Mechano-chemical Control of Human Endothelium Orientation and Size

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Abstract. Human umbilical vein endothelial cells (EC) were grown on elastic silicone membranes subjected to cyclic stretch, simulating arterial wall motion. Stretching conditions (20% amplitude, 52 cycle/min) stimulated stress fiber formation and their orientation transversely to the strain direction. Cell bodies aligned along the same axis after the actin cytoskeleton. EC orientation response was inhibited by the adenylate cyclase activator, forskolin (10^{-5} M), which caused stress fiber disassembly and the redistribution of F-actin to the cortical cytoplasm. Preoriented EC depleted of stress fibers by forskolin treatment retained their aligned state. Thus, stress fibers are essential for the process of EC orientation induced by repeated strain, but not for the maintenance of EC orientation.

The monolayer formed by EC grown to confluence in conditions of intermittent strain consisted of uniform elongated cells and was resistant to deformation. In contrast, the monolayer assembled in stationary conditions was less compliant and exposed local denudations on initiation of stretching. When stretched in the presence of 10^{-5} M forskolin it rapidly (3–4 h) reestablished integrity but gained a heterogeneous appearance since denuded areas were covered by giant cells. The protective effect of forskolin was because of the stimulation of EC spreading. This feature of forskolin was demonstrated while studying its action on EC spreading and repair of a scratched EC monolayer in conventional culture. Thus mechanical deformation and adenylate cyclase activity may be important factors in the control of endothelium morphology in human arteries.

Two major morphologic changes occur in human arterial endothelium with aging; it loses its orientation, and it becomes morphologically heterogeneous (19, 23). These events are believed to be related to atherogenesis. Several approaches are proposed to obtain insight into the mechanism of these phenomena. Endothelial heterogeneity is studied on *en face* preparations and in primary culture that successfully reproduces the situation in situ (3). However, the oriented state of endothelial cells (ECs)[1] in the artery fails to be modeled by conventional cell growing techniques. It requires vectoral factors to be introduced to the culture conditions. Among these factors, blood flow and the periodic stretch of the artery wall are the most obvious. It was demonstrated that shear stress produced by the flow of liquid leads to the elongation and aligning of cultured endothelial cells in the direction of flow (10). Microfilament bundles revealed in the endothelium subjected to the shear stress in vivo and in vitro exhibited orientation parallel to the blood flow (25) and to the cells' long axis. These findings suggested a close association between actin cytoskeleton dynamics and the response of endothelium to mechanical stimulation.

Periodic strain and relaxation produces similar orientation of the endothelial monolayer as flow does (15). To provide information on the mechanism of stretch-induced orientation, we have grown human endothelium on elastic silicone substrata cyclically stretched to simulate artery wall motion. In the present work, we report that EC orientation is mediated by actin cytoskeleton rearrangement. The increase of intracellular cAMP levels produced by forskolin results in stress fiber disassembly, F-actin redistribution and blocking of the EC orientation response. The combined action of elevated cAMP and repeated strain rapidly (3–4 h) converts the uniform endothelial monolayer into a heterogeneous one. We propose that heterogeneity may develop when ECs spread to repair local denudations brought about by deformation of the monolayer.

Materials and Methods

Materials

Medium 199 (Earle salts), FCS, Dulbecco's PBS, L-glutamine, penicillin, streptomycin, and sodium pyruvate were from Gibco Laboratories (Paisley, Scotland). Sylgard 184 silicone elastomer kit was purchased from Dow Corning Corp. (Midland, MI). Human platelet myosin was a generous gift of Dr. J. R. Sellers and IgG fraction of rabbit antiserum to human platelet

1. *Abbreviation used in this paper*: EC, endothelial cells.

myosin was presented by Professor R. S. Adelstein (National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD). Antivimentin monoclonal antibody V9 was kindly donated by Professor K. Weber and Professor M. Osborn (Max Planck Institute for Biophysical Chemistry, Gottingen, FRG). TRITC-phalloidin was purchased from Sigma Chemical Co. (St. Louis, MO). Forskolin (Calbiochem-Behring Corp., San Diego, CA) was dissolved in ethyl alcohol (E. Merck, Darmstadt, FRG) and stored as a stock solution in aliquots at −80°C. Before use, aliquots were diluted with growth medium (medium 199 supplemented with 25 mM Hepes, 2 mM L-glutamine, 1 mM pyruvate, penicillin at 100 U/ml, streptomycin at 0.1 mg/ml, 20% [vol/vol] FCS) and sterilized by filtration through 0.22 μm filters (Nalge Co., Rochester, NY). All other compounds were of reagent grade.

Cell Culture on Silicone Membranes

Primary cultures of human umbilical vein EC were prepared according to Antonov et al. (2). 100-μm-thick transparent elastic membranes (20 mm × 50 mm, 10 cm^2 growth area) were solvent cast on a microscope slide using Sylgard 184 kit (Dow Corning Corp.) according to the manufacturer's instructions. The lateral borders of the membrane were thickened to 200 μm to reduce narrowing of the central part during stretching. To the shorter sides 30-mm-long pieces of injection needle were attached to ensure hooking of membrane in the stretching apparatus. Membranes were hydrophylized by 1 min of immersion in concentrated sulfuric acid. After extensive washing, membranes were sterilized under UV light (2 h) and coated with a sterile 0.1 mg/ml solution of human fibronectin in PBS. After 30 min of incubation, unbound fibronectin was washed out, and ECs were plated at a density of 5–8 × 10^4 cells/cm^2 in a growth medium supplemented with endothelial cell growth factor (100 μg/ml). EC grown on unstretched membranes looked morphologically indistinguishable from endothelium cultured on plastic. The stretching apparatus (Fig. 1) was a modified version of the device described by Dartsch et al. (9).

