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Organization of actin cytoskeleton in normal and regenerating arterial endothelial cells

(stress fibers/actin polymerization/blood vessels/atherosclerosis)

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ABSTRACT **The distribution of actin stress fibers in normal and regenerating (after endothelial denudation by means of a balloon catheter) rabbit aortic endothelial cells has been studied by means of immunofluorescence with human actin autoantibodies on *en face* endothelial cell preparations. Our results show that: (*i*) under normal conditions actin is accumulated as a network at the periphery of endothelial cells. Stress fibers are present only in endothelial cells located immediately below intercostal artery branches; (*ii*) stress fibers develop in endothelial cells early during regeneration and persist after the end of endothelial mitotic and motile activities; and (*iii*) the orientation of stress fibers within the cytoplasm follows the direction of blood flow, with the exception of stress fibers situated in cells at the edge of the wound, when endothelial cell progression toward the denuded area as well as mitotic activity have ceased. We conclude that stress fibers are an organelle present in endothelial cells *in vivo* and that they reorganize during endothelial cell adaptation to unfavorable or pathological situations.**

Endothelial continuity depends on the maintenance of an extremely thin single cell layer. When arterial endothelium is removed by mechanical abrasion, the wound is repaired by endothelial cell replication and movement (1). When the endothelial cell loss is small (few cells in width) repair is accomplished essentially by movement without replication (2). Cell movement results probably from the activity of cytoskeletal and cytocontractile elements (3), among which actin is the most abundant. Cellular actin (4, 5) is organized in the form of G actin, a microfilamentous network, and microfilamentous bundles or stress fibers, which are typical of most adherent cultured cells, including endothelial cells. Stress fibers have been shown to contain actin, myosin, and regulatory proteins (6–9). Little information is presently available on the distribution of stress fibers *in vivo* (5).

We have studied the organization of stress fibers in endothelial cells of rabbit aorta and carotid artery under normal conditions and during regeneration after ballooning-induced endothelial denudation (10). For this purpose, we have developed a technique of immunofluorescent staining with antiactin antibodies (AAAbs) on *en face* endothelial cell preparations. Our results show that under normal conditions stress fibers are present only in some aortic endothelial cells—namely, those submitted to high shear stress; they become common in regenerating endothelial cells and persist long after motile and mitotic activities have ceased.

MATERIALS AND METHODS

Male rabbits, ≈3 kg of body weight, were used for the experiments. The endothelium was removed from the thoracic aorta or the left carotid artery by using the technique originally described by Baumgartner and Studer (10) with minor modifications. Evans blue (4 ml of a 1% solution in 0.9% NaCl) was injected intravenously 20 min prior to sacrifice to label denuded areas (blue areas). Two rabbits were sacrificed 2 hr and 2, 7, 15, and 40 days after aorta ballooning and 2, 5, and 7 days after carotid ballooning, together with untreated controls. Human AAAbs were obtained from the serum of a patient with chronic aggressive hepatitis and were purified by affinity chromatography as described (11).

For the *en face* preparations of aortic intima, we modified the protocol previously described (12) for the study of endothelial cell thymidine index and density. Rabbits were perfused through the left carotid artery for 5 min, at a constant pressure of 100 mm of Hg, with 3.7% formaldehyde, which was freshly prepared from paraformaldehyde in phosphate-buffered saline (P_i/NaCl) containing Ca^{2+} and Mg^{2+}. The aorta and the carotid artery were dissected and were immersed for an additional 10 min in 3.7% formaldehyde and for 2 hr in P_i/NaCl containing 0.1 M glycine. After washing in P_i/NaCl and carefully stripping the adventitia, the arteries were opened on their ventral part and rectangular segments (of about 5 × 7 mm for the aorta and 3 × 4 mm for the carotid) were cut. A small incision was made in the superior right portion of each segment to orient the specimen. The specimens then were made permeable in absolute ethanol at −20°C for 1 min, washed in P_i/NaCl, and incubated for 30 min with AAAbs. They were washed three times for 10 min in P_i/NaCl and were incubated with a fluorescein-conjugated IgG fraction of goat antiserum against human IgG (Miles) for 30 min and then were washed in P_i/NaCl. The specimens then were immersed again in freshly prepared 3.7% formaldehyde for at least 12 hr and were processed for the *en face* preparations as described (12). Control preparations were incubated with normal human IgG in amounts similar to those of AAAbs.

