

Growth Factors Are Released by Mechanically Wounded Endothelial Cells

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Abstract. Growth factors may be required at sites of mechanical injury and normal wear and tear *in vivo*, suggesting that the direct action of mechanical forces on cells could lead to growth factor release. Scraping of cells from the tissue culture substratum at 37°C was used to test this possibility. We show that scraping closely mimics *in vitro* both the transient plasma membrane wounds observed in cells subject to mechanical forces *in vivo* (McNeil, P. L., and S. Ito. 1989. *Gastroenterology*. 96:1238-1248) and the transient plasma membrane wounds shown here to occur in endothelial cells under normal culturing conditions. Scraping of endothelial cells from the culturing substratum released into the culture medium a potent growth-promoting activity for Swiss 3T3 fibroblasts. Growth-promoting activity was released rapidly (within 5 min) after scraping but was not subsequently degraded by the endothelial cells for at least 24 h thereafter. A greater quantity of growth-promoting ac-

tivity was released by cells scraped 4 h after plating than by those scraped 4 or 7 d afterwards. Thus release is not due to scraping-induced disruption of extracellular matrix. Release was only partially cold inhibitable, was poorly correlated with the level of cell death induced by scraping, and did not occur when cells were killed with metabolic poisons. These results suggest that mechanical disruption of plasma membrane, either transient or permanent, is the essential event leading to release. A basic fibroblast growth factor-like molecule and not platelet-derived growth factor appears to be partially responsible for the growth-promoting activity. We conclude that one biologically relevant route of release of basic fibroblast growth factor, a molecule which lacks the signal peptide sequence for transport into the endoplasmic reticulum, could be directly through mechanically induced membrane disruptions of endothelial cells growing *in vivo* and *in vitro*.

POLYPEPTIDE growth factors activate cell growth, motility, and a variety of other processes in cells *in vitro*. While in most cases their role *in vivo* remains to be established definitively, it is clear that growth factors are potential molecular mediators of (a) repair of injured tissues (for reviews see Huang et al., 1988; Banks, 1988; Fox, 1988; Assoian, 1988); and (b) cell renewal in those tissues, such as gut and skin epithelium, where the rate of cell turnover is normally very rapid. Any mechanism to explain such putative growth factor functions *in vivo* must identify the specific growth factor released, the cell types that release the growth factor, the cell types that respond to it, the stimulus for release, and the cellular route of release. It should also explain how release is accurately targeted to the injured site and/or appropriate tissue.

Endothelial cells are known to synthesize both basic fibroblast growth factor (bFGF)¹ and PDGF *in vitro*, but undisturbed cultures release only PDGF at readily detectable

levels into their culturing medium (Vlodavsky et al., 1987). Moreover, no stimuli promoting bFGF release *in vitro* have been identified to date, although several are now known which promote release of PDGF (for reviews see DiCorleto and Fox, 1988; DiCorleto et al., 1987). Indeed, the cellular and molecular mechanisms for release of bFGF, acidic fibroblast growth factor (aFGF), and also interleukin-1 (IL-1) from cells have not been elucidated: bFGF (Abraham et al., 1986), aFGF (Jaye et al., 1986), and IL-1 (Auron et al., 1984; Lomedico et al., 1984) all lack the signal peptide sequence normally considered a prerequisite for protein secretion via the exocytotic pathway within a membrane-bound compartment (for review see Walter and Lingappa, 1986).

There has recently been much speculation that bFGF, aFGF, and IL-1 could be released from "dead," "damaged," or "injured" cells by "leakage" or "lysis" (Young et al., 1988; Klagsbrun and Vlodavsky, 1988; Thomas, 1988). In fact, the concept of a growth-promoting "wound hormone" released by cells upon tissue injury is not a new one (cf. Abercrombie, 1957). However, there is no direct experimental evidence that addresses these possibilities or better defines the potential mechanisms and nature of the cell injuries that might promote the postulated leakage, death, and damage. An *in vitro* model for accurately mimicking the wounds or injuries

1. *Abbreviations used in this paper:* aFGF, acidic fibroblast growth factor; BAEC, bovine aortic endothelial cells; BCEC, bovine capillary endothelial cells; bFGF, basic fibroblast growth factor; CMF PBS, calcium- and magnesium-free PBS; DB, DME containing 0.1% BSA; FDx, FITC-labeled dextran; HRP, horseradish peroxidase; IL-1, interleukin-1.

likely to be experienced by cells *in vivo* will be required for directly addressing these questions.

Mechanical forces that could act to disrupt plasma membranes could provide both a biologically relevant stimulus for and a mechanism of bFGF release from endothelial cells. Cells grown in culture are apparently rigorously protected from mechanical forces. This is not the case for cells *in vivo*, where mechanical forces can destroy tissue integrity at sites of severe traumatic injury, causing lethal wounds to many cells. Cells exposed to less severe mechanical forces in mildly injured or in undisturbed but motile tissues *in vivo* might also be wounded at their plasma membranes. However, such membrane wounds would probably not be detected by conventional morphological methods because of the cell's seemingly well-developed ability to reseal and thus survive them.

Using novel methods for detecting cell membrane wounding *in vivo*, we found that many cells in lightly traumatized intact gut tissue are wounded but survive wounds to their plasma membrane (McNeil and Ito, 1989). Such cells are thus transiently permeabilized by the injury. More importantly, we also provided evidence that mechanical forces generated by normal gut motility alone can wound individual cells of gut epithelium. We have proposed that membrane disruption or wounding is a normal and common, but overlooked, event in certain organs and tissues *in vivo*. We also propose that its occurrence suggests a previously unrecognized route for molecular traffic into and out of cell cytoplasm that bypasses the conventional endocytotic and exocytotic membrane-bound pathways (McNeil and Ito, 1989).

