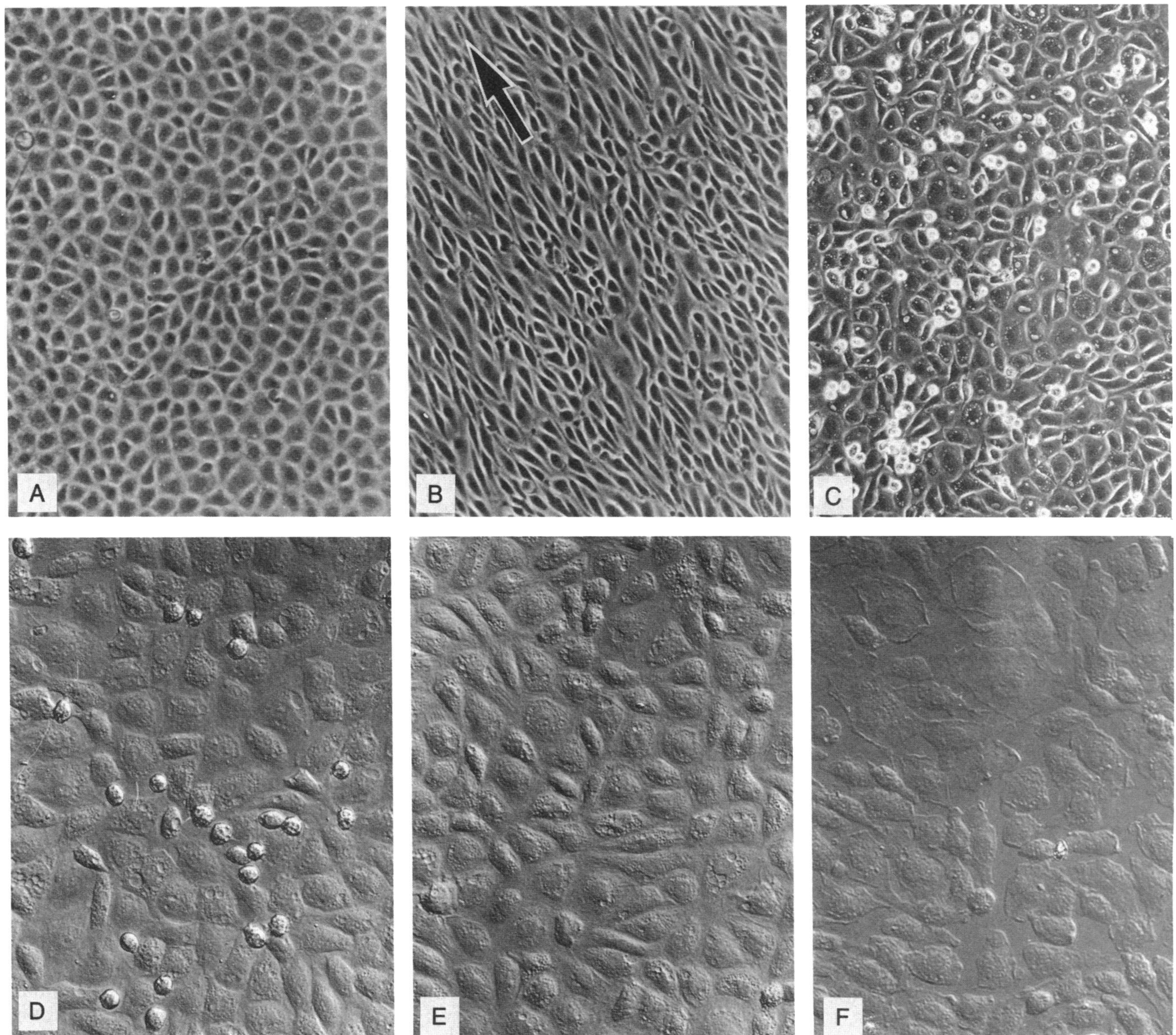

FIG. 1. Morphological changes induced in confluent bovine aortic endothelial cells by exposure to shear stress in laminar and turbulent flow. (*A*) Monolayer in static culture. Under no flow conditions, cells exhibit a polygonal configuration with no preferred orientation. (Phase-contrast microscopy; ×280.) (*B*) Alignment of cells in a confluent endothelial monolayer after 24 hr of exposure to shear stress at 8 dynes/cm^2 in laminar flow. Note ellipsoidal shape change. Arrow indicates direction of flow. (Phase-contrast; ×280.) (*C*) Confluent endothelial monolayer after 16 hr of exposure to shear stress at 1.5 dynes/cm^2 in turbulent flow. Cell shape in the monolayer is more variable than in *A*, no alignment is apparent, and significant numbers of rounded cells can be seen attached to the upper surface of the monolayer. Similar effects were noted after 16 hr of exposure to 3, 5, and 14 dynes/cm^2 in turbulent flow. (Phase-contrast; ×300.) (*D*) Higher-power Nomarski image of rounded cells attached to the monolayer under the effects of low shear stress in turbulent flow (1.5 dynes/cm^2; 16 hr). Most appeared to remain attached to adjacent cells. (×600.) (*E*) Nomarski image after 5 hr of exposure to shear stress at 14 dynes/cm^2 in turbulent flow showing a confluent monolayer without evidence of cell–cell retraction. Forty percent of these cells, however, go on to synthesize DNA. (×580.) (*F*) Appearance of gaps in the cell monolayer reflecting cell retraction and cell loss after 24 hr of exposure to shear stress at 14 dynes/cm^2 in turbulent flow. (Nomarski; ×600.)

monolayer (Fig. 1*C*). Most of these rounded cells, however, appeared to remain attached (Fig. 1*D*). Cell retraction and cell loss was not detectable at earlier time points (3, 5, 8 hr), even at a relatively high level of turbulent shear stress (mean, 14 dynes/cm^2; Fig. 1*E*). Prolonged exposure to turbulent flow (24 hr or longer), however, resulted in the development of gaps in the monolayer (Fig. 1*F*), reflecting cell retraction and cell loss (see below).

Endothelial cell turnover was substantially increased in the monolayers exposed to turbulent flow compared to laminar flow over a comparable range of shear stresses (Fig. 2). Exposure to relatively high shear stresses in laminar flow for up to 24 hr caused no significant change in the percentage of [^{3}H]thymidine-labeled cell nuclei. In turbulent flow, however, relatively short exposure (3 hr) to shear stress as low as 1.5 or as high as 14 dynes/cm^2 stimulated significant increases in DNA synthesis (17% and 44%, respectively), indicating that the cell cycle was initiated in the absence of discernible cell–cell retraction. It was estimated by flow cytofluorography of DNA content that by 16 hr (14 dynes/cm^2), 15.0% of the cell population were in S phase of the cell cycle and 25.2% had entered G_2/M phase. Thus, rounded, surface-attached cells visible after 16 hr may correspond to cells in mitosis. When the