CYTOCHALASIN B INHIBITION OF ENDOTHELIAL

PROLIFERATION AT WOUND EDGES IN VITRO

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ABSTRACT

Cytochalasin B prevents both migration and wound-associated replication when applied to wounded monolayers of bovine endothelium in tissue culture. The normal low background rate of replication in undisturbed areas of the cultures is not inhibited by cytochalasin B. These results suggest that some form of movement may be required for initiation of wound-associated replication by endothelial cells.

KEY WORDS cell growth control · cell migration · tissue culture · 3T3 cells · contact inhibition of movement · density dependent inhibition of growth

When a wound is made in a cellular layer, both migration and proliferation are stimulated in the remaining cells (12, 39). This is true both in vivo and in vitro. This observation has led to the suggestion that movement and proliferation are causally related. In support of this, a wide range of culture conditions which stimulate proliferation is also associated with increased cell movement (2, 4, 6, 12, 17, 37, 39, 40, 43). On the other hand, it is also possible to design culture conditions which allow cells to move but do not permit replication (15, 23). This, of course, does not eliminate the possibility that movement is required before cells are stimulated to divide. Two studies have examined the effects of inhibitors of cell movement. Yarnell and Schnebli (45) found that colcemide prevented movement of Balb/c 3T3 cells into a wound but did not prevent the stimulation of growth seen when cultures were treated with insulin. They did not determine the effect of colcemide on cell replication at the wound edge itself. Stoker and Piggott (37) used cytochalasin B

to inhibit cell movement across a wound line in Balb/c 3T3 cultures. Even though movement was inhibited, cells near the wound edge were stimulated to synthesize DNA. The authors interpreted this experiment as evidence that initiation of replication at the wound edge is independent of the ability of cells to move away from the intact cell sheet. Balb/c 3T3 cells, however, represent an established, aneuploid cell line with a poorly defined cell of origin (38). In this paper, we present evidence that, in cultures of bovine aortic endothelium (BAE), a euploid cell strain, when movement at wound edges is inhibited by use of the drug cytochalasin B, the usual proliferative response is also inhibited.

MATERIALS AND METHODS

Materials

Bovine aortic endothelial cells were obtained as previously described (32). These experiments used cells which had undergone <15 doublings in vitro. Cells were maintained at 37°C in 4% CO_2/air. Unless otherwise stated, the growth medium was Waymouth's complete medium with 20% fetal calf serum, and cells were grown in 1.6-cm diameter multiwell plates (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.).

Wounding Procedure

For all experiments, cells were first grown to stationary density. This density, ~1.5 × 10⁵ cells/cm², was previously established as producing a population of cells that grows no further (32). Wounds were made by drawing laterally across the dish surface a fragment of a stainless steel razor blade (3). This marks the plastic slightly, indicating the edge of the intact sheet. Wells were rinsed once with phosphate-buffered saline (PBS) to remove nonadherent cells. Fresh medium was then added. For autoradiography, [³H]TdR (tritiated thymidine 6.7 Ci/mmol, New England Nuclear, Boston, Mass.) was added to cultures to a final concentration of 1 μCi/ml, from a 10× solution in tissue culture medium. [³H]TdR was given either for the duration of the experiment or for the 2 h before termination of the experiment. Autoradiograms were made using routine procedures (21, 33). The emulsion used was NTB2 (Kodak); exposure was for 1 wk; nuclei were stained with hematoxylin.

Cytochalasin B

Cytochalasin B (Aldrich Chemical Co., Milwaukee, Wisc.) was dissolved in dimethyl sulfoxide (DMSO) (2 mg/ml); 0.125% DMSO was added to control wells. The effective dose of cytochalasin B was determined by comparing inhibition of migration, using the wound system described above, with evidence of cytotoxic effects including rounding up or arborization of cells. Cells were plated in multiwells and grown to stationary density. Cytochalasin B was added after the cultures were wounded. At 20 h, distances of migration were measured and the percentage of cells showing cytotoxic effects was determined.

