Abstract. The repair of small endothelial wounds is an important process by which endothelial cells maintain endothelial integrity. An in vitro wound model system was used in which precise wounds were made in a confluent endothelial monolayer. The repair process was observed by time-lapse cinemicrophotography. Using fluorescence and immunofluorescence microscopy, the cellular morphological events were correlated with the localization and distribution of actin microfilament bundles and vinculin plaques, and centrosomes and their associated microtubules. Single to four-cell wounds underwent closure by cell spreading while wounds seven to nine cells in size closed by initially spreading which was then followed at ~1 h after wounding by cell migration. These two processes showed different cytoskeletal patterns. Cell spreading occurred independent of centrosome location. However, centrosome redistribution to the front of the cell occurred as the cells began to elongate and migrate. While the peripheral actin microfilament bundles (i.e., the dense peripheral band) remained intact during cell spreading, they broke down during migration and were associated with a reduction in peripheral vinculin plaque staining. Thus, the major events characterizing the closure of endothelial wounds were precise in nature, followed a specific sequence, and were associated with specific cytoskeletal patterns which most likely were important in maintaining directionality of migration and reducing the adhesion of the cells to their neighbors within the monolayer.

The presence of an intact endothelial monolayer is important in maintaining normal blood vessel integrity. It appears that small denuding injuries occur in vivo (Reidy and Schwartz, 1984) and these reendothelialize very rapidly without requiring endothelial proliferation (Reidy and Schwartz, 1983, 1984). This rapid repair is thought to occur by a process in which endothelial cells respond to focal loss of an adjacent cell by the extrusion of lamellipodia which undermine dead cells and rapidly cover the denuded area (Hansson and Schwartz, 1983; Reidy and Schwartz, 1983; Wong and Gotlieb, 1984).

Cytoskeletal fiber systems are important in regulating endothelial cell shape change during repair processes (Wong and Gotlieb, 1984). Microfilaments appear to play a role in the force-generation machinery of the cytoskeleton (Kreis and Birchmeier, 1980) and in cell substratum adhesion (Singer, 1982; White et al., 1983; Wong et al., 1983), while the centrosome and its associated microtubules have a role in the directional migration of endothelial cells during large endothelial wound repair (Gotlieb et al., 1981, 1983). Although the cytoskeleton is important during endothelial repair, little is known about the sequence of cytoskeletal events which occur during rapid reendothelialization.

The present experiments were designed to characterize the sequence of cytoskeletal events occurring in those endothelial cells participating in the rapid repair of a small in vitro endothelial wound. Since the micropipette wounding model system has many similarities to in vivo small wound repair (Wong and Gotlieb, 1984), it was used to study reendothelialization of discrete small wounds of known cell size (Wong and Gotlieb, 1984). Fluorescence and immunofluorescence microscopy and time-lapse cinemicrophotography were used to correlate the organization of microfilaments, microtubules, and centrosomes with the motile activity of the cells occurring during closure of the wound.

Materials and Methods

Endothelial Cell Cultures

Cells were isolated and cultured by the enzyme dispersion method as previously described (Gotlieb and Spector, 1981). Cells were grown on 22 × 22-mm glass coverslips in 35-mm Falcon cell culture dishes (Becton Dickinson, Oxnard, CA) in M199 with 5% FBS (Gibco Laboratories, Grand Island, NY). Cultures were fed every 2 d and 24 h before experimentation.

Endothelial Wounding

Individual endothelial cells were removed from confluent cultures using a micropipette guided by a micromanipulator as previously described (Wong and Gotlieb, 1984). No partial cell fragments were left behind and all cells adjacent to the wound were able to participate in wound closure.
Cinemicrophotography

The motile activity of the cells was recorded by 16-mm time-lapse cinemicrophotography. The cells were observed under a constant temperature of 37°C and humidified in 5% CO2 and air in an environmental chamber, as previously described (Wong and Gotlieb, 1984). Still 35-mm photographs were taken at 15-min intervals using 2415 Technical Pan film (Kodak Canada, Toronto, Ontario). Two frames were taken at each time point, one focused on the lamellipodia to clearly delineate the cell shape, and the other on the centrosome which is visible in endothelial cells as a dark perinuclear area under phase-contrast optics (Gotlieb et al., 1981). Centrosomes in living cells were best visualized by stopping down the condenser diaphragm to about one-third open. The 2415 film was used at an ASA rating of 125 and processed with Kodak D-19 developer for 4 min as per the manufacturer's instructions. 16-mm time-lapse cinemicrophotography was carried out at a rate of one frame every 15 s using Kodak 7276. The film was commercially processed by PFA Laboratories (Toronto, Ontario).