Microscopy

At the end of the stretching experiment, membranes were briefly rinsed with PBS at 37°C and fixed for 5 min in 2.5% glutaradehyde in PBS for silver staining or 3.7% formaldehyde for fluorescence. Silver staining of EC borders was conducted as described previously (4, 5, 27). TRITC-phalloidin was used to reveal F-actin in EC permeabilized with 1% Triton X-100. The cells were photographed on a microscope (Diaphot-TMD; Nikon Inc., Garden City, NY) through a 10 × objective and on a microscope (Photo III; Carl Zeiss Inc., Thornwood, NY) through a 40 × Planapo objective, using film (Tri-X Pan; Eastman Kodak Co., Rochester, NY), and developed (in Diafine; Acufine, Inc., Chicago, IL).

Other Methods

EC shape and orientation were analyzed on silver nitrate-stained monolayers using a MOP-3 digitizer (Reichert Jung, Vienna, Austria) as described (2). Triton-insoluble cytoskeletons were prepared from EC grown to confluence on 60-mm petri dishes according to the protocol described by Holzapfel et al. (14) using 0.2% Triton X-100. Cytoskeletal proteins were analyzed by SDS-PAGE in 7.5% gels (16). Immunoblotting was conducted according to Towbin et al. (21). For each step of sample preparation, the integrity of the monolayer remained unaltered. *En face* preparations of human aorta were made as previously described (2).

Results

Cyclic Stretching Causes EC Orientation

EC from human umbilical vein grown on silicone elastic membranes stretched at 52 cycle/min and 20% amplitude for 48 h exhibited significant morphologic changes. Cells became elongated and uniformly oriented (Fig. 2 *A*). Silver nitrate staining revealed the intactness of EC monolayer formed on the stretched membranes (Fig. 2 *C*). The distinct visualization of cell borders obtained by this type of staining allowed us to quantitate EC orientation. It was found that the majority of cells aligned at 80°-120° to the strain direction

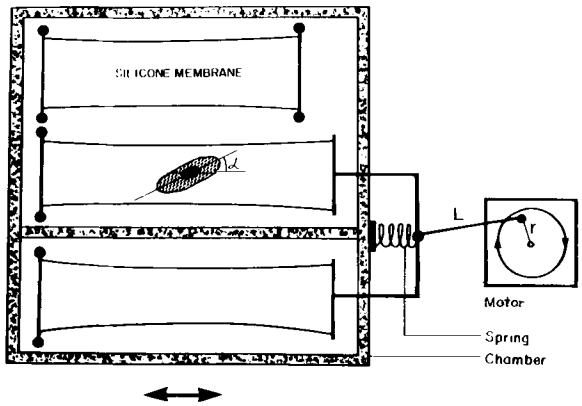

Figure 1. The scheme of the apparatus for cyclic stretching of cultured cell on elastic membranes. The chamber contained three membranes in two separate compartments. Two membranes were stretched identically, and the third one served as a stationary control. The amplitude of stretching was regulated by fixing eccentrically metal rod (*L*) on the motor driving shaft at a desired distance (*r*) from the axis. The frequency of stretching was adjusted by means of the motor speed control. The relative elongation of silicone rubber was uniform across the whole membrane area. The lateral thinning did not exceed 1% at 20% stretching amplitude. No surface structures, which may cause cell orientation, appeared on the membranes after prolonged repeated stretching.

(Fig. 3 *B*). EC in control experiments maintained a random orientation regardless of whether membranes were unstretched or fixed in a stretched position (Figs. 2, *B* and *D*, and 3 *A*). Morphologic changes of the endothelial monolayer brought about by periodic stretching and relaxation of the substratum were not peculiar to human umbilical vein EC only. Human aorta and lung artery EC in primary culture demonstrated the same behavior in this experimental model (data not shown).

The Actin Cytoskeleton Is Involved in the Orientation Reaction

The actin cytoskeleton of EC grown on unstretched membranes was similar to that observed in conventionally cultured endothelium (26). It was represented by the cortical array of microfilaments and a moderate amount of stress fibers (Fig. 2 *F*). In the course of cyclic stretching, marked orientation of stress fibers coincident with the cell's long axis was observed (Fig. 2 *E*). Stress fibers appeared as intensively stained uninterrupted cables running in parallel accord throughout the cytoplasm. The cortical system of the microfilaments was reduced to a single but prominent band. After brief exposures of preconfluent EC to repeated strain (~10–15 min) the presence of cells with unusual distribution of stress fibers was observed. Actin cables in these cells run parallel to each other and already perpendicular to the strain direction (Fig. 4 *A*). However, they showed no coincidence with the cell principal axis. The number of EC demonstrating this type of actin cytoskeleton organization rapidly decreased with time of mechanical stimulation. Later on, only EC possessing coordinated orientation of the cell body and actin cables were found on the silicone membranes.