The pictures were taken with a Zeiss ultraviolet photomicroscope equipped with epi-illumination and a specific filter for fluorescein, by using a Plan Apo-Chromate ×40/1.2 objective on Agfa-Pan vario XL black-and-white film or Ektachrome 400 color slide film.

RESULTS

Incubation with AAAbs of normal aortic *en face* preparations showed two main patterns of actin immunofluorescent staining. The first and more frequent consisted of a polygonal type of distribution at the periphery of every cell (Fig. 1 *a* and *b*). This staining corresponds to cell flaps, as seen by scanning electron microscopy (13), and to the filamentous network visible within these peripheral flaps by means of transmission electron mi-

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Abbreviations: AAAb, antiactin antibody; P_i/NaCl, phosphate-buffered saline.

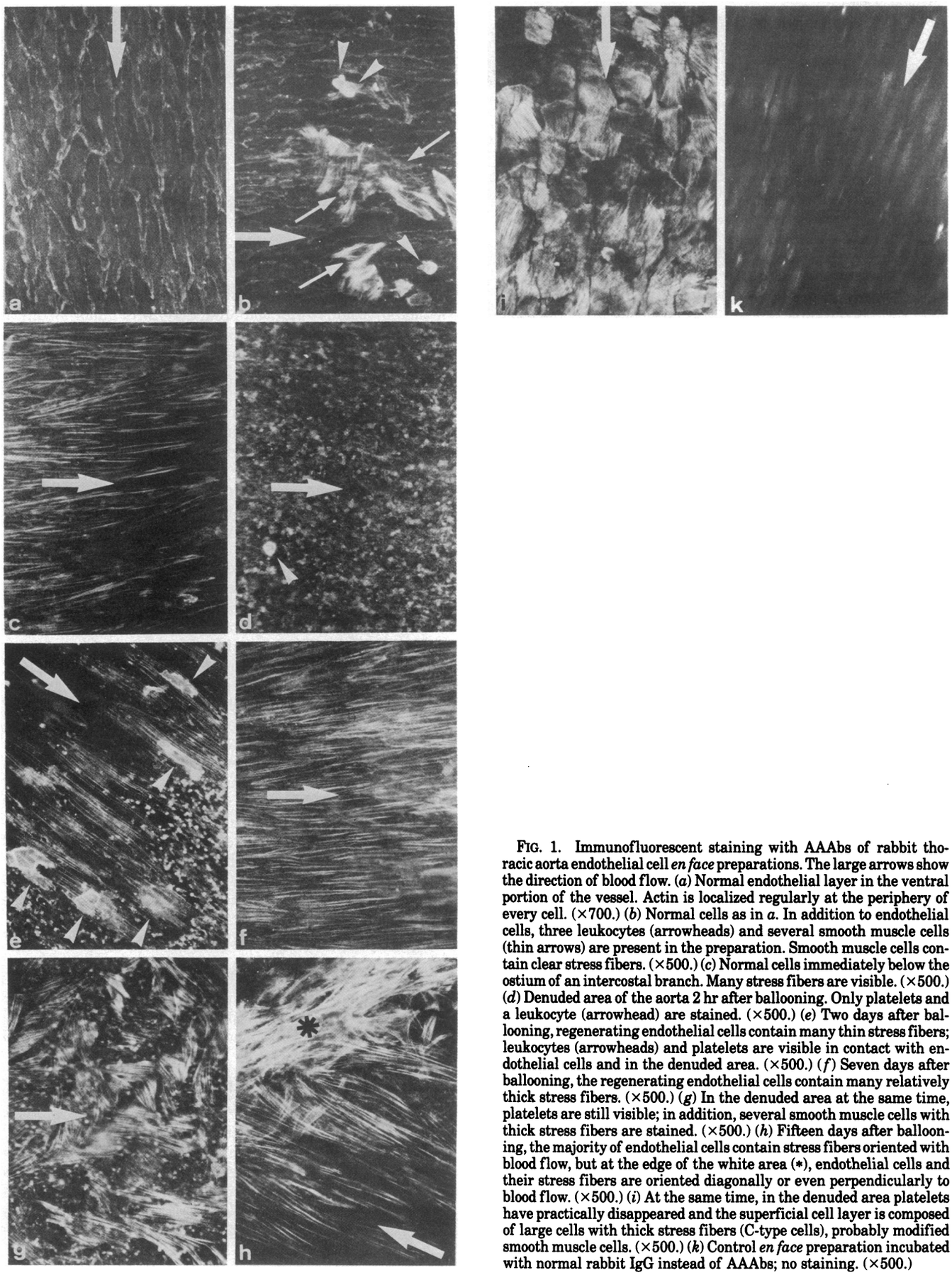

FIG. 1. Immunofluorescent staining with AAAbs of rabbit thoracic aorta endothelial cell *en face* preparations. The large arrows show the direction of blood flow. (*a*) Normal endothelial layer in the ventral portion of the vessel. Actin is localized regularly at the periphery of every cell. (×700.) (*b*) Normal cells as in *a*. In addition to endothelial cells, three leukocytes (arrowheads) and several smooth muscle cells (thin arrows) are present in the preparation. Smooth muscle cells contain clear stress fibers. (×500.) (*c*) Normal cells immediately below the ostium of an intercostal branch. Many stress fibers are visible. (×500.) (*d*) Denuded area of the aorta 2 hr after ballooning. Only platelets and a leukocyte (arrowhead) are stained. (×500.) (*e*) Two days after ballooning, regenerating endothelial cells contain many thin stress fibers; leukocytes (arrowheads) and platelets are visible in contact with endothelial cells and in the denuded area. (×500.) (*f*) Seven days after ballooning, the regenerating endothelial cells contain many relatively thick stress fibers. (×500.) (*g*) In the denuded area at the same time, platelets are still visible; in addition, several smooth muscle cells with thick stress fibers are stained. (×500.) (*h*) Fifteen days after ballooning, the majority of endothelial cells contain stress fibers oriented with blood flow, but at the edge of the white area (*), endothelial cells and their stress fibers are oriented diagonally or even perpendicularly to blood flow. (×500.) (*i*) At the same time, in the denuded area platelets have practically disappeared and the superficial cell layer is composed of large cells with thick stress fibers (C-type cells), probably modified smooth muscle cells. (×500.) (*k*) Control *en face* preparation incubated with normal rabbit IgG instead of AAAbs; no staining. (×500.)