Assessment of Wound Repair

All time-lapse cinemicrophotographic recordings were started 15 min before wounding and continued until 60 min after wound closure. In preliminary experiments, at least six wounds of each wound size from 1-12 cells were studied. Since we found that one- to four-cell wounds closed by cell spreading alone while those greater than six cells in size closed by spreading followed by cell migration, we studied three sizes of wounds: single cell wounds, three- to four-cell, and seven- to nine-cell wounds. 15 wounds were analyzed for each wound size. In five additional long-term experiments for each of the three wound sizes, cultures were continuously observed from 3 h before wounding to 24 h after wound closure.

Visualization of Endothelial Cell Cytoskeleton

30 wounds in each group were double stained at 30 min, 1, 2, and 3 h after wounding. For the colocalization of microfilaments and microtubules, cells were fixed in 3% formaldehyde, washed with PBS, and then treated sequentially with rabbit antiserum raised against tubulin (a generous gift of Dr. V. I. Kalnin, Department of Anatomy, University of Toronto, Toronto, Canada) (Connolly et al., 1977) followed by a fluoresceinated goat IgG raised against rabbit IgG (Miles-Yeda, Toronto) and then with rhodamine-labeled phalloidin (Molecular Probes, Junction City, OR). The coverslips were gently washed five times with PBS between each step for a total of 25 min. After the final wash, the coverslips were mounted in glycerol/PBS, 1:1. Cells were also stained with each reagent separately. To colocalize F-actin with vinculin, coverslips were first treated with 0.2% Triton X-100 in 0.1 M KCl/0.01 M Tris-HCl, pH 7.5 for 2 min at 4°C before fixation (Wong and Gotlieb, 1984). Controls included preabsorption of the tubulin antibody with tubulin, staining with the secondary antibody alone, and competition with excess phalloidin (Wong and Gotlieb, 1984).

Analysis of Centrosome Position

Since the location of the centrosome has been identified in the live endothelial cell through the use of phase-contrast optics (Gotlieb et al., 1981), we were able to locate and track the centrosome in the cells participating in wound closure.

Wound closure was analyzed by projecting the time-lapse cine film, frame by frame, onto a calibrated grid. The sequential position of cells and their centrosomes was traced out onto a series of clear acetate sheets. By lining up and overlaying these sheets, the association between centrosome redistribution and cell activity was assessed. Cell migration was defined as the forward displacement of both the leading and trailing edge of the cell. The trailing edge was defined as the part of the cell circumference facing away from or at 180° to the leading lamellipodia.

To numerically assess the proportion of cells which showed centrosomal redistribution, the wounds were fixed within 10 min after wound closure and stained for tubulin. These wounds were photographed, printed, and the location of the centrosome determined using the nucleus and the center of the closed wound as reference points (Rogers et al., 1985, 1986). The centrosome in each endothelial cell participating in wound repair was classified as being "Toward," "Away," or "Middle" with respect to the nucleus and the center of the wound. A centrosome classified as Toward was located between the nucleus and the side of the cell facing away from the wound. A centrosome in the Middle was located along the side of the nucleus. For the analysis of the control intact monolayer, the photograph of the intact monolayer was overlaid on a numbered grid, transluminated, and the reference point determined by random number selection. Nine 3-4-cell wounds, 17 7-9-cell wounds, and nine non-wounded cultures were examined in this fashion. The numbers were grouped into Toward, Middle, or Away categories and an analysis of variance was carried out. If the null hypothesis (i.e., control = small wound = large wound) was rejected, then a Newman-Kuels test was carried out to determine the experimental protocols that were significantly different from each other (Zar, 1974).