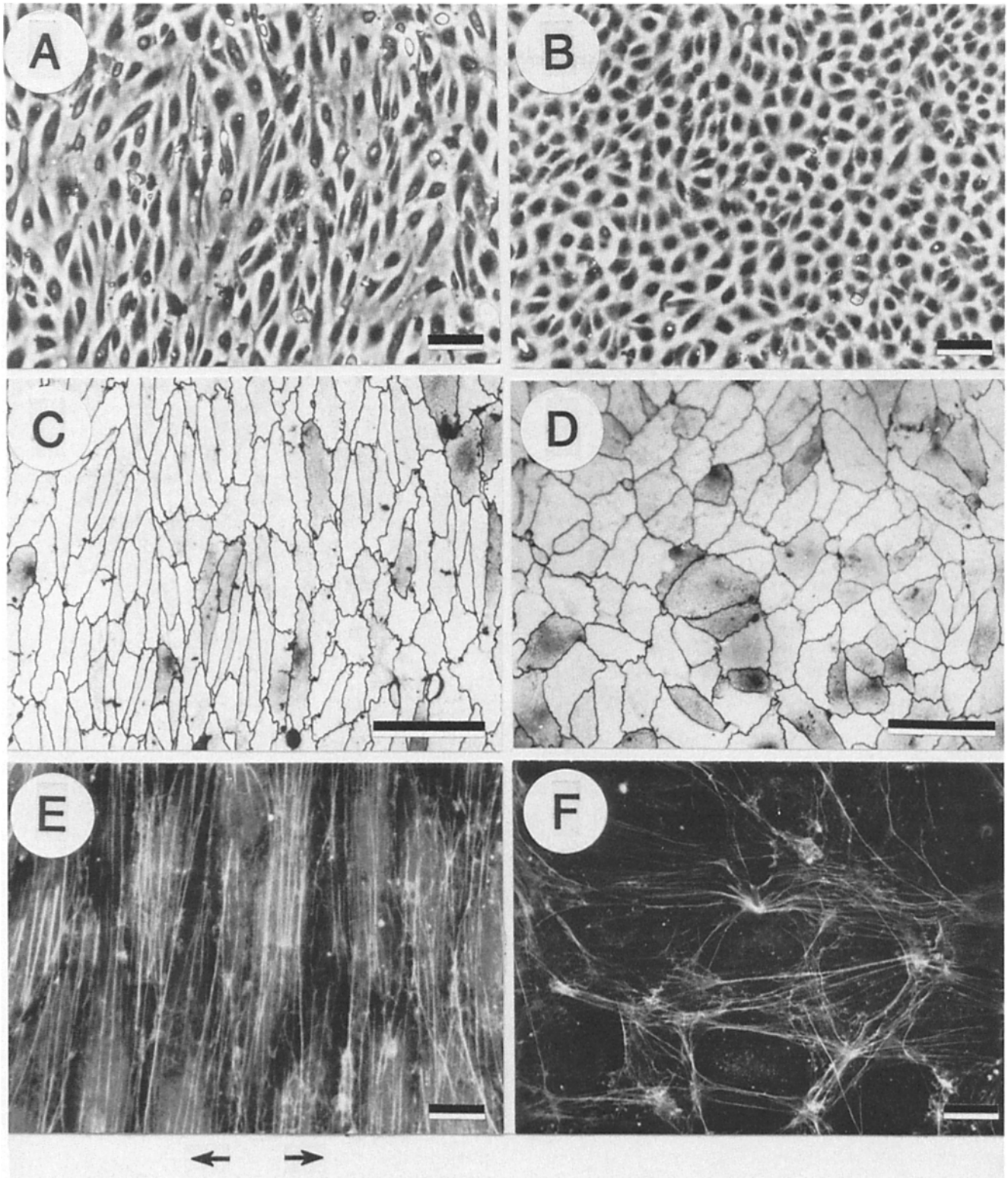

Figure 2. The effect of cyclic stretching on EC orientation and actin cytoskeleton organization. EC were grown on silicone membranes subjected to stretches and relaxations at 20% amplitude and 52 cycle/min for 48 h (*A*, *C*, and *E*); EC on unstretched membranes served as a stationary control (*B*, *D*, and *F*). (*A* and *B*) phase contrast; (*C* and *D*) silver nitrate staining. (*E* and *F*) TRITC-phalloidin fluorescence. Arrows show the direction of strain. Bars, (*A–D*) 100 μm, and (*E* and *F*) 20 μm.