Effects of Cytochalasin B on Incorporation of [³H]TdR

Cells were plated at a density of 25 × 10³/cm² in multiwells and grown for 18 h. The medium was then changed to include cytochalasin B in doses shown on Fig. 3. [³H]TdR, 1 μCi/ml, was also added to the culture medium. A second set of wells also received cytochalasin B, but [³H]TdR was added only for the final 2 h of the incubation. At the end of 26 h, cells were briefly rinsed with 0.54 mM EDTA, 0.02 M Tris HCl (pH 7.4), then detached by a 20-min incubation in 0.5 ml of the rinsing solution plus trypsin (Difco Laboratories, Detroit, Mich.) 0.5 mg/ml, and transferred to a centrifuge tube. To this tube were added: a 0.5-ml phosphate-buffered saline rinse of the well, 0.5 ml of 2% bovine serum albumin in H₂O, and 1 ml of 5% trichloroacetic acid. The resulting precipitate was centrifuged for 10 min at 500 g, then washed three times with 1 ml of 5% trichloroacetic acid. The precipitate was dissolved in 1 ml of 0.1 N NaOH. An aliquot was mixed with 10 ml of Bray's solution (New England Nuclear) and counted for 10 min in a liquid scintillation spectrometer.

Quantitation of Results

Data were collected using a microscope with an eyepiece micrometer grid enclosing an area of 0.014 mm² at ×400. This system was used to measure cell density, thymidine index, and mitotic index. The cell density was determined by counting nuclei and is expressed as cells per cm². The labeling index is expressed as a percentage. For estimates of the mitotic index, cells in metaphase, anaphase, and early telophase were counted as mitotic cells. The rate of migration was determined by measuring the distance from the wound line out to the nucleus of the farthest migrated cell at several locations along the wound line.

In a set of experiments designed to reproduce the experimental conditions used by Stoker and Piggott (37), the locations of interest in cytochalasin B-treated cultures were the wound edge and the undisturbed monolayer. Nuclei in five random fields adjacent to the wound line and five random fields in the monolayer were observed. In controls, nuclei in five random 435 μm-diameter fields in the wound adjacent to the wound edge and in five random 0.014-mm² fields in the monolayer were counted. Six replicate experiments were observed.

All values are expressed as means ± standard error (SE). The statistical significance was tested by Student's unpaired t-test, or the c-test where applicable.

RESULTS

Cell Density Changes and Regeneration Zone

The sequence of changes following injury to the endothelial monolayer closely parallels events described for several other cell types in tissue culture (12, 17, 39, 40). BAE cells begin to spread processes into the wound area by ~4 h after wounding. By 8 h, cells have begun to move across the line of injury. By 22 h, over 80% of the leading cells are incorporating [³H]TdR as demonstrated by autoradiography (Fig. 1b). Except near the leading edge, cells in the regenerating sheet, as seen by phase microscopy, maintain contact with one another.

Cytochalasin B Dose Response

Doses of 0.5-1.0 μg/ml cytochalasin B stop cell movement in several cell types (1, 5, 37). Higher doses (10 μg/ml) cause nuclear extrusion (5), rounding up of cells, and an arborized cell morphology (1). For BAE, we found that migration was almost completely inhibited at a dose of 3 μg/ml cytochalasin B although significant movement remains at doses of 1-2 μg/ml (Fig. 2). We chose
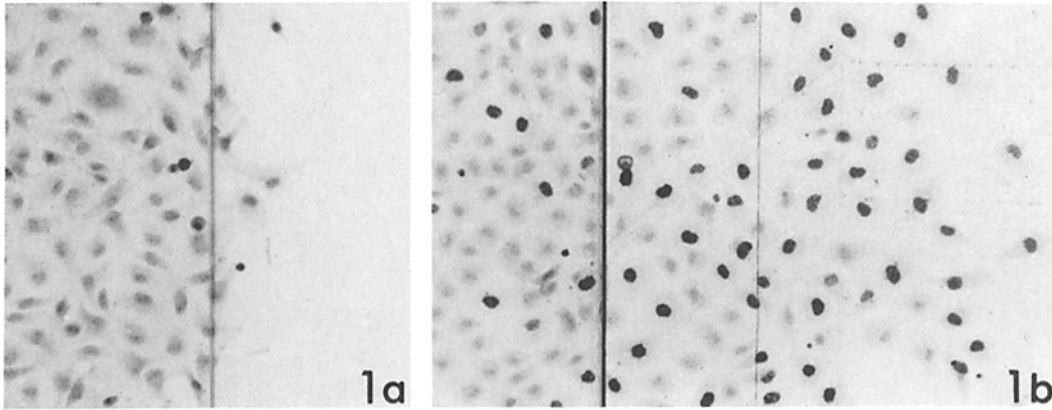
FIGURE 1 Cytochalasin B effect 22 h after wounding. (a) Cytochalasin B. The wound line is at the center of the figure. There is virtually no cell migration, and typically few cells are labeled. Autoradiogram of [³H]TdR incorporation. (b) Control. The wound line is in the left third of the figure. The highest proportion of labeled cells is toward the right, at the leading edge of the migration. Autoradiogram of [³H]TdR incorporation. × 130.