Results

Endothelial Wound Repair

The morphology of single cell wound closure has been previously characterized using time lapse cinemicrophotography (Wong and Gotlieb, 1984). Briefly, the cells facing the wound underwent retraction after removal of the single cell. Focal cell membrane ruffling with extension of small filopodia into the wound occurred within 5 min after wounding. This ruffling became generalized, involving the entire side of the cell abutting upon the wound. Thereafter the extrusion of the broad flat lamellipodia was observed. The sides of the cell remaining in contact with the monolayer did not show marked ruffling activity.

Circular three- to four-cell wounds underwent closure in a fashion similar to that of single cell wounds in that extrusion of lamellipodia from all of the cells abutting upon the wound occurred. No cell migration or cell mitosis was observed. Cells immediately behind the first row of cells bordering on the wound did not participate in wound closure.

The removal of seven to nine cells from the confluent culture was followed by retraction of all the cells abutting upon the wound (Fig. 1A). Within 5 min, cell ruffling and the beginning of lamellipodia extrusion was observed. By ~30 min, broad flat lamellipodia appeared (Fig. 1B) and became more prominent over the next 30 min. By 60-90 min, cell elongation became apparent (Fig. 1C) and cell migration occurred usually within the next 60 min. Wound closure occurred within 90 min after the onset of migration (Fig. 1D). Observations of intact monolayers before wounding did not show any cell migration.

Organization of Microfilaments, Microtubules, and Centrosomes During Repair

The repair of one- to four-cell wounds by lamellipodia extrusion was not accompanied by centrosomal redistribution. Cells with their centrosome facing away from the wound behaved in the same manner as those with their centrosome facing toward the direction of lamellipodia extrusion. The analysis of centrosome position after the repair of three- to four-cell wounds revealed a distribution similar to that of intact controls (Table I) in that the proportion of cells with centrosomes in the Toward, Middle, or Away position did not differ significantly from those of intact, nonwounded controls. No centrosomal redistribution was observed in closed one- or three- to four-cell wounds even after 24 h after wound closure.

In seven- to nine-cell wounds the initial period of cell retraction and lamellipodia extrusion showed no centrosomal redistribution. After 60-90 min, as the cells began to elongate all the cells abutting upon the wound began to posi-
Figure 1. Reendothelialization of a nine-cell wound. Phase-contrast micrograph of sequential observations of wound repair after the removal of nine cells at time points: (A) zero, (B) 30, (C) 90, and (D) 150 min. Extrusion of prominent lamellipodia was present (B) at 30 min and cell elongation (C) became discernible by 90 min. (D) Note the redistribution of the centrosome which began at ~60 min giving rise to a situation where all the centrosomes, including those which were originally on the opposite side of the nucleus, are facing the center of the closed wound. Small arrows in B and C demarcate the margins of the wound and larger arrows in B and D point out the location of the centrosome which is adjacent to the nucleus of the cell. Bar, 20 μm.

Redistribution of their centrosomes toward the direction of eventual cell movement (Fig. 1).

Cinephotographic observations made during redistribution of the centrosome showed that the centrosome either moves around a stationary nucleus, or the nucleus and centrosome rotate together as a unit. In the former method of redistribution, the centrosome may move either around the nucleus or over the nucleus but not under it. The localization of centrosomes in closed wounds by tubulin staining (Fig. 2) showed a significant shift of the distribution toward the front of the cell (Table 1).

Rhodamine-phalloidin staining of cells facing the wound edge showed marked splaying of that part of the dense pe-

Table 1. Centrosome Distribution at Wound Closure

|                      | Toward | Middle | Away   |
|----------------------|--------|--------|--------|
|                      | X* SEM |        |        |
| Control, nonwounded  | 23 ± 3.1 | 39 ± 4.7 | 38 ± 5.7 |
| Small wound          | 28 ± 7.5 | 41 ± 6.8 | 31 ± 6.9 |
| Large wound          | 64± ± 4.3 | 35 ± 4.2 | 1.01± ± 0.4 |

* Percent cells, mean of experiments at each size (see text).

† p > .001 large wound vs. control and small wound, Newman Keuls multiple range test.
ripheral band (DPB) associated with the extruding lamellipodia. As shape change began to occur there was a progressive reduction in the DPB in the rest of the periphery of the cell and some cells showed the presence of central microfilament bundles parallel to the long axis of the cell (Fig. 3).