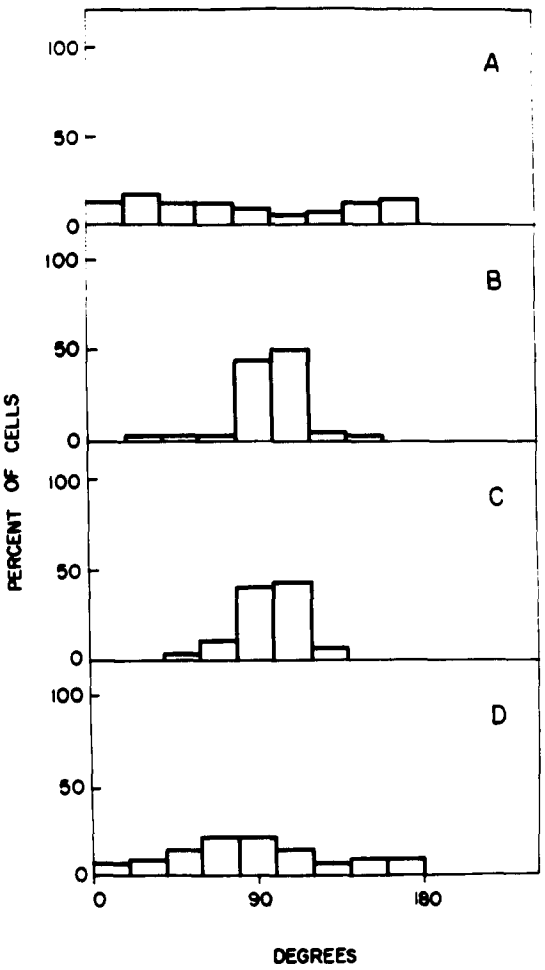

Figure 3. Histograms showing EC orientation in response to cyclic stretching. Percent of EC is plotted as a function of the angle between cell long axis and the strain direction. (*A*) stationary control; (*B*) EC after 24-h cyclic stretching; EC stretched for 1.5 h in the absence (*C*) and in the presence (*D*) of 10^{-5} M forskolin.

Forskolin Inhibits Orientation of EC and Affects Stress Fibers

The stretch-induced orientation response of EC may be effectively counteracted by the addition of forskolin. Preincubation of endothelium with 10^{-5} M forskolin for 1.5 h followed by a further 1.5 h of stretching in the presence of forskolin significantly inhibited the orientation reaction. EC retained a rather random orientation (Fig. 3 *D*), while most of the cells stretched in the absence of forskolin for the same time aligned at angles of 80°-120° to the strain direction (Fig. 3 *C*). Examination of the actin cytoskeleton in forskolin-treated stretched and unstretched EC revealed a similar picture (Fig. 4 *B*). Actin bundles disappeared from the central part of the EC, leaving only the cortical array of microfilaments. SDS-PAGE revealed several protein bands in Triton X-100 insoluble cytoskeletons of EC (Fig. 5). Polypeptides with relative molecular masses of 200 and 43 kD were identified as myosin heavy chain and actin on the basis of their comigration with human platelet myosin and skeletal muscle actin, respectively. A 55-kD protein band cross reacted with vimentin antibody on immunoblots (data not shown). Using these electrophoretic techniques, we did not find substantial changes in the amount of actin and vimentin associated with Triton-insoluble cytoskeleton after exposure of EC to forskolin (Fig. 5). On the contrary, the intensity of the myosin band decreased about four times, as calculated from densitometric scans of electrophoretic bands shown in Fig. 5. Immunoblotting confirmed the results of electrophoresis and demonstrated that the staining of the myosin band with antiplatelet myosin antibodies was markedly reduced in forskolin-treated EC (Fig. 5). The total amount of the proteins mentioned above did not change in EC during the time of experiments (data not shown). When oriented EC were further stretched in the presence of 10^{-5} M forskolin for 1.5 h, the amount of stress fibers was reduced as in stationary culture, but orientation of EC remained unchanged (Fig. 4 *C*).

Cyclic stretching conditions were found to induce the elaboration of actin bundles. EC were pretreated with forskolin to remove existing stress fibers. Repeated strain of these cells for 3 h resulted in the massive formation of stress fibers and the orientation response (Fig. 4 *D*). No actin bundles appeared in forskolin-pretreated but mechanically unstimulated EC over this period of time. Cells looked similar to those shown in Fig. 4 *B*. The induction of microfilament bundles in the course of cyclic stretching may be observed in EC without their special pretreatment though not so obvious (Fig. 2, *E* and *F*).

Combination of Cyclic Stretching and Forskolin Stimulates EC Heterogeneity

Preconfluent EC (5 d) growing under the action of intermittent strain assembled in a uniform, oriented monolayer that maintained integrity in spite of stretching (Fig. 6 *A*). This monolayer showed close similarity to the in situ organization of the endothelial lining in a child's aorta (Fig. 6 *B*). In contrast, the monolayer assembled in stationary conditions (Fig. 2, *B* and *D*) could not resist repeated mechanical deformation and displayed local denudations in zones of high cell density. However, in the presence of 10^{-5} M forskolin it rapidly (3–4 h) regained integrity but became heterogeneous, containing giant cells (Fig. 6 *C*). This monolayer reproduced the heterogeneous appearance of the endothelium in adult human aorta (Fig. 6 *D*). The large size of the cells covering denudations was probably because of their spreading. To test this hypothesis, we studied the effect of forskolin on EC behavior in stationary culture. Fig. 7 shows the process of EC spreading in the multiwell chamber 30 min after plating. In the presence of 10^{-5} M forskolin, cells have already spread (Fig. 7 *B*), while in control wells spreading was considerably delayed (Fig. 7 *B*). 1 h later, EC in all wells were completely spread and indistinguishable from each other (data not shown). Fig. 7 (*C–F*) depicts the process of monolayer repair. At zero time, a scratch was made on the monolayer with the tip of pasteur pipette (Fig. 7, *C* and *D*) and repair was monitored by phase-contrast microscopy. When forskolin (10^{-5} M) was present in the medium, the scratch was completely covered by EC within 3 h after its application (Fig. 7 *F*). Cells covering the deendothelized area were mononucleated and of larger size than EC located far from the repaired zone. The control scratch remained uncovered

