Reduced In Vitro Repair In Endothelial Cells Harvested From the Intercostal Ostia of Porcine Thoracic Aorta

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Abstract—The ability of large-vessel endothelium to repair itself rapidly after injury is important in the maintenance of its barrier function and in limiting the development and progression of atherosclerosis. Because dysfunctional repair may be involved in the pathogenesis of some atherosclerotic plaques, including those at the ostia of aortic branches, linear mechanical denuding wounds were made in confluent monolayers of endothelial cells harvested by scraping from the flow divider, the upstream wall of the intercostal branch and unbranched regions in the thoracic aorta. The extent of wound closure was significantly lower in cells derived from either side of the intercostal branches, compared with cells from unbranched areas. The wound edge of cells harvested from the flow divider and its opposite wall closed by 22 ± 0.084 μm and 22 ± 1.3 μm, respectively, versus control, unbranched endothelial cells (30 ± 2.2 μm) at 24 hours and by 48 hours, 48 ± 3.4 μm and 47 ± 3.6 μm compared with control (61 ± 3.4 μm). Extent of wound closure in cells harvested by scraping from unbranched regions was comparable with collagenase-harvested endothelial cells at 24 and 48 hours. Distribution of F-actin microfilaments, tubulin and centrosomes have been shown to be disrupted at the wound edge in poorly migrating cells. In our study, however, no differences were observed in cytoskeletal distribution between cells from branched, unbranched and control areas. Thus, aortic endothelial cells from the intercostal branch region show a reduced ability to repair wounds compared with cells harvested from unbranched aorta. The mechanism for this difference is currently unknown. (Arterioscler Thromb Vasc Biol. 1999;19:665-671.)

Key Words: endothelium ■ migration ■ repair ■ atherosclerosis

The ability of the large-vessel endothelium to repair itself rapidly after injury is important in the maintenance of its barrier function and in limiting the development and progression of atherosclerosis. Our laboratory has shown that the disruption of vascular integrity in vitro and in vivo promotes cytoskeletal reorganization, cell spreading, migration, and proliferation to effect rapid and efficient wound repair.1–7

The intimal cushion present on the flow divider wall at branches in vivo is thought to be a physiological response to altered flow dynamics that may eventually become a precursor lesion of atherosclerosis. Modifications of flow implicated in lesion development include increased or decreased flow velocity or wall shear stress, flow separation, and departures from unidirectional laminar flow, including both orderly, nonlinear flow patterns and turbulence.8 Fluctuations in blood flow in experimental animals, particularly at sites of branching, can affect endothelial cell morphology,9,10 cause separation of intercellular junctions,11 and denudation of cells with the subsequent adherence of platelets.12 Such factors may account for the preferential location of plaques at the entrances of branches.

In vivo, cells within regions of eccentric intimal thickening such as the intimal cushion, an area of thickening present on the downstream margins of branch point openings, have been found to show altered function compared with cells from adjacent, thinner areas of the intima including the presence of focal patterns of increased cell proliferation with high permeability to Evans Blue dye at flow divider intimal cushions in the intercostal ostia of normocholesterolemic young pigs.13 Increased permeability of the endothelial lining to plasma lipids has also been observed in rabbit models of atherosclerosis.14

We used cell cultures to study the endothelial cells harvested from areas at branches and away from branches in the thoracic aorta to assess their ability to repair in vitro denuding wounds. Because dysfunctional repair is thought to be important in the pathogenesis of at least some atherosclerotic plaques, it was hypothesized that a decreased capacity for repair would be observed in endothelial cells harvested from atherosclerosis-prone regions around branches.

Materials and Methods

Endothelial Cell Cultures

Three porcine thoracic aortas were obtained from a local slaughterhouse within 15 minutes of death. They were transported to the laboratory in sterile PBS containing antibiotics, gently cleaned of fat and adventitia, then opened longitudinally to expose the lumen.

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Endothelial Repair at Intercostal Ostia In Vitro

Cells from 4 regions in each thoracic aorta were collected (Figure 1): the first 3 intercostal branch point flow dividers (flow divider, [fd]), the area distal to these intercostal branches (opposite flow divider, [ofd]), unbranched tissue opposite the branch point (unbranched opposite intercostal, [uboi]), and unbranched tissue above the first intercostal but below the aortic arch (unbranched above intercostal, [ubai]). In order to harvest endothelial cells from these 4 regions, each region was gently scraped using a number-22 scalpel blade. Each scrape was washed from the blades into separate wells of 24-welled Falcon trays containing 1 mL medium M199 (Gibco) containing 5% fetal bovine serum (Gibco), 2% penicillin/streptomycin, and 2% fungizone (Gibco) fed through a 10-mL syringe and 21-gauge needle. Cell cultures were fed twice weekly thereafter until cultures were established. They were then plated into 35-mm dishes and grown to confluency, at which time cells harvested from the same region for each of the 3 aortas were pooled and then plated into 100-mm dishes. Confluent cells were then passaged once more into 35-mm dishes in preparation for wounding. Analyses of wound repair kinetics and growth curves were performed on harvested endothelial cells between passages 3 and 5. Collagenase-harvested thoracic aortic endothelial cells, harvested as previously described,6 were fed in the same manner as scraped cells and were used as a control in the following experiments.