croscopy (14, 15). Only exceptionally thin and short AAAb-positive bundles were seen in these cells. Clear AAAb-positive stress fibers were seen constantly in the smooth muscle cells that were occasionally stripped with the endothelial cells during the *en face* preparation (Fig. 1*b*). The second pattern of AAAb staining was localized exclusively in triangular areas having their bases on the distal portion of each ostium of intercostal arteries and their vertices in the aorta at a distance of about 8–10 cells. These cells showed in their cytoplasm several (two to five) AAAb-positive bundles (Fig. 1*c*), always oriented with blood flow. AAAb-positive round cells, probably leukocytes, were rarely present underneath endothelial cells in the areas with polygonal staining, but were more frequent around branches and particularly in areas with stress fiber-containing endothelial cells. Endothelial cells from the uninjured carotid artery only showed the polygonal pattern of AAAb staining.

One hour after ballooning, the denuded aortic surface was covered with AAAb-positive platelets and several leukocytes (Fig. 1*d*). Two days after ballooning, small white areas of regeneration were macroscopically visible around intercostal ostia. Endothelial cells at this location contained several thin stress fibers (Fig. 1*c*), always aligned with blood flow. In general, this pattern of alignment persisted through the different time intervals (see below). Seven days after ballooning, the areas covered by endothelial cells were larger than at 2 days, and stress fibers were greater in number and thickness (Fig. 1*f*). In the areas not covered by endothelium, AAAb immunofluorescent staining showed the presence of several platelets, leukocytes, and some smooth muscle cells that were distinguishable by the presence of thick stress fibers which were randomly oriented as compared to those of endothelial cells (Fig. 1*g*). Fifteen days after ballooning, stress fibers were still present and, in most cells, were still oriented with the blood flow. However, those cells located at the edge of white areas showed stress fibers oriented tangentially to the direction of outgrowth, thus diagonally or even perpendicularly to the blood flow (Fig. 1*h*). The direction of stress fibers always appeared to be along the long axis of each endothelial cell when examined by means of phase-contrast or Nomarski interference-contrast optics (data not shown). At this time, most platelets and leukocytes had disappeared from blue areas. The denuded surface was covered with many flat, thick cells with short stress fibers oriented randomly with respect to the direction of blood flow (Fig. 1*i*). These cells corresponded to the previously described pseudoendothelial "C-type cells" by means of scanning and transmission electron microscopy (16, 17). Forty days after ballooning, the white area had not increased significantly when compared to that of 15 days (18) and the immunofluorescent staining of endothelial cells and of blue areas was very similar to that observed at 15 days.