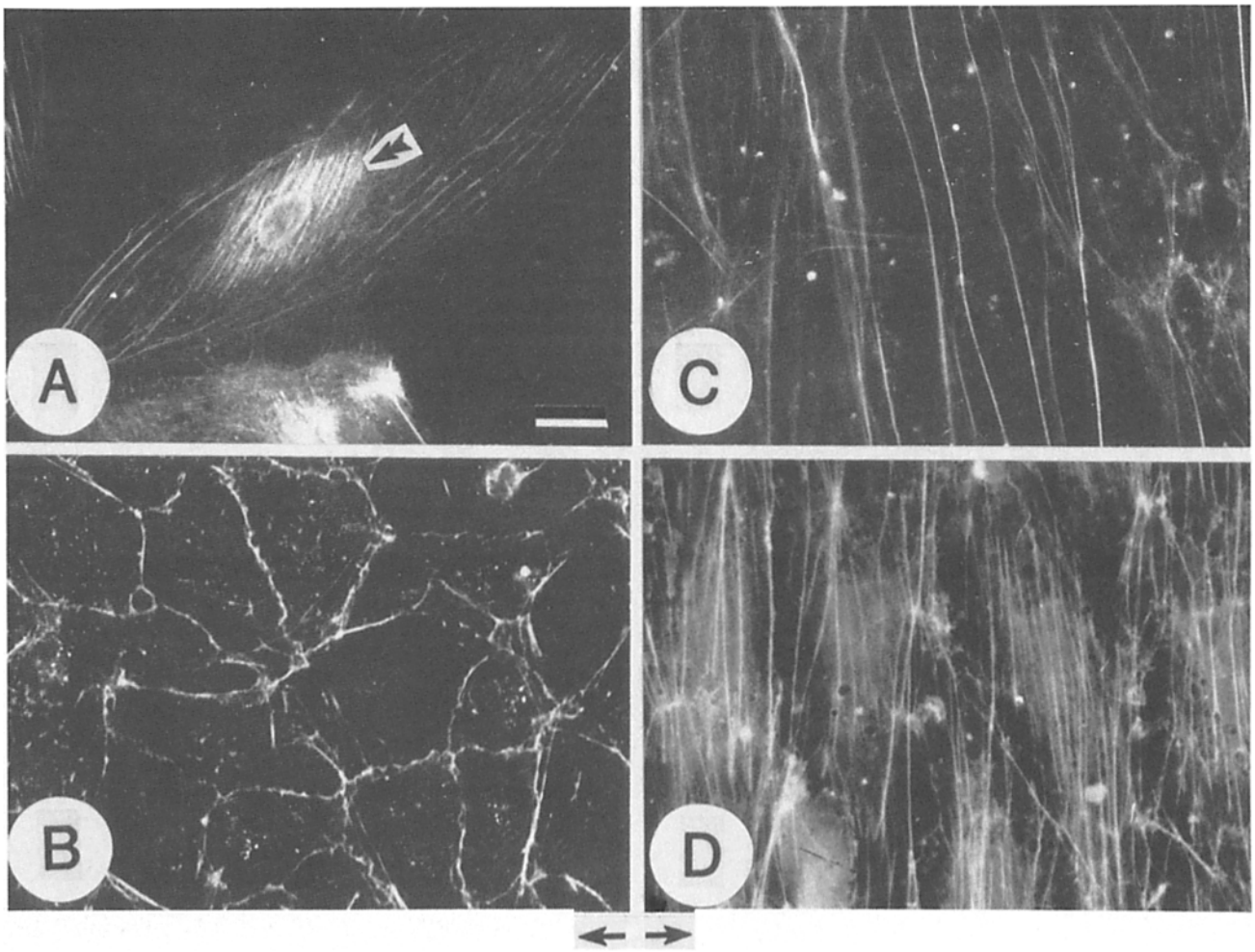

Figure 4. EC actin cytoskeleton rearrangement in the course of cyclic stretching. (*A*) The example of preconfluent endothelial cell demonstrating the unusual distribution of stress fibers (*double arrowhead*) after 15 min of cyclic stretching; (*B*) unstretched EC treated with 10^{-5} M forskolin for 3 h; (*C*) the disappearance of stress fibers from oriented EC after 10^{-5} M forskolin addition; (*D*) induction of stress fibers in forskolin pretreated EC by pulsatile strain for 3 h. TRITC–phalloidin fluorescence. Arrows show the direction of stretching where applied. Bars, 20 μm.

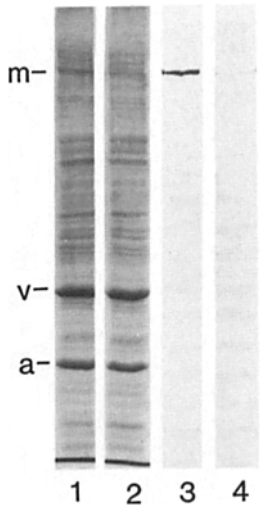

Figure 5. The effect of forskolin addition on F-actin, myosin, and vimentin content in Triton-insoluble cytoskeleton of EC. SDS-PAGE of Triton-insoluble EC proteins before (lane *1*) and after (lane *2*) 1.5-h preincubation of EC with 10^{-5} M forskolin at 37°C; (lanes *3* and *4*) immunoblots of lanes *1* and *2*, respectively, stained with antibodies to human platelet myosin; *m*, myosin; *v*, vimentin; and *a*, actin.

after 3 h (Fig. 7 *E*) and was finally "healed" 24 h later (data not shown).