Cell Identification
Didl-conjugated acetylated LDL has been used as a marker for endothelial cells.13 Acetylated-LDL (Biomedical Technologies Inc) was incubated with harvested cells for 2 hours at 5 μg/mL in culture medium to demonstrate that cells were endothelial in nature. Collagenase-harvested porcine thoracic endothelial and smooth muscle cells served as controls. Harvested and control cells on glass coverslips were also labeled using immunofluorescent rhodamine phalloidin (Molecular Probes, 1:20) to demonstrate F-actin, along with an alpha smooth muscle cell actin mouse monoclonal primary (1:400) followed by an FITC-conjugated goat anti-mouse secondary (Sigma Chemical Co, 1:100) to confirm their endothelial characteristics.

Wound Closure Experiments
Cells were grown to confluency on coverslips in 35-mm dishes at which time a linear wound measuring approximately 2 to 2.5 mm was made down the center of the coverslip using a plastic spatula. An orienting scratch and 3 guide scratches were made on the periphery of the coverslip with a diamond-tipped pencil, then wounded cultures were rinsed with PBS and fed with fresh standard medium M199 containing 5% fetal bovine serum. Media were not changed for the duration of the experiment. The distance between the 2 edges of the wounds was measured under phase microscopy using a 1-mm micrometer at each of 3 scratch marks for each of 3 dishes per location at the time of wounding, and after 24 and 48 hours in culture. Collagenase-harvested thoracic aortic endothelial cells which are routinely used in the laboratory were used as a control. Experiments were performed in triplicate and analyzed using a factorial ANOVA to determine significance. If significance was observed, then a Fisher’s PLSD post hoc test was run (Statsview 4.5, Power Macintosh 7200/90).

Growth Curves
Growth curves were carried out during a 7-day period for cells from the 4 regions of the aorta (fd, ofd, uboi, and ubai). Confluent cultures were washed twice with warmed PBS, then 2 mL of trypsin was added. Once cells detached from the culture dish, 3 mL of medium was added to neutralize the trypsin. Cells were washed off by gently pipetting the medium/trypsin around the dish. A 0.5-mL sample was drawn for counting, then the remaining 4.5 mL was collected and centrifuged. The medium/trypsin was then aspirated, leaving <0.5 mL in each tube. Fresh media were added to each tube in proportions that would achieve a final concentration of 15 000 cells per 300-μL aliquot and cell pellets were resuspended. Ninety-six 35-mm dishes without coverslips (24 per location) were plated with 15 000 cells initially. A total of 3 dishes per location for each of 8 time points (5 hours, 1 day, 2, 3, 4, 5, 6, and 7 days) were plated. Cells were fed every 2 days with standard medium M199 containing 5% FBS.

Three dishes were selected at random from each of the 4 locations at each time point. Each dish was washed twice with warm PBS, followed by addition of 1 mL of warmed trypsin and incubated at 37°C for 2 minutes. Cells were dispersed by pipetting, and a 0.5-mL aliquot was withdrawn for counting on a Coulter counter (Coulter Industries Inc, Model 7163Z). Each sample was counted 3 times and an average value taken. Results shown represent experiments performed in triplicate.

Figure 1. Schematic of the porcine thoracic aorta and the first intercostal branch point. The areas under study are noted. fd indicates flow divider; ofd, opposite flow divider; uboi, unbranched opposite intercostal; and ubai, unbranched above intercostal.

Figure 2. Phase-contrast photomicrographs showing cell morphology of A, subconfluent, flow divider-harvested cells 4 days after plating; B, collagenase-harvested confluent endothelial cells; and C, subconfluent medial smooth muscle cells. Magnification ×10, bar=10 μm.
formed in triplicate and analyzed using a factorial ANOVA to determine significance.