a dose of 2.5 μg/ml to minimize cell arborization and retain inhibition of migration.

Cytochalasin B Effect on Incorporation of [³H]TdR

For this experiment, cells were plated at preconfluent density (25 × 10³/cm²). After 18 h, the medium was changed to include cytochalasin B. [³H]TdR was added either at this time or 24 h later for the final two h of the 26-h exposure to cytochalasin B. Fig. 3 shows the dose-related inhibition of [³H]TdR uptake into BAE.

Cytochalasin B Effect on Labeling Index

Cytochalasin B was used to examine whether stimulation of DNA synthesis was related to cell migration. [³H]TdR was added at 20 h and cultures were fixed 22 h after wounding. In control cultures, 47.6% of cells that had entered the wound were labeled (Fig. 4a). There was virtually no migration in the cytochalasin B-treated cultures (Fig. 1a). As a result, we chose to measure the labeling index in the intact cell sheet within 120 μm of the edge. Only 6.5% of the cells in this zone were labeled (Fig. 4a). This was not significantly higher than the 3.6% labeling in undisturbed areas of the same cultures (Student's t-test [p > 0.05, n = 8]). The labeling index in intact, undisturbed areas of control cultures was 1.5%, not significantly different from the value for the same areas in cytochalasin B-treated cultures (p > 0.05, n = 8). In summary, cytochalasin B selec-
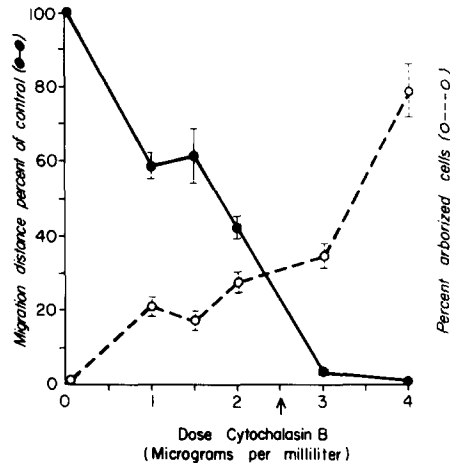

FIGURE 2 Cytochalasin B dose/response curve 20 h after wounding. Solid line: mean distance from the wound line reached by the farthest migrated cell in several 340 μm-wide segments along the wound edge, expressed as a percent of control (±SE). Dashed line: Change in morphology was determined by counting the number of arborized cells in several fields in undisturbed areas of the culture, expressed as a percent of cells observed (±SE). Each experiment was performed in two replicate wells.

tively inhibited the increase in labeling normally associated with the response of cells at the wound edge, whereas replication away from the wound edge was unaffected by this drug.