Discussion

In vitro and in situ experimental data suggest that longitudinal orientation of endothelium in the arteries is attributed to the combined action of shear stress produced by the flow of blood and stretching of the artery wall that accompanies blood pulsation (10, 15, 13). Other cellular constituents of the vessel wall like smooth muscle cells and fibroblasts are not normally exposed to the shear stress but also get oriented in response to cyclic stretching (6, 7, 8, 9). The amplitude of stretching used in different studies, including this work, varied from 2% to 20%, affecting the velocity and the final angle of orientation of the cultured cells (8). Since comparable amplitudes of stretching are achieved in the arteries in vivo (18), one can suppose that cyclic stretching plays an important role in creating the architecture of arterial wall.

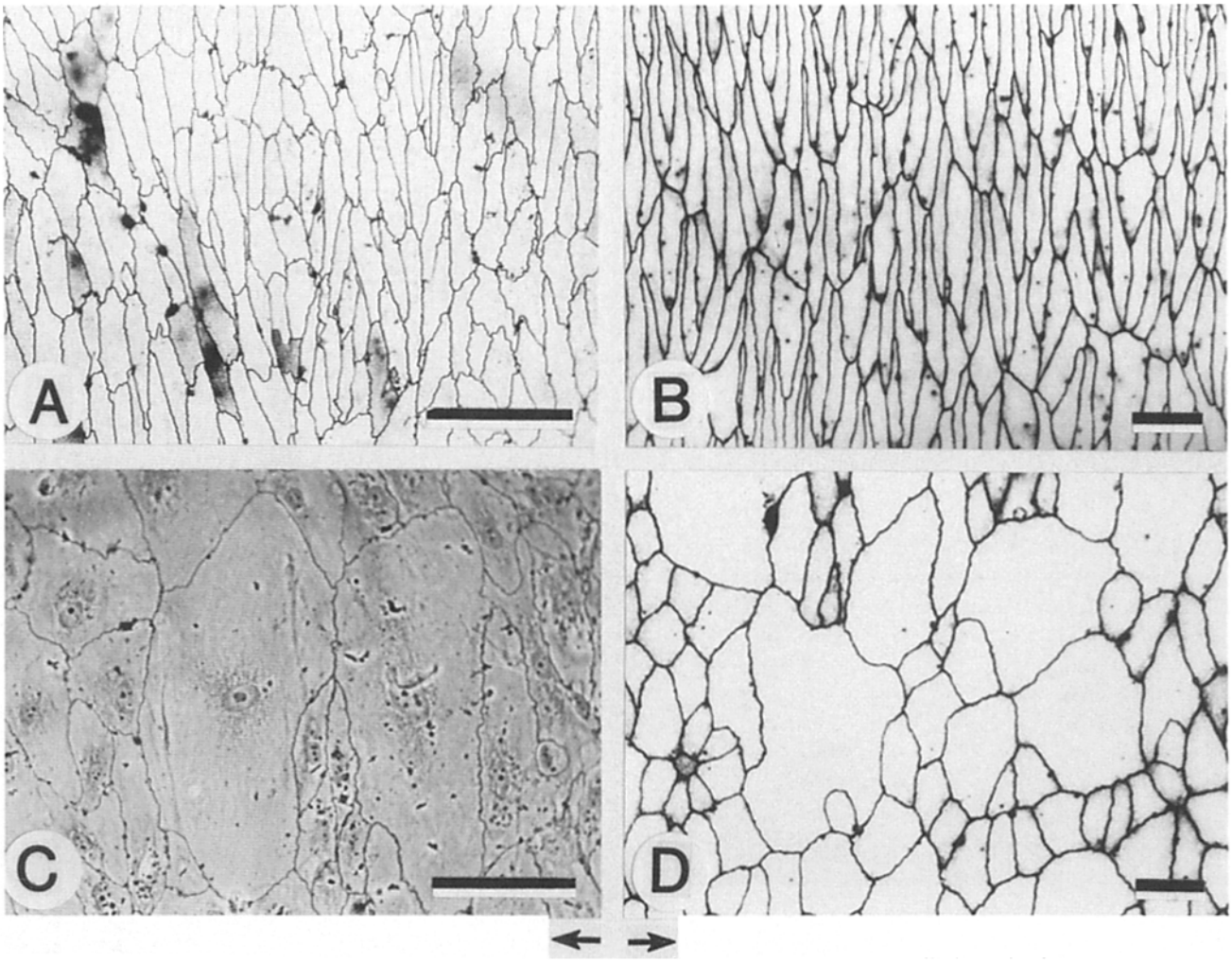

Figure 6. The morphologic appearance of EC monolayer produced by cyclic stretching shows the similarity to in situ organization of endothelium in human aorta. (*A*) Homogeneic, oriented monolayer formed after 48-h periodic stretching of preconfluent EC; (*B*) the endothelial lining of child's aorta; (*C*) heterogeneous, oriented monolayer is formed as a result of the combined action of cyclic stretching and 10^{-5} M forskolin on the confluent EC; and (*D*) endothelial lining of adult human aorta. (*A*, *B*, and *D*) Silver nitrate staining. (*C*) Combination of silver nitrate staining and phase contrast; a single nucleus is visible in the center of each giant cell. Arrows show the direction of strain applied to the silicone membrane and the vessel wall. Bars, 40 μm.