**F-actin and Tubulin Expression in Wounded Cultures**

Wounded cells on glass coverslips at 0, 24, and 48 hours postwounding were rinsed with warm PBS, then fixed for 20 minutes with warm 3% paraformaldehyde, after which time they were rinsed and permeabilized with 0.1% triton for 3 minutes. Cells were double-labeled using monoclonal mouse anti-α-tubulin (Sigma Chemical Co) applied in a 1:500 dilution for 1 hour, followed by an FITC-conjugated goat anti-mouse secondary (Sigma Chemical Co, 1:100), and rhodamine phalloidin (1:25, 30 minutes) to demonstrate microtubules and F-actin, respectively. Coverslips were inverted, mounted in 1:1 glycerol/PBS, and cells from the leading edge of wounds were analyzed under laser scanning confocal microscopy. Cells at the leading edge were scanned to depths of 2.5 μm.

**Results**

**Identification of Harvested Cells**

Under phase-contrast microscopy, all cell scrapes were observed to grow as islands of cells, typical of endothelial cell growth in culture (Figure 2A). They attained cobblestone morphology on reaching confluency (Figure 2B), unlike smooth muscle cells obtained from the underlying medium (Figure 2C). Dil-conjugated acetylated LDL uptake occurred to the same extent in collagenase-harvested endothelial cells (Figure 3A) and cell scrapes (Figure 3C through 3F) at 2 hours, but to a much lesser degree in collagenase-harvested smooth muscle cells (Figure 3B). F-actin staining showed the presence of the dense peripheral band in collagenase-harvested and cell-scrape–harvested endothelial cells but not in smooth muscle cells. Alpha smooth muscle cell actin staining was observed in smooth muscle cell cultures and not in collagenase-harvested endothelial cells or in cells harvested from cell scrapes (not shown).

**Wound Closure**

Percent wound closure refers to the degree to which wounds have closed at the time point measured, with 100% referring to the closure of the gap between wound edges. Significantly greater wound closure was measured in collagenase-harvested unbranched endothelial cells and cells from unbranched areas compared with cells derived from either side of intercostal ostia. Wound edges of cells harvested from the flow divider and cell-scrape–harvested cells from the flow divider and wall opposite the flow divider (fd, 62 ± 3.0 μm; ofd, 65 ± 3.4 μm) closed by 22 ± 0.084 μm and 22 ± 1.3 μm, respectively (n = 3), versus cells harvested from collagenase-harvested unbranched endothelial cells (30 ± 2.2 μm) and scrape-harvested unbranched regions (uboi, 33 ± 2.0 μm; ubai, 31 ± 2.0 μm) at 24 hours. By 48 hours, the difference in wound closure seen at 24 hours was even more pronounced. Cells from branch regions again showed a significantly decreased extent of wound closure (fd, 48 ± 3.4 μm; ofd, 47 ± 3.6 μm) compared with cells from collagenase-harvested (ec: 61 ± 3.4 μm) and scrape-harvested unbranched regions (uboi, 65 ± 3.4 μm; ubai, 62 ± 3.0 μm). Cells harvested from branch point ostia, examined by phase-contrast microscopy at both 24 and 48 hours, appeared less elongated than cells harvested from unbranched regions and collagenase-harvested cells, which was consistent with the reduced degree of wound closure observed. The wound closure data are summarized in Figure 4.

**Growth Curves**

Cell culture growth for 7 days is shown in Figure 5. Plating efficiency averaged 99% at 5 hours postplating and cell numbers grew exponentially from day 2 (30 211 ± 3577) and thereafter through day 4 (126 294 ± 16 092), after which time proliferation slowed by 50%, presumably caused by cell quiescence as monolayers approached confluency. No differences were observed between cell numbers between regions
on any given day during 7 days culture (final count: 275 140 ± 6 24 354), suggesting that differential rates of cell proliferation are not likely to account for the differences in wound closure observed in earlier experiments.

F-actin and α-Tubulin Expression

Cells from branched and unbranched areas were similar at 24 and 48 hours in terms of F-actin and α-tubulin distribution (collagenase-harvested endothelial cells, Figure 6; flow divider–harvested endothelial cells, Figure 7; cells from unbranched aorta opposite the intercostals, Figure 8). At the time of wounding, a dense peripheral band of actin microfilaments was present around cell borders and cells contained few central microfilament bundles in collagenase-harvested endothelial cells (Figure 6). Microtubules spanned the entire area of cells in a fibril-like network in cells along the wound edge and centrosomes were observed to be located randomly around the cell nucleus (Figure 6).

