In Vitro Endothelial Wound Repair
Interaction of Cell Migration and Proliferation

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Most re-endothelialization requires both cell migration and cell proliferation. To study the association between cell migration and the initiation of DNA synthesis in an in vitro wound model, confluent cultures of porcine aortic endothelial cells grown on glass cover slips were scraped to remove half of the monolayer. Treatment with 1 ng/ml of taxol, a microtubule stabilizing drug, for 24 hours resulted in no visible change in F-actin or microtubule organization as assessed by fluorescence and immunofluorescence microscopy. There was, however, a reduction of wound re-endothelialization and an associated reduction in the proportion of cells with centrosomes redistributed toward the wound edge. No significant differences, however, were seen in the labeling indices for the first two rows of cells at the wound edge as revealed by 3H-thymidine autoradiography. Labeling of nuclei in Rows 3 to 8 and in a zone deeper within the monolayer was reduced in treated cultures. The data suggest that endothelial proliferation in cells within an area bordering a wound is dependent on both denudation, which is sufficient to promote maximal proliferation in the two rows adjacent to the wound, and cell migration, which is required for the propagation of proliferation in cells further away from the wound edge.

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The relationship between cell migration and proliferation during endothelial wound repair is not well understood. Previous work has emphasized the importance of cell spreading1 and cell translocation2,3,4 in regulating wound repair. The importance of centrosomal re-orientation for commencement of the directed cell migration, which precedes cell proliferation, has been described,2,5 as has the loss of the dense peripheral band of actin microfilaments.1

To dissect the relationship between cell migration and cell proliferation, we developed a model that selectively affects cytoplasmic microtubules and thus cell migration, but does not affect DNA synthesis and other cellular processes. We used the drug, taxol, a plant-derived compound that stabilizes and promotes assembly of microtubules in the cytoplasm.6,7 Taxol does not bind DNA, nor does it have any specific effects on DNA synthesis, thymidine transport, or protein synthesis.7,8,9

Using very low concentrations of taxol, we were able to separate two processes involved in wound repair, cell migration and cell proliferation. Our results indicate that monolayer denudation was sufficient to initiate proliferation in the cells adjacent to the wound, while those cells farther back in the monolayer required cell migration at the wound edge for initiation of proliferation.

Methods

Cells

Porcine aortic endothelial cells were isolated from aortas obtained at a local abattoir as previously described.2 Briefly, cells were harvested by collagenase dispersion and were grown in complete media consisting of M199 plus 2% penicillin, streptomycin, and fungisone and 5% fetal bovine serum (GIBCO, Grand Island, NY). The cells were incubated at 37°C in a humid atmosphere containing 5% CO2 and 95% air. When primary cultures reached confluence, the cells were passed by using trypsin ethylenediaminetetraacetic acid (EDTA) dispersion. The cells from the second to the fifth passage were used.

Taxol

A stock solution of taxol in sterile dimethyl sulfoxide (Fisher Scientific, Fair Lawn, NJ) consisting of 5 mg/ml of taxol was used. Aliquots were added to complete media to produce working concentrations from 1 to 1000 ng/ml of taxol. After preliminary experiments at various concentrations, experiments were carried out using either 1 ng/ml or 5 ng/ml. The former allowed us to carry out critical experiments in which cell migration and proliferation were separated, while the latter showed some effects on proliferation as well as on migration. Control experiments were done with media containing an equivalent volume of sterile dimethyl sulfoxide.
Growth Curves

Endothelial cells were plated at 2 × 10⁴ cells per 35 mm culture dish containing serum-free media. After 24 hours, some dishes were randomly selected for cell counting, and the remainder were fed complete media containing 0, 1, or 5 ng/ml of taxol for up to 72 hours. Cultures were trypsinized at intervals of 24 hours and were counted electronically (Coulter Electronics, Hialeah, FL). The results were expressed as the means of three dishes for each time point. The experiments were repeated three times.