Fig. 4b shows the results of a second set of experiments closely following the conditions used
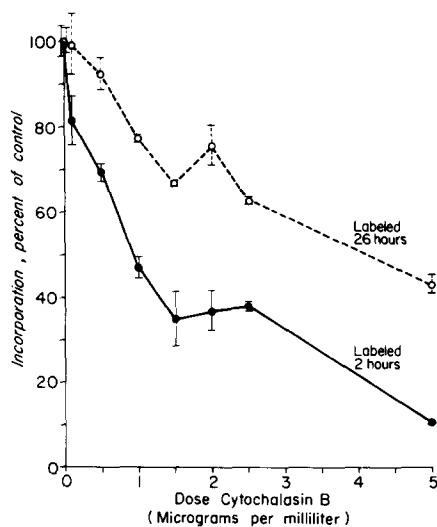
FIGURE 3 Effect of cytochalasin B on [³H]thymidine incorporation. Solid line: Cells were labeled for the final 2 h with 1 μCi/ml [³H]thymidine, 6.7 Ci/mM. Dashed line: Cells were labeled for the entire 26-h interval following addition of cytochalasin B. Acid-precipitable activity per culture is expressed as a percent of mean incorporation in three control cultures. Values for cytochalasin B-treated cultures are the mean of two replicate experiments (±SE).

by Stoker and Piggott to study Balb/c 3T3 cells (37). Their procedures differ from ours in two ways: first, the serum concentration used was 4% rather than 20%; second, cells were exposed to [³H]TdR from the time of wounding to termination rather than being labeled only during the final 2 h. Again, cytochalasin B had no apparent effect on labeling in the intact sheet.

Cytochalasin B Effect on Mitotic Index

Fig. 3 suggested the possibility that the absence of response to the wound edge in cytochalasin B-treated cultures might have been an artifact due to inhibition of S-phase uptake of [³H]TdR, as Everhart and Rubin found for Chinese hamster ovary cells (13). Changes in mitotic index were examined to determine whether the drug actually inhibited cell replication. Mitoses per 2,000 cells were counted in two replicate cultures sacrificed at 30 h. The mitotic index within 120 μm of the wound edge in cytochalasin B-treated cultures was 0.3 ± 0.12%. This is significantly less than the mitotic index of 1.6 ± 0.3% found in the wound in control cultures (p < 0.05). In undisturbed fields >4 mm from the wound edge, there were
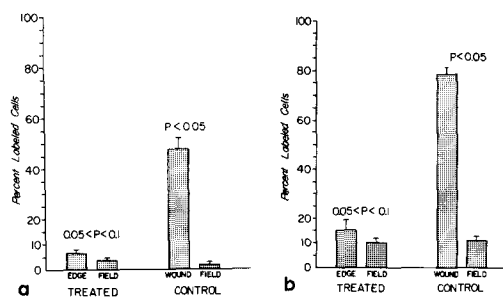

FIGURE 4 Cytochalasin B effect on [³H]thymidine labeling index. In cytochalasin B-treated cultures, there was no migration, therefore counts were made in fields immediately adjacent to the wound edge (EDGE). In control cultures, cells had migrated into the wound; the average labeling of cells in the wound was determined (WOUND). In treated and control cultures, the labeling was quantitated in undisturbed areas >4 mm from the wound edge (FIELD). Results are expressed as means ±SE. (a) 22 h after wounding. Cultures were [³H]TdR-labeled for the final 2 h (1 μCi/ml, 6.7 Ci/mM [³H]TdR), four replicate wounds. (b) 24 h after wounding. Cultures were labeled for 24 h with the same dose of [³H]TdR, six replicate cultures.
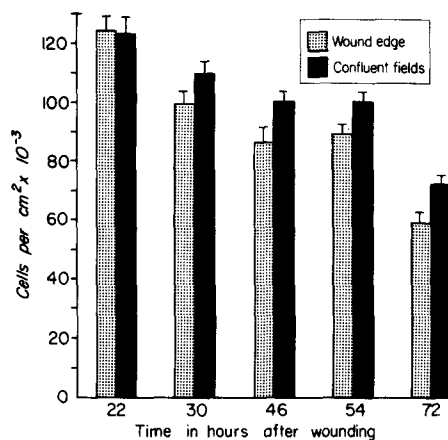

FIGURE 5 Cell densities in cytochalasin B-treated cultures. Cell densities in cytochalasin B-treated cultures are plotted as a function of time after wounding and addition of fresh medium containing cytochalasin B in dimethyl sulfoxide or dimethyl sulfoxide alone. Overall cell density decreased with time, presumably due to the arborization effect (see Fig. 2). Cell densities were measured in twenty 0.014 mm² fields adjacent to the wound edge or in random fields >4 mm from the wound edge in two replicate cultures at each time point. Results are expressed as means ±SE.