The mechanisms of EC orientation in response to mechanical stimulation remain obscure. Several studies pointed to the involvement of the actin cytoskeleton in this process. Shear stress was reported to induce elaboration of actin stress fibers and their further orientation along the long axis of EC (11). In the present work, we demonstrated that similar events occur to the actin cytoskeleton of EC repeatedly stretched on the silicone films (Figs. 2 *E* and 4 *D*). However, in this case actin bundles oriented perpendicularly to the strain while in shear-stressed EC they aligned parallel to the shear force. Different behavior of the actin cytoskeleton in EC experiencing repeated strain or shear stress is probably related to the pattern of force distribution in these two circumstances. Shear stress acts on the apical surface of EC and tends to tear them off from the substrate. Hence, the stress fibers oriented along the flow counteract the action of shear stress most effectively. On the contrary, cyclic stretching exerts its action on the whole cell body, including membrane–substratum attachment sites. It selectively breaks stress fibers that run parallel to the strain but leaves intact actin bundles lying perpendicular to the strain. For the same reasons, the elaboration of the new stress fibers is now restricted to be perpendicular to the direction of external force. On the basis of their findings, Franke et al. (11) have postulated that microfilament bundle aligning is not directly related to the action of shear stress and takes place after EC orientation (11). Our data suggest that in the case of EC stretching actin bundle aligning may precede the cell body orientation. After very short exposures to the pulsatile strain (10–15 min), we were able to observe cells with transverse or oblique distribution of stress fibers with respect to the cell long axis (Fig. 4 *A*). We refer these cells to the short-living intermediates that then quickly gain the coordinated orientation of the stress fibers and cell axis. Similar cell images cross-striated with stress fibers were described by Dartsch and Hammerle (8) in the course of cyclic stretching of vascular smooth muscle cells. They were also regarded as the transient forms.