Double staining for microtubules and microfilaments in cells demonstrating centrosomal redistribution during the cell elongation stage showed that this redistribution occurs before the complete loss of the DPB (Fig. 4).

Colocalization of microfilaments and vinculin showed that during repair, the loss of the DPB was associated in time and place with the reduction of vinculin plaques. Thus in the same cell abutting on the wound, the reduction of the DPB along the sides of the cell was associated with a reduction in vinculin plaques, while the persistence of the DPB at the back of the cell was associated with prominent vinculin plaques.

Discussion

These experiments show for the first time the sequence of major cytoskeletal events which occur during the process of small wound reendothelialization in vitro. By using two sizes of in vitro small wounds, we have been able to identify a spreading event and a migration event, and characterize each with respect to specific organizational changes in the cytoskeleton. The cells adjacent to the wound first extrude prominent lamellipodia to close the wound. This is a spreading activity without translocation of the rest of the cell and without a change in the shape of the whole cell. In this process the dense peripheral band of actin microfilaments remains intact and centrosomes do not undergo relocation. After ~1 h, if the wound remains open, a series of events occur in which the centrosome relocates to the front of the cell, the DPB becomes reduced or absent, and the cell elongates and translocates toward the wound.

It is important to note that some of the cytoskeletal events described in our study have been shown to occur in vivo at an endothelial wound edge. After wounding, the peripheral actin is reduced (Gabbiani et al., 1983) and centrosome redistribution does occur (Rogers et al., 1985).

The centrosome plays a major role in the determination of cell polarity. Data from various biological processes including cytotoxic T-cell killing (Geiger et al., 1982; Kupfer et al., 1983), mammary epithelial cells (Dylewski and Keenan, 1984), and chemotaxis of neutrophils (Malech et al., 1977; Schliwa et al., 1982; Anderson et al., 1982) and macrophages (Nemere et al., 1985) bear out this relationship. In the specialized case of cell migration, several studies have shown that the position or redistribution of the centrosome

1. Abbreviation used in this paper: DPB, dense peripheral band.
determined the direction of cell movement in amoebae (Swanson and Taylor, 1982), 3T3 cells (Albrecht-Buehler and Bushnell, 1979), fibroblasts (Kupfer et al., 1982), and endothelial cells in vitro (Gotlieb et al., 1981, Mascardo and Sherline, 1984) and in aortic organ culture (Rogers et al., 1986). It has been shown in endothelial cells that redistribution occurs before cell migration (Gotlieb and Wong, 1988). Although it is accepted that the centrosome plays a role in the formation of microtubules, especially after depolymerization by colchicine-like compounds (de Brabander, 1982a, b; Dustin, 1984), the mechanism of interaction between centrosome redistribution and polarized cell activities, such as cell migration, is not well defined. Centrosomal redistribution in endothelial cells however can occur independent of cell migration (Gotlieb et al., 1983). The presence of a wound edge, even if migration is inhibited, is enough to induce centrosome redistribution. It should also be noted that centrosomal redistribution requires intact microtubules (Gotlieb et al., 1981) and that there may be microtubule-microfilament interactions that enhance the redistribution (Schliwa et al., 1982; Gotlieb et al., 1983; Euteneuer and Schliwa, 1984; Pollard et al., 1984). It has been recently suggested on the basis of morphological and immunocytochemical studies that microtubules in migrating fibroblasts select and stabilize focal long-lived contacts which function to nucleate the assembly of stress fibers (Rinnerthaler et al., 1988), especially toward the leading lamellipodia.

Figure 3. Photomicrograph of nine-cell wound cultures fixed and stained with rhodamine-phalloidin at specific times after wounding. (A) At 75 min after injury, the initial lamellipodia extrusion has occurred and cell elongation is beginning. Note the splaying of the DPB (large arrow) at the site of lamellipodia extrusion, the attenuation of the rest of the DPB at the cell periphery, and the continuing presence of the DPB in cells behind those participating in the wound response (arrowhead). (B) Cells fixed immediately after wound closure, 2.5 h after wounding. Cells are elongated towards the center of the closed wound and some cells show central microfilaments (small arrow) oriented parallel to the direction of cell translocation. Arrowheads, DPB. Bar, 30 μm.