Migration Assay

Endothelial cells were cultured on glass coverslips until confluent. The monolayer was wounded by scraping approximately one-half of the coverslip with a Teflon spatula to remove the cells. A small scratch was made with a diamond pencil at the wound edge of the monolayer to measure the distance of re-endothelialization. The distance from the scratch to the edge of the wound was measured under a 10x phase objective of a light microscope. Measurements were done after wounding and at 24-hour intervals until the end of the given experimental time period. Complete media containing taxol (0, 1, or 5 ng/ml) was added at wounding. Each group consisted of three sets of experiments, each consisting of three coverslips. The results were expressed as the mean distance migrated at each time point for each treatment group.

³H-Thymidine Autoradiography

Endothelial cells were plated at 4 × 10⁴ onto tissue culture well slides (2 wells/slide, Miles Scientific, Naperville, IL) and were fed complete media until confluent. One well was used for the control and one for the experiment. Cultures were then wounded and incubated in complete media containing 1 µCi/ml of ³H-thymidine (Du Pont, Montreal) and the appropriate taxol concentrations. After incubation at 37°C for 24 or 48 hours, cultures were rinsed with four changes of phosphate-buffered saline (PBS) for 15 minutes each, were fixed with 3% paraformaldehyde for 20 minutes, were rinsed in PBS, and were then dehydrated in ethanol and air dried. Unwounded monolayers were also treated with taxol and ³H-thymidine as above to determine the background proliferation.

Air-dried slides were dipped in melted NBT-2 nuclear tract emulsion (Eastman Kodak, Rochester, NY), diluted 1:1 with warm distilled water, were air dried in complete darkeness, and were then stored in dessicant-containing metal tins at 4°C for 2 days. Slides were developed on the third day in freshly made cold D-19 for 5 minutes, were rinsed in cold tap water for 30 seconds, and were then fixed for 10 minutes in cold Kodak Fixer. After fixing, slides were washed for 20 minutes in running tap water and stained in Meyer's hematoxylin for 5 to 10 minutes. They were then rinsed again in tap water, dehydrated in ethanol and xylene, and coverslipped with Permount (Fischer Scientific, Fair Lawn, NJ).

Slides were examined with a Nikon light microscope under a 40x objective, and the mitotic index of cells at the wound edge was obtained by counting the number of labeled cells present along the wound edge. Cells in the first row, in the second row, and in rows 3 to 8 were counted across the length of the wound. In cultures treated for 24 hours, this method of counting resulted in the counting of the entire proliferative zone at the wound edge. However, cultures incubated for 48 hours had larger zones of labeled nuclei. Thus, additional counts were performed to quantify the mitotic response to taxol treatment deeper within the monolayer. The inner border of the proliferative zone in control cultures (0 ng/ml taxol) at 48 hours was identified, and its distance from the edge of the wound was measured. Counts were made at this distance from the edge of the wound in cultures incubated with 0, 1, or 5 ng/ml of taxol and were called "deep" mitotic counts. The labeling index was calculated as the percent of labeled cells per total number of cells. The results were expressed as the mean labeling index in each group of cells for each treatment group.

Immunofluorescence

Endothelial cells were plated onto glass coverslips and fed complete media until grown to confluence. Cultures were then wounded, fed with appropriate media, and incubated for various times. For tubulin staining, the coverslips were rinsed with PBS and fixed with ice-cold methanol and acetone. Coverslips were further rinsed with PBS and stained with antibodies to chicken brain tubulin raised in rabbits (CN Immunobiologicals, Lisle, IL) and swine anti-rabbit IgG conjugated to fluorescein (Dakopatts, Denmark) with standard techniques. For F-actin visualization, coverslips were fixed with 3% paraformaldehyde, were permeabilized with 0.1% Triton X100 for 15 minutes, were rinsed in PBS, and were then dehydrated in ethanol and air dried. Unwounded monolayers were also treated with taxol and ³H-thymidine as above to determine the background proliferation.