culture medium was filtered after 24 hr of turbulent shear stress (14 dynes/cm^2), intact cells and cell debris were trapped on the filter, indicating cell loss from the monolayer. Monolayer cell density after 24 hr declined to 89.5% of that in static controls ($P < 0.05$). These results are in marked contrast to those obtained in laminar flow, where we were unable to detect cell loss following 24 hr of laminar flow at comparable mean shear stresses, and the cell density after alignment in laminar flow was unchanged compared with static control monolayers.

DISCUSSION

Our experiments demonstrate that endothelial cell turnover *in vitro* is considerably more sensitive to relatively low shear stresses in turbulent flow than to much higher shear stresses applied in laminar flow, implying endothelial cell susceptibility to the flow characteristics rather than to the magnitude of shear stress alone. Forces acting on the endothelium in both types of flow have the same tractive hemodynamic component, shear stress, but in turbulent flow both the duration of the shear stress applied to the cell and its direction are fluctuating. In contrast, the shear stress signal received by the cell subjected to laminar flow was unidirectional and nonfluctuating. The specific characteristics of turbulent flow that induce endothelial cell turnover in these experiments are not known. As we have reported previously, oscillating laminar flow at frequencies up to 1 Hz, with shear oscillations from 3 to 13 dynes/cm^2, did not elicit this response (22). We postulate that it is the small-scale, high-frequency fluctuation and rapidly changing direction of turbulent shear stress that induces cell turnover. No definitive measurements of the spectrum of turbulence in a cone-and-plate apparatus have yet been published. Preliminary observations by Tse have demonstrated a broad spectrum with root-mean-square fluctuation amplitudes in the range of 25–50% for the conditions of the present experiment.[§] Flow visualization failed to demonstrate any dominant frequencies.[§] Estimates based on the turbulent scaling laws suggested by Corrsin (27) suggest that the turbulent integral scale [L] is approximately 0.1 of the local cone-plate gap height at the cell location in our apparatus. For a cone angle of 5°, the value of L is 500 μm and the turbulent Kolmogorov scale of the order of 100 μm, a dimension within a factor of 5 of the size of an individual endothelial cell. This relationship implies that the smallest turbulent eddies containing significant energy approach the dimensions of a single cell and infers the existence of significant gradients in shear stress over distances comparable to cellular dimensions. Such unbalanced forces may initiate endothelial turnover by means of unknown mechanisms. We postulate that critical flow-induced shear stress frequencies may be common to lesion-susceptible locations *in vivo* and in the turbulent flow conditions responsible for enhanced endothelial cell turnover *in vitro*.