None of the previous studies have demonstrated the chemi-

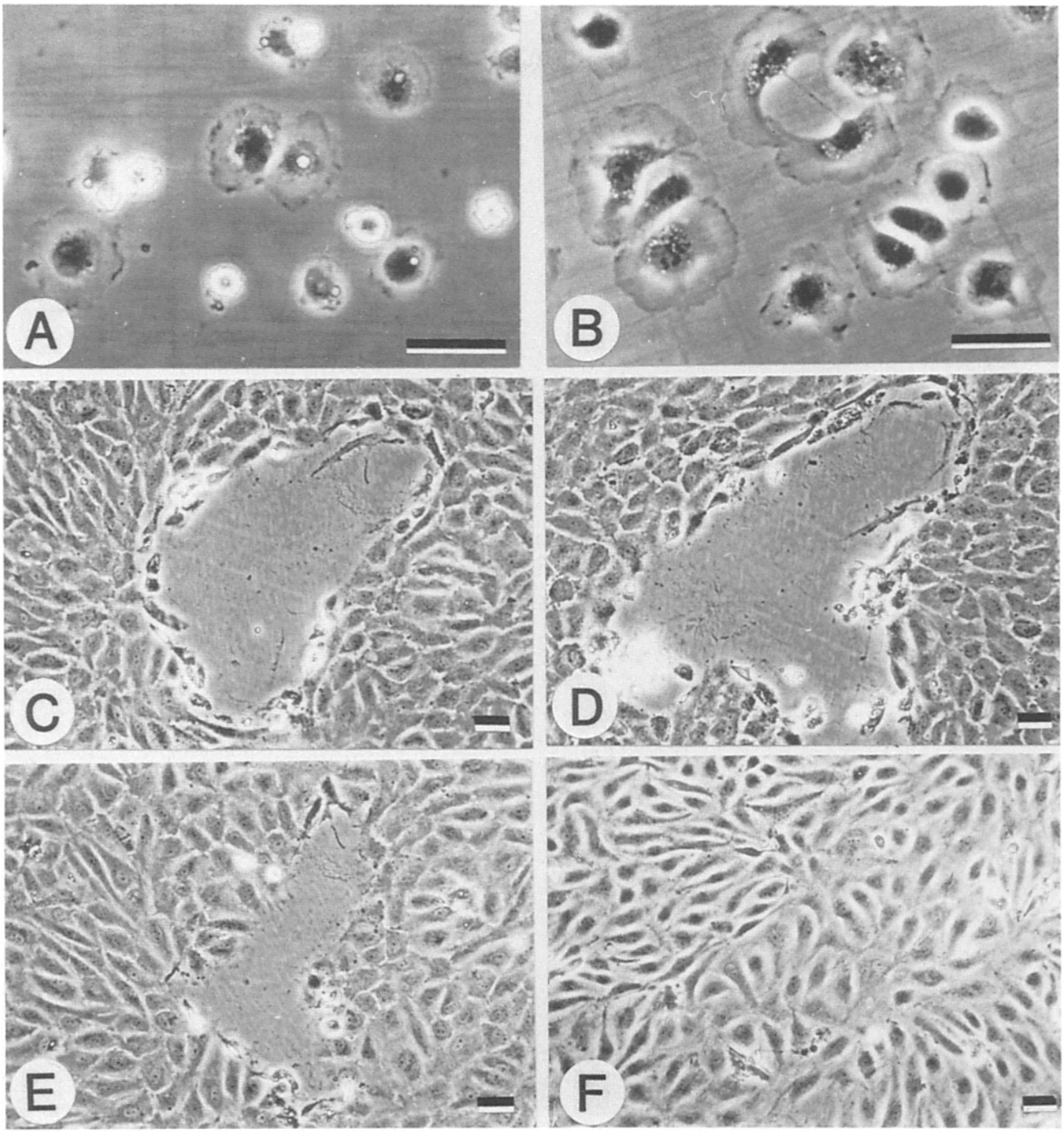

Figure 7. Forskolin accelerates EC spreading and repair of the scratched monolayer. Spreading of EC in the multiwell chambers 30 min after plating in the absence (*A*) and in the presence (*B*) of 10^{-5} M forskolin. (*C* and *D*) The hole was made in the monolayer by scratching it with the Pasteur pipette. 3-h time point of the scratch covering in the absence (*E*) and in the presence (*F*) of 10^{-5} M forskolin is shown. Phase contrast. Bars, 50 μm.

cal way to regulate mechanically induced orientation response of cultured cells. In the present work, we showed that adenylate cyclase activator forskolin that causes cAMP elevation in EC (2) was able to inhibit endothelium aligning induced by cyclic stretching (Fig. 3). We observed that the treatment of EC with forskolin affects the organization of the actin cytoskeleton. Forskolin causes the disappearance of stress fibers and dense packing of the cortical microfilaments (compare Fig. 2 *F* and Fig. 4 *B*). The drug is equally effective in the stationary and repeatedly stretched EC (Fig. 4, *B* and *C*). However, already oriented cells depleted in stress fibers (Fig. 4 *C*) do not lose orientation for at least several hours. Thus, our results support the involvement of the actin cytoskeleton in stretch-induced orientation response of EC and, possibly, its primary aligning with respect to that of cell body. On the other hand, stress fibers are apparently not required for the maintenance of oriented state of EC.

While decreasing the number of stress fibers, forskolin

does not significantly reduce the amount of F-actin. This was demonstrated by SDS-PAGE of Triton X-100–resistant cytoskeletons of EC (Fig. 5). The amount of actin in the samples of the cytoskeletons was independent of pretreatment of EC with forskolin. Perhaps, forskolin activates in EC the cAMP-regulated pathway of stress fiber disassembly and redistribution of the filamentous actin to the cortical cytoplasm.

The differential action of forskolin on the cortical microfilaments and stress fibers suggests that the elevated cAMP does not affect F-actin itself but rather some of the actin-binding proteins that stabilize microfilament associations. One of the candidates for this role could be myosin which colocalizes with stress fibers in nonmuscle cells but is frequently absent from the cell periphery (12). It was found that concomitantly with the stress fiber disappearance, the amount of myosin associated with EC cytoskeletons decreased about fourfold (Fig. 5). Perhaps the treatment of EC with forskolin, via cAMP-dependent inhibition of myosin light chain kinase (1), induces the depolymerization of nonmuscle myosin filaments (20, 22) and subsequent disassembly of stress fibers. Thus, the elevation of cAMP in EC inhibits their ability to orient in response to mechanical stimulation, and this inhibition may be explained by the cAMP-dependent alterations in the actin cytoskeleton.

In contrast to the endothelial monolayer formed from preconfluent cells in the course of cyclic stretching, the EC monolayer assembled in the stationary conditions has the reduced tolerance to the strain. It displayed the local denudations on initiation of stretching especially in the zones of high cell density. We noticed that in the presence of forskolin the integrity of periodically stretched monolayer was readily regained, but its morphology changed drastically. Cells became heterogeneous in size (compare Fig. 2 *D* and Fig. 6 *C*). We hypothesized that giant ECs originated from the cells of normal size, stimulated by forskolin to spread over the denuded areas. In additional experiments conducted in the absence of repeated strain, we confirmed the ability of forskolin to accelerate EC spreading from the rounded state (Fig. 7, *A* and *B*) as well as the repair of the scratched monolayer (Fig. 7, *C–F*). Thus, our data point to the important role of adenylate cyclase in the reparation of the defects in endothelial monolayer via increasing size of individual cells. Morphologically heterogeneous endothelium is widely present in aorta of adult human (Fig. 6 *D*) whereas it is not normally found in child's aorta (Fig. 6 *B*) and animal arteries. Heterogenization of human endothelium is believed to be related to atherogenesis. However, the origin of human endothelium heterogeneity is currently unknown. One hypothesis explains heterogeneity by the accumulation of the large multinuclear EC (3). On the basis of our results with forskolin-stimulated EC spreading, we propose that along with polyploidization of EC another mechanism may account for the endothelium heterogeneity in situ. It involves the rapid cAMP-dependent spreading of the mononuclear EC over the local denudations in endothelial lining of the artery that may be produced by various causes. Among the possible factors damaging the endothelium integrity, one may name the nonuniform stretching and the turbulent flow in the vicinity of developing atherosclerotic plaques. Recent observations that mechanical deformation may really cause the reversible loss of contacts between EC in situ (13) support the possibility of this mechanism.

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