Two days after ballooning of the carotid artery, endothelial cells at the edge of white areas had many thin intracytoplasmic stress fibers, similar to those of growing aortic endothelial cells. Stress fibers were present in several rows of endothelial cells behind the wound edge 5 and 7 days after ballooning. Changes in blue areas were similar to those observed in the aorta at the same time intervals after ballooning. Control preparations incubated with human gamma globulins instead of AAAbs were always negative (Fig. 1*k*).

DISCUSSION

Our results show that actin stress fibers similar to those of cultured cells are present only in those endothelial cells of the normal rabbit aorta that are submitted to particularly high shear forces (19). Our findings agree with two recent preliminary reports (20, 21). It has been suggested that stress fibers *in vivo* are present in normal fibroblasts of the scale of the common goldfish *Carassius auratus* (22) and, by means of electron microscopy, in myofibroblasts of granulation tissue in several species (23), in regenerating rat epidermal cells (23–25), and in rat aortic endothelial cells during hypertension (14, 15). The present work shows that they become common in aortic endothelial cells during regeneration. The experiment of carotid artery ballooning indicates that stress fibers can develop *de novo* in cells having a polygonal peripheral pattern of actin distribution. Taken together, these findings suggest that stress fibers represent the reaction to unfavorable or pathological situations *in vivo* as well as to tissue culture.

There is evidence that *in vitro* stress fibers are related to isometric contraction and adhesion to the substrate rather than to isotonic contraction and cell movement (26), despite the fact that isolated stress fibers can contract under appropriate conditions (27, 28). In our experiments, stress fibers appear in moving and replicating cells but persist long after endothelial cell progression toward the denuded area and replication have ceased (18). During active regeneration these fibers parallel the direction of cell movement—that is, the axis of the vessel (18). Interestingly, cells at the edge of the outgrowth have fibers oriented tangentially to the direction of cell regeneration. However, this pattern does not appear until regeneration has stopped (18), suggesting that there is a correlation between the loss of the ability to move, the loss of the ability to replicate, and reorientation of the cytoskeleton. The persistence of prominent stress fibers after replication and movement have stopped implies that these structures are not directly related to cell movement or cell replication.

On the other hand, it is conceivable that the development of stress fibers is somehow related to an increased need for adhesion to the substrate (5, 26). Mitotic cells, at least *in vitro*, show lessened ability to adhere (29). This could be particularly critical for the endothelium because the lining of the aorta is subject to focal shear forces as high as 200 dynes/cm (19). The maintenance of stress fibers in regenerated endothelium *in vivo* and in endothelium *in vitro* could reflect the failure of the regenerated cells to develop a substrate equivalent to the substrate that develops in the subendothelium during normal conditions. One implication of this hypothesis is that we have yet to design a physiologic culture system for the endothelium. It is important to note that even under the best culture conditions, endothelial cells show a high turnover rate, reflected by the rate of cell replication at saturation density that is 10–100 times as high as the rates of 10^{-3} to 10^{-4} cells per day observed *in vivo* (30). The observation that in normal aorta stress fibers are present only in cells submitted to a high degree of shear forces suggests that stress fibers *in vivo* represent, at least in part, an endothelial reaction to high degrees of stress. However, this does not necessarily apply to stress fibers of regenerating cells and, of course, to stress fibers of cultured cells.

Cultured cells do not spread and do not form stress fibers when plated on a weakly adhesive substrate, but they do spread and form prominent stress fibers when the substrate is strongly adhesive (31). Adhering cells generate tension on the substrate as has been shown by culturing them on flexible substrates (32). Under these conditions, the cells that induce wrinkling beneath them show large stress fibers (5). These observations suggest that the interactions between cell surface and substrate are important for the formation of stress fibers *in vitro* (5). It is conceivable that the development of stress fibers in endothelial cells *in vivo* is regulated, at least in part, by factors similar to those playing a role *in vitro*. Further work is needed to clarify these questions. In any event, it appears that stress fibers are an organelle present in endothelial cells *in vivo* and that they can re-

organize during endothelial cell adaptation to unfavorable or pathological stimuli.

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