Loss of the dense peripheral band in cells along the wound edge was observed in all cells at 24 hours postwounding along with prominent central microfilaments oriented perpendicularly to the wound edge in elongated cells. Microtubule distribution, which again spanned the length of cells and extended into lamellipodial extrusions, was more condensed. Centrosomes in cells at the leading edge of the wound were oriented toward the wound edge. At 48 hours, cells were similar to those at 24 hours in terms of actin microfilament orientation. Microtubule distribution resembled that seen at 24 hours.

Discussion

Analysis of in vitro wound healing in confluent monolayers of endothelial cells harvested from different regions of the porcine thoracic aorta indicated that cells from the branch region showed a reduced capacity to repair wounds compared with cells harvested from unbranched aorta at 24 and 48 hours postwounding. The data presented herein support the concept of heterogeneity of function within intimal cells of the vessel wall, even within microenvironments relatively close to one another. Although at present the mechanism for this difference is undetermined, several cellular processes may likely be involved, including differences in cell proliferation, cytoskeletal function, and cell–cell and cell–substratum adhesion.

One possibility to account for the relatively decreased capacity of branch-harvested cells to migrate may be a delay in growth factor-influenced cytoskeletal reorganization. A number of growth factors released at various times during wound healing including platelet-derived growth factor,17 epidermal growth factor,18,19 the interleukins,20 and basic fibroblast growth factor21,22 play roles in the stimulation/inhibition of cell proliferation and migration. Platelet-derived growth factor–induced cell motility via the reorganization of actin microfilaments is thought to involve the PI3-kinase pathway.23 Basic fibroblast growth factor, known to be required for centrosome redistribution to the front of the cell in anticipation of cell migration,6 has recently been implicated in the expression and synthesis of biglycan-rich matrix in migrating endothelial cells after wounding.24 Additionally, levels of growth factor signaling are dependent on the temporal and spatial activation of extracellular matrix receptors and the modulation of classical signal transduction pathways including protein tyrosine phosphorylation25 and the downstream activation of protein kinase C.26 The activation of these pathways has also been shown to result in the enhancement of cytoskeletal reorganization and subsequent cell motility.25,26 However, analysis of F-actin microfilament distribution and the associated tubulin network in actively migrating endothelial cells at the wound edge did not reveal obvious distinctions between harvested cells. Loss of the dense peripheral band and the appearance of central stress fibers oriented perpendicularly to the wound edge, conditions which are characteristic of porcine endothelial cell migration,16 were similar among cells at both 24 and 48 hours.

Differences in the rates of wound closure observed between cells from branch regions and cells from unbranched regions may also reflect differences in proliferation. This is
difficult to assess because proliferation at the wound edge is closely related to migration, and a reduction in migration has been shown to result in a reduction in cell proliferation. Thus, we assessed growth in the cell populations by growth curve analysis, to ensure that there was not a general defect in cell proliferation in any of the cell populations when compared with each other and to normal cells. There were no differences between the 4 regions. It is arguable, however, whether the initial plating of cells for growth curve measurements is a proliferative stimulus comparable with that effected in cells at the leading edge by mechanical wounding. Pepper and colleagues have demonstrated that intercellular communication in mechanically wounded bovine microvascular cell cultures is significantly greater at 24 hours compared with that observed in sparse and preconfluent cell monolayers; however, investigators also found that increased

Figure 6. Confocal photomicrographs of wounded control endothelial cell cultures stained for F-actin and α-tubulin. A, times zero; C, 24 hours; and E, 48 hours postwounding of F-actin-stained cells. B, times zero; D, 24 hours; and F, 48 hours postwounding of cells showing α-tubulin expression. Magnification ×60, bar=25 μm.
junctional communication did not correlate with bovine microvascular cell proliferation.

In addition to cell migration and proliferation, repair after injury also requires controlled remodeling of the extracellular matrix. The use of culture systems has provided ample evidence to support a role for the extracellular matrix in influencing the rate of cell migration in vascular endothelial cells. Specific matrix proteins such as laminin in particular are known to influence endothelial proliferation29,30 and differentiation.31 In vivo, cells located in areas of high mechanical strain produce more of the extracellular matrix proteins tenascin and collagen VII.32 It is therefore possible that endothelial cells harvested from different locations in the aorta evolve varying ratios of the various extracellular matrix proteins on injury, which may confer to harvested cells the different reparative capacities observed in culture. In any event, wound repair requires a continually evolving network of interactions among cells and the extracellular matrix in which they reside. The integrin family of cell surface receptors is well known for their role in mediating cellular adhesion to extracellular matrix proteins. Indeed, Liaw and colleagues33 have reported that the upregulation of osteopontin in injured rat arteries stimulates the directed migration of bovine aortic endothelial cells through interactions with the αvβ3 receptor.

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References
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