Results

Cell Morphology

There were no specific morphological differences between control cultures and those treated with 1 ng/ml of taxol for up to 96 hours (Figures 1A and 1B). After 24 hours, the lamellipodia extrusion was not as prominent in cultures treated with concentrations of taxol of 5 ng/ml or higher, and there was detachment of cells from the substrate (Figures 1C, 1D, and 1E). These effects were enhanced with longer treatment times.
Figure 1. Phase-contrast micrographs of cultures 24 hours after wounding of confluent monolayer. A. Control. B. 1 ng/ml of taxol. C. 5 ng/ml of taxol. D. 100 ng/ml of taxol. E. 1000 ng/ml of taxol. The direction of cell migration is toward the top of the page. Lamellipodia extrusion was not as prominent in cultures treated with 5 ng/ml taxol or more, and there was rounding up of cells and cell detachment from the substrate (arrows). These effects were enhanced with longer incubation times and higher concentrations. ×100

**Cell Cytoskeleton**

Control cultures and those treated with 1 ng/ml of taxol showed no qualitative differences in microtubule organization (Figures 2A and 2B). The cultures treated with higher concentrations of taxol had large cytoplasmic structures composed of bundles of microtubules (Figure 2C); the microtubule network did not extend to the edge of the cell but was condensed in the perinuclear region in cultures treated with 100 or 1000 ng/ml (Figures 2D and 2E). Taxol did not disrupt F-actin in these cells. Control cultures and those treated with 1 ng/ml of taxol showed cells at the wound edge with prominent cytoplasmic stress fibers and no obvious dense peripheral band of F-actin (Figures 3A and 3B). Cells at the wound edge of cultures treated with higher doses of taxol have fewer stress fibers and more prominent dense peripheral bands (Figures 3C, 3D, and 3E).

**Cell Growth**

The data from growth curve experiments showed that cells treated with 1 ng/ml of taxol had growth similar to nontreated control cultures, while those treated with 5 ng/ml of taxol experienced little increase in numbers (Figure 4).

**Wound Re-endothelialization**

Wound re-endothelialization was affected by taxol treatment. Cultures incubated with 1 ng/ml of taxol also resulted in a significant reduction in wound repair, while 5 ng/ml of taxol gave a large reduction in monolayer re-endothelialization (Figure 5).

**Centrosome Redistribution in Wound**

The position of the centrosome in relation to the wound edge in the first row of cells was examined by using immunofluorescence staining of monolayers cultured with 1 ng/ml of taxol and fixed after 12, 24, or 48 hours of incubation (Table 1). There was a significant reduction in the proportion of cells with re-oriented centrosomes in the cultures treated with taxol at all time points.

**Cell Proliferation In Wound**

The cultures were incubated with \(^{3}\)H-thymidine in the presence of taxol for 24 or 48 hours after wounding. After 24 hours, the width of the zone of proliferating cells (labeled nuclei) was reduced to 85% of control values in cultures treated with 1 ng/ml taxol and to 32% of control values in...
Figure 2. Immunofluorescence micrographs of cells stained for tubulin at 24 hours after wounding of confluent monolayers. A. Control. B. 1 ng/ml of taxol. C. 5 ng/ml of taxol. D. 100 ng/ml of taxol. E. 1000 ng/ml of taxol. The direction of cell migration is toward the top of the page. Control cultures and those treated with 1 ng/ml of taxol showed no qualitative differences in microtubule organization. Cultures treated with higher concentrations of taxol had apparent microtubule bundles in their cytoplasm. The mitotic spindle was also disrupted in these cultures; the rounded-up cells seen in D were cells in metaphase arrest (arrowheads). In addition, the microtubule network did not extend to the edge of the cell but condensed in the perinuclear region in cultures treated with 100 and 1000 ng/ml of taxol (large arrows). Note the centrosomes oriented toward the wound edge (small arrows). ×420

5 ng/ml cultures (Figure 6). After 48 hours, this proliferative zone was reduced in cultures treated with 1 ng/ml of taxol to 75% of control and was 55% of control in cultures treated with 5 ng/ml of taxol (Figure 6).