0.18 ± 0.1% mitotic cells in control cultures and 0.15 ± 0.09% mitotic cells in cytochalasin B-treated cultures (p > 0.05). There was no signifi-

cant difference between the mitotic indices of cells in the undisturbed fields and those of cells near the wound edge in cytochalasin B-treated cultures ($p > 0.05$).

A third independent measure of response to the wound is cell density change. If cells at the wound edge were proliferating but not migrating, there would be an increase in nuclei per unit area in the cell sheet adjacent to the wound edge. Fig. 5 shows cell density in the field and at the wound edge at five time points from 22–72 h in cytochalasin B-treated cultures. In none of the cultures does edge cell density exceed field cell density. Overall, cell density decreases with time, perhaps reflecting the arborization phenomenon described above.

Reversibility of Cytochalasin B Effect

We performed an experiment to determine the reversibility of the effects of cytochalasin B. Cultures were wounded as usual, then 2.5 μg/ml cytochalasin B in medium containing 20% fetal calf serum was added. After 20 h, these cultures were rinsed three times with fresh medium, then were fed medium without cytochalasin B. Parallel unwounded cultures not exposed to cytochalasin B were wounded at this time to serve as controls. Cultures were labeled for 2 h with [^{3}H]TdR before fixation at 2, 10, and 24 h after removal of cytochalasin B and wounding of the control cultures. The maximum distance of migration was indistinguishable when treated-released cultures were compared to control cultures at the final, 24-h labeling point ($440 \pm 14,428 \pm 28$ μm, $p > 0.05$, n = 6). An increase in labeling index was not detected in cells at the wound edge or on the wound surface at 2 or 10 h, but the labeling index at 24 h was increased in both cultures (cytochalasin B: 50.33 ± 1.4; control: 50 ± 2.1; $p > 0.05$, n = 6).

DISCUSSION

When endothelium in tissue culture is treated with cytochalasin B, both migration and proliferation are inhibited at wound edges. In contrast, the conclusion from similar experiments with other cell types has been that initiation of proliferation is not dependent on the ability of cells to move.

Effects of Cytochalasin B

Cytochalasin B has several effects other than inhibition of cell movement. At sufficient concen-trations, cytochalasin B inhibits uptake of [^{3}H]TdR by cells entering S-phase (13), causes nuclear extrusion and changes in cell shape (1), and prevents cytokinesis (5, 31). We have confirmed these effects for BAE (Figs. 2 and 3). In addition, cytochalasin B has been shown to inhibit transport of simple sugars such as 2-deoxyglucose and glucosamine (20, 26), to affect membrane receptor mobility (27), and to inhibit endocytosis (1, 10).

The most important of these actions of cytochalasin B in the present study is the effect on uptake of [^{3}H]TdR. Fig. 4 shows that cytochalasin B did not decrease the labeling index in the intact, undisturbed monolayer. This is not surprising since the thymidine labeling index is a measure of the number of labeled cells rather than the amount of label incorporated per cell. Thus, the decreased rate of incorporation of [^{3}H]TdR does not appear to have affected the estimate of numbers of replicating cells. The mitotic index data and the cell density data for wound edges in cytochalasin B-treated cultures (Fig. 5) confirm the labeling index data and show that there is selective inhibition of proliferation at wound edges in cytochalasin B-treated cultures (Fig. 4).