Endothelial cells in a confluent monolayer are growth-inhibited by contact with their neighbors. They are unresponsive to known mitogens and only agents that disrupt the continuity of the monolayer appear capable of stimulating cell growth. Thus, scraping or "wounding" of the monolayers, or retraction of individual cells by drug-induced changes in cytoskeletal components (28), can trigger cell cycle entry. In laminar flow, alignment of the cells occurred without increased cell turnover, indicating that retraction is not necessary for realignment and that the extensive cytoskeletal reorganization that occurs during alignment (29) is not a sufficient stimulus to initiate the cell cycle. Although cell retraction was a consistent morphological feature of cells exposed to turbulent flow *in vitro* for 16 hr or longer, shorter periods of exposure resulted in little morphological change yet a subsequent large increase in cell turnover. It is unclear whether turbulent shear stresses induce subtle cell retraction, causing loss of contact inhibition of growth, or whether they can stimulate other, unknown, mechanisms that initiate cell cycle entry. Once committed to divide, however, cells that reach mitosis are less tightly attached to the substratum (30). An increased cell cross-sectional area presented to turbulent flow may have precipitated cell detachment during the period between 16 and 24 hr. Whatever the precise initiating mechanisms of endothelial turnover in our experiments, it is clear that the confluent endothelial lining is very sensitive to physical forces associated with turbulent flow characteristics at very low levels of shear stress. In a related study, Langille and colleagues (31) have recently reported increased endo-

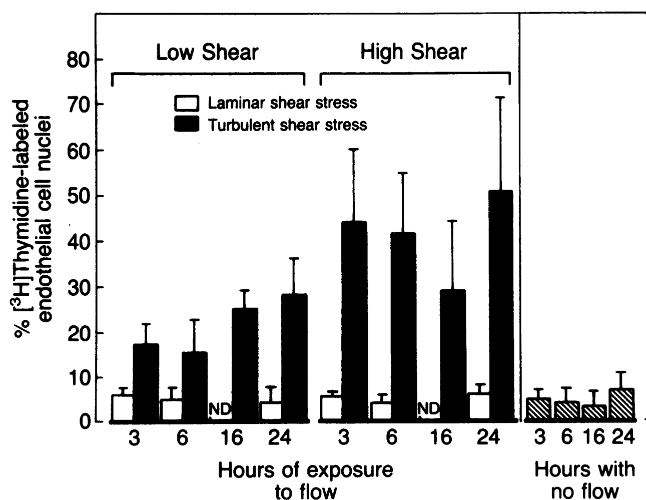

FIG. 2. Endothelial cell turnover *in vitro* in laminar and turbulent flow. Confluent endothelial cell monolayers were subjected to low or high shear stress in laminar or turbulent flow for various periods. The nuclear labeling of DNA [^{3}H]thymidine during a subsequent 24-hr period was determined by autoradiography. "Low" shear stress was 1.0 dynes/cm^2 in laminar flow and 1.5 dynes/cm^2 in turbulent flow. "High" shear stress was 15 dynes/cm^2 in laminar flow and 14 dynes/cm^2 in turbulent flow. These values represent the upper and lower limits of turbulent shear stress amplitude in this cone-plate apparatus. ND, not determined.

§Tse, R. (1981) Bachelor of Science Thesis (Massachusetts Institute of Technology).

thelial cell turnover in regions of disturbed flow *in vivo*. Although the shear stress characteristics could not be precisely measured, their findings support our conclusions that disturbed flow significantly influences endothelial turnover and compromises the integrity of the endothelial monolayer. Such effects appear to be causally linked to the development of focal atherosclerosis.

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