Thymidine labeling indices were obtained to determine which cells at the wound edge were synthesizing DNA. After 24 hours, treatment with 1 ng/ml of taxol showed that in the first two rows at the wound edge, mitotic indices were identical to the control, but that cells farther back from the wound edge had significantly reduced labeling indices (Figure 7A). Treatment with 5 ng/ml of taxol resulted in significant reductions of mitotic indices as compared to controls. After 48 hours of taxol and 3H-thymidine incubation, there were no significant indices in labeling indices in row 2 and rows 3 to 8, as compared to controls (Figure 7B). However, there was a significant reduction of the deep mitotic indices of cultures treated with 1 or 5 ng/ml of taxol for 48 hours, as compared to controls (Figure 7B). Labeling in the deep zone of 5 ng/ml treated cultures was not significantly different from background labeling in unwounded cultures (see Table 2). Labeling indices obtained from unwounded monolayers showed no significant differences between taxol-treated and control values at either 24 or 48 hours of incubation (Table 2).

Discussion

Wound repair after endothelial denudation in vitro is achieved by a predictable sequence involving cell spreading, cell migration (translocation), and cell proliferation. Small, punctate denudations are repaired by cell spread-
Figure 3. Fluorescence micrographs of cells stained for F-actin with rhodamine-conjugated phallolidin at 24 hours after wounding of confluent monolayers. A. Control. B. 1 ng/ml of taxol. C. 5 ng/ml of taxol. D. 100 ng/ml of taxol. E. 1000 ng/ml of taxol. The direction of cell migration is toward the top of the page. In cultures treated with low concentrations of taxol (A to C), peripheral bands of actin were lacking from cells at the wound edge, and cytoplasmic stress fibers (arrows) were more prominent. Cells at the wound edge of cultures treated with higher doses of taxol had fewer stress fibers and more prominent dense peripheral bands (arrowheads). ×380

Figure 4. Growth curves from cultures initially plated at low densities, then incubated with taxol for up to 72 hours. Cells treated with 5 ng/ml of taxol experienced little increase in cell numbers, while those treated with 1 ng/ml of taxol had proliferation profiles similar to those of control cultures.

Figure 5. Re-endothelialization (cell migration and proliferation) of wounded monolayers treated with taxol. Cultures incubated with 5 ng/ml of taxol had a large reduction in monolayer re-endothelialization. Treatment with 1 ng/ml of taxol also resulted in a significant reduction in wound repair (p<0.05).

This study demonstrates that a reduction of cell migration in an in vitro, wounded endothelial monolayer does not result in a concomitant reduction of cell proliferation in cells close to the edge of the wound. Treatment of wounded monolayers with a low dose of taxol (1 ng/ml) reduces migration and delays centrosome reorientation, while the high rate of DNA synthesis at the immediate wound edge is not affected. In contrast, cells farther back in the monolayer...
Table 1. Orientation of Centrosome in First Row of Cells at Wound Edge in Control and in Treated Cultures

<table>
<thead>
<tr>
<th>Location of centrosome</th>
<th>Time after wounding</th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 hrs</td>
<td>24 hrs</td>
<td>48 hrs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toward wound edge</td>
<td>Control 75.7 (0.6)</td>
<td>Taxol</td>
<td>Control 79.5 (1.6)*</td>
<td>Taxol 69.4 (1.4)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19.9 (1.9)</td>
<td></td>
<td>16.1 (1.2)</td>
<td></td>
<td>23.9 (1.3)</td>
</tr>
<tr>
<td>Middle of cell</td>
<td>Control 26.0 (1.4)</td>
<td>Taxol</td>
<td>Control 40.0 (0.8)</td>
<td>Taxol 47.6 (1.5)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.4 (1.3)</td>
<td></td>
<td>9.0 (1.3)</td>
<td></td>
<td>6.7 (0.7)</td>
</tr>
<tr>
<td>Away from wound edge</td>
<td>Control 4.3 (0.5)</td>
<td>Taxol</td>
<td>Control 9.0 (1.1)</td>
<td>Taxol 4.4 (0.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.4 (0.3)</td>
<td></td>
<td>9.0 (1.3)</td>
<td></td>
<td>6.7 (0.7)</td>
</tr>
</tbody>
</table>

Values are the means (SE).
Treated cultures were treated with 1 ng/ml of taxol.
*Significantly different from control values (p<0.05).