Figure 4. Photomicrograph of a nine-cell wound fixed and double stained at 75 min after wounding to localize (A) tubulin and (B) F-actin. (A) The majority of cells abutting the wound possess centrosomes (c) directed towards the wound as opposed to Middle or Away, and (B) show a reduction of the DPB, especially along the sides of the cells. n, nucleus. Bar, 30 μm.
In wound repair, the effector capacity of the centrosome is thought to be related to its close association with the Golgi apparatus (Rogalski and Singer, 1984; reviewed by Dustin, 1984). Using a transcribed viral protein as a probe for Golgi function, it has been shown that cell membrane insertion of this protein occurs preferentially in the area in front of the centrosome (Bergman et al., 1981, 1983). Although biochemical function of the Golgi apparatus and the quantity of protein inserted into the membrane remained unchanged after disruption of the centrosome-Golgi apparatus association, polarized addition of the protein was impaired (Rogalski et al., 1984). Thus, the centrosome may play a central role in directing Golgi apparatus substances toward the cell periphery.

In our study, centrosome redistribution did not occur during the directional lamellipodia extrusion occurring to repair wounds of one to four cells in size. Only when cell translocation was to occur did this mechanism come into play. Several implications arise from these observations. It is possible that centrosome-directed Golgi flow toward the membrane can occur in a manner independent of centrosome location by using preexisting microtubules which already extend toward the area of lamellipodia extrusion. An alternative explanation is that the directional extrusion of lamellipodia is an event independent of centrosome location. The cell membrane participating in cell spreading may come from the numerous blebs and folds found on the surface of cells (Follet and Goldman, 1970; Erickson and Trinkaus, 1976; Soni et al., 1980). During cell translocation however, active cell cycling becomes necessary because the membrane reservoir has been depleted by the preceding cell spreading. At present there is no direct evidence to support either concept.

The DPB is markedly reduced in cells undergoing elongation and translocation. This attenuation of the DPB is accompanied by a concomitant loss of peripheral vinculin. Since the presence of peripheral vinculin plaques may be important in the ability of endothelial cells to maintain close cell-cell contact (Wong and Gotlieb, 1986), it is noteworthy that peripheral vinculin is lost during cell migration. Although this might reduce the ability of the endothelium to maintain a tight monolayer (Shasby et al., 1982), one might speculate that this occurs to allow each endothelial cell to move less hindered by its neighbors. In fact, our cells in confluent monolayers do not show any significant migration whereas at the large wound edge migrating endothelial cells readily move in an independent fashion.

The central microfilaments also redistribute from a random orientation to one in which the microfilaments are often lined up parallel to the cell's axis of movement. Similar observations have been reported in our laboratory using an in vitro large scrape wound model system (Gotlieb et al., 1984). Redistribution of central microfilament bundles have also been observed in vivo injury to corneal (Gordon et al., 1982) and rabbit aortic endothelium (Gabbiani et al., 1983). The reason this occurs during cell translocation is not clear, however several possibilities have been proposed. These fibers may serve to pull the cell body forward during cell locomotion by using actomyosin mechanisms anchored at cell adhesion plaques (discussed by Buckley, 1981). Alternatively, Albrecht-Buehler (1977) has suggested from his studies on 3T3 cells that such structures act as intracellular positioning elements.

This study shows that the repair of defects in an in vitro endothelial monolayer is a multistep process involving a spreading event and, if necessary, a migration event, each characterized by specific distribution of cytoskeletal systems. Since one of the major initial events in the pathogenesis of atherosclerosis involves endothelial cell repair of monolayer defects, understanding these cytoskeletal events is important to the understanding of the atherogenic process. Studies have shown that centrosome redistribution may be modulated using blood-derived products (Mascardo and Sherline, 1984; 1987). Since platelet-derived growth factor has been shown to remove vinculin from adhesion plaques (Herman and Pledger, 1985), the release of platelet-derived growth factor at endothelial injury sites might also act to enhance the migration phase of repair. Having characterized the specific cytoskeletal events that occur, it is now possible to begin a systematic study of the regulation of the cytoskeletal modulators of endothelial repair.

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