If we assume that this effect of cytochalasin B is a result of its effect on the cells' motile apparatus, it is important to note that the inhibition of replication at wound edges could be the result of drug action on some form of movement other than movement of cells themselves. For example, cytochalasin B has been shown to inhibit endocytosis (1, 10). Increased endocytosis is found in relation to initiation of proliferation including the increased proliferation at the edges of cell sheets (9, 41, 42), and it is reasonable to assume that endocytosis may be required for the utilization of medium components required for growth. Alternatively, movement might be related to cell replication via changes in cell shape (14) or via as yet undefined changes in the distribution of cell surface elements. Finally, if movement of the cells themselves is required for replication at wound edges, this raises the possibility that cell contacts are involved. Studies both in vivo (24) and in vitro (44) have suggested that cell contact or direct interaction between cells may be important in growth control. This, however, can not be the only mechanism of density-dependent inhibition of growth, since cells may become density inhibited even when they are not in contact (16), and there is a poor correlation between the extent

of cell contact and the probability of an individual cell's dividing (25). On the other hand, Whittenberger and Glaser recently reported that plasma membranes isolated from 3T3 cells were able to inhibit replication of this cell type (44). Thus, the issue of growth control by some form of interaction between cell surfaces remains open.

Comparative Biology of 3T3 and BAE

The differences between our results and those reported by Stoker and Piggott (37) could be due to the cell types studied. The 3T3 cell lines used in earlier studies are aneuploid with unlimited replicative lifespan. The cells were originally derived from whole mouse embryos, and the exact cell types from which these strains derive are not known (38). In contrast, BAE has a normal karyotype, has a limited replicative lifespan, retains immunochemical markers distinctive for endothelial cells, and grows as a monolayer sheet in tissue culture similar in morphology to the same tissue in the intact animal (32). Thus, BAE may be a better model for normal tissues.

Endothelium also differs from 3T3 cells in its sensitivity to growth factors. If medium containing 30% serum is changed frequently, Swiss 3T3 cells pile up on top of each other, growing to ten times confluent density (19). The saturation density of Balb/c 3T3 cells depends on the serum concentration (11). Unlike 3T3 cells, confluent endothelial cells do not proliferate in response to frequent changes of fresh medium (18), and saturation density is not dependent on serum concentration (35). Moreover, growth of endothelial cells is independent of the presence or absence of epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin, or the platelet-derived growth factor (30, 35, 43). These factors have a strong effect on the growth of 3T3 cells. Dependence on growth factors may be an important determinant of how growth is controlled in some cell types but not in others. For example, Dulbecco (11) has described a number of differences in growth properties of two epithelial lines, BSC-1 and CV-1, as compared with 3T3. In particular, the cell density of the epithelial lines at saturation density was independent of serum concentration, while the saturation density of 3T3 cells was a function of the amount of serum present. Thus, growth control in 3T3 cells may well be dependent on availability of growth factors, while growth control in the epithelial lines or endothelium may depend

more on some as yet undefined interaction between cells. The role of cell movement in growth control in epithelial cell lines is not yet known.

Comparison with Studies In Vivo

The availability of endothelium in culture offers a valuable opportunity to compare results of experiments in vivo with those in vitro. We have recently presented data from an in vivo system, the aortic endothelium of the intact rat (34). In that system, the response to wounding included increased replication as far as 100 cells back from the wound edge, consistent with the results of a number of studies of wounded epithelia in vivo (7, 8, 22, 28, 29). In addition, regenerating cells established a hyperplastic zone with cell densities two to three times normal (34). In contrast, in our in vitro studies, only those cells migrating onto the wound surface were labeled with [³H]TdR, and there was no hyperplastic zone. These results are identical to those obtained with other cell types studied in culture (36, 39, 40). Differences between earlier in vivo and in vitro studies may reflect differences in cell types studied since most in vivo studies used epithelia while in vitro studies used 3T3 cells or fibroblasts. Our studies, however, used aortic endothelium both in vivo and in vitro. The differences between results in vitro and in vivo may reflect special properties of cultured cells or problems of methodology. For example, the wound line in vitro can be marked on the substrate; in vivo there is no way of marking the substrate, and some method must be used to estimate the location of the line of injury. There are also obvious differences in the composition of the medium, the nature of the substrate, and the presence or absence of flow. At present, the most we can do is express caution in the interpretation of any results in vitro.

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