Figure 6. Autoradiographs of wounded monolayers at 24 hours (top row) and at 48 hours (bottom row) after wounding. A. Control. B. 1 ng/ml of taxol. C. 5 ng/ml of taxol. The direction of cell migration is toward the top of the page. Note that taxol treatment resulted in a reduced zone of proliferating cells (labeled nuclei). × 50
show reduced DNA synthesis relative to control untreated wounds, suggesting that cell migration does have a role in the propagation of endothelial cell replication.

We used taxol because microtubule depolymerizing agents such as colchicine, vinblastine, or nocodazole, although they do induce reduced cell migration, are not suitable to explore the relationship between migration and proliferation during re-endothelialization; these are known to have a stimulatory effect on DNA synthesis in a variety of in vitro systems. This effect is mediated via microtubule depolymerization. In our study, taxol at 1 ng/ml had no effect on cell proliferation in low density endothelial cell cultures. High concentrations of taxol (0.125 to 20 μg/ml) are thought to affect cell proliferation by producing a late G2 or early M block in cultured cells. Taxol at sufficient concentrations disrupts the microtubule/free tubulin equilibrium in the cytoplasm, preventing sufficient microtubule depolymerization to initiate DNA synthesis. There appear to be no primary effects on DNA synthesis in cells that have already entered the S phase, as they continue to synthesize DNA normally. High concentrations (1 to 10 μg/ml) of taxol also promote the assembly of new, stable microtubules, which polymerize in the cytoplasm, free from the organizing capacity of the centrosome or kinetochores. These appear under immunostaining techniques to be aggregated into bundles of several microtubules. In our study, the nature of the interaction of taxol with endothelial microtubules was not examined. However, at the 1 ng/ml concentration used to carry out our critical experiments, such effects were not apparent since the microtubules appeared normal by immunofluorescence. Only at higher concentrations, including 5 ng/ml, were some thick bundles of microtubules apparent.

The nature of the signal(s) involved in propagating endothelial cell replication and how it might be related to cell migration is unknown. In our study, there was a reduction in wound re-endothelialization and a concomitant delay in centrosomal reorientation in cells at the wound edge of cultures treated with 1 ng/ml taxol. With respect to locomotion of endothelial cells, centrosome reorientation, a rapid event which precedes cell translocation, is considered a signaling mechanism for directed cell migration. Such a change in cell polarity can be altered by external agents, including insulin, and hyperglycemic media. Whether changes in cell polarity can act as a signal that can be transmitted from cell to cell by some mechanism is unknown. As well, growth factors including fibroblast growth factor and transforming growth factor (β) have been shown to alter wound re-endothelialization and should be considered in future studies as substances which may play a role in the regulation of cell proliferation by cell migration. Previous studies have shown that there is a propagation of signals within the repairing endothelial monolayer, presumably through gap type junctions. We have shown that within the cells of the wound edge, redistribution of the actin microfilaments occurs before any migration of the cells involved and before any redistribution of the actin microfilaments in these cells. Thus, propagation of information appears to be an important mechanism to organize coordi-

### Table 2. \(^{3}H\)-Thymidine Labeling Indices in Unwounded Monolayers Treated with Taxol

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Labeling indices</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hrs</td>
</tr>
<tr>
<td>Control</td>
<td>3.34 (0.31)</td>
</tr>
<tr>
<td>1 ng/ml taxol</td>
<td>3.53 (0.06)</td>
</tr>
<tr>
<td>5 ng/ml taxol</td>
<td>3.18 (0.22)</td>
</tr>
</tbody>
</table>

Values are means (SE). There were no significant differences in labeling indices for taxol-treated vs. control cultures at any time point.
nated activity within the wound edge. With respect to cell proliferation, we now observe similar processes. The cells at the edge of the wound respond to the loss of contact inhibition, while the cells deeper within the wound edge depend on propagation of information to act in a coordinated fashion to promote repair. It appears that the propagation of information is associated with cell migration.

Acknowledgment
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References
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