In this paper, we have explored the possibility that release of bFGF by endothelial cells might be through membrane disruptions such as those we have documented for cells *in vivo*. Here we provide evidence that endothelial cells can be wounded and that they reseal these membrane wounds under normal culturing conditions. We compare scrape-loading (McNeil et al., 1984) as a method for rapidly, reproducibly, synchronously, and efficiently wounding endothelial cell membranes *in vitro* with other techniques for disrupting membranes, such as freezing and thawing or sonicating, and conclude that scrape-loading appears to mimic closely the mechanically induced membrane wounds we have observed *in vivo* and *in vitro*. Using scrape-loading as an *in vitro* model, we show that growth factors, including a bFGF-like molecule, are released from endothelial cells upon mechanical wounding of their plasma membranes.

Materials and Methods

Cell Culture

Bovine aortic endothelial cells (BAEC-11), a gift from Bill Atkinson (Brigham and Women's Hospital, Boston, MA), were used from passage 24 to 39. Bovine capillary endothelial cells (BCEC) were used from passage 11 to 15. Swiss 3T3 (CCL 92), *Potorous tridactylis* kidney cells (PtK-2) (CCL 56), and human embryo lung fibroblast-like cells (MRC-5) (CCL 171) were obtained from American Type Culture Collection (Rockville, MD). Madin-Darby canine kidney (MDCK) cells were a gift from Karl Matlin (Harvard Medical School, Boston, MA).

Cells were cultured at 37°C in 75-cm² flasks in Eagle's Basal Medium (MRC-5 cells) or in DME (all other cell types) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin (except MRC-5 cells), a crude extract of retinas (BCEC) (Gitlin and D'Amore, 1983), and 10% (vol/vol) calf serum (Swiss 3T3 cells and BCEC) or FBS (all other cells). All media and supplements were obtained from Gibco Laboratories (Grand Island, NY).

Cultures were passaged at confluence with trypsin-EDTA in Hanks' balanced salt solution (Gibco Laboratories). For experiments, cells were plated into 35-mm (1.15 × 10⁵ cells for BAEC and BCEC; 10⁵ cells for all others), 60-mm (3.4 × 10⁵ cells) dishes, or 24-well plates (2.1 × 10⁴ cells for Swiss 3T3 cells; 10⁴ cells for BAEC and BCEC).

Wounding by Scraping at 37°C

7d after plating, endothelial cells to be used for medium conditioning were washed twice with DME followed by incubation in DME containing 0.1% BSA (DB) for 15 min at 37°C, unless otherwise stated. The cells were then scraped from the substratum with a rubber policeman in the presence of DB. A "1×" concentration of conditioned medium will be defined in this paper as 2 ml of DB conditioned by a 35-mm dish of confluent endothelial cells. Wounded cells were allowed to condition the medium for 20 min at 37°C after which the suspension was centrifuged at 2,500 rpm at 20°C for 5 min. The pellet was discarded and the supernatant was assayed for growth-promoting activity. Control cultures left undisturbed in their dishes were allowed to condition DB for 20 min. This medium was then assayed for growth-promoting activity (generally negligible) and the counts per minute obtained were subtracted from those of experimental cultures.

Chemical Induction of Cell Death

Cells were rinsed as above and treated in one of the following three ways with DB containing 3 mM sodium arsenite and 10 mM iodoacetate (both from Sigma Chemical Co., St. Louis, MO): (a) cells were incubated in poison for 1.5 h; (b) cells were incubated for 1.5 h in the poison and then scraped in its presence; and (c) cells were rinsed and incubated in DB as usual followed by scraping in the presence of the poison. As a positive control cells were scraped in the presence of DB. The supernatant (equivalent to a 4× concentration) from each treatment was collected and dialyzed against three changes of DME (Spectrapor dialysis tubing; 6,000–8,000 mol wt cut off). As controls for dialysis, samples of DB plus poison and of DME plus 10% calf serum plus poison were also dialyzed. The samples were then sterile filtered and assayed for growth-promoting activity.

Growth Activity Assay

Swiss 3T3 cells were washed 6 d after plating with DME and incubated in DB for 24 h. Conditioned medium or control medium was added to Swiss 3T3 cells (1 [24-well plates] or 2 ml [35-mm dishes]). Positive controls received an equivalent volume of DME plus calf serum and negative controls received DB. ³H-Thymidine (New England Nuclear, Boston, MA) (1 [35-mm dish] or 0.2 μCi [24-well plate]) was added and the cultures were incubated for 24 h. They were then washed twice with PBS and incubated twice in 5% TCA for 5 min each. TCA-insoluble material was harvested in 0.8 ml of 0.25 M NaOH (DiCorleto, 1984), transferred into scintillation vials, mixed with 5 ml Aquasol scintillation cocktail (DuPont Co., Wilmington, DE), and samples were counted in a scintillation counter (model LS 3801; Beckman Instruments, Inc., Fullerton, CA).

Assessment of Cell Viability

Aliquots of cell suspensions of wounded cultures were diluted two-fold with a 5-mg/ml solution of fluorescein diacetate (Sigma Chemical Co.). Viable cells were fluorescent. A total of 80–90 cells was counted and the percentage of viable cells was calculated.

Autocrine Response of Endothelial Cells

BAEC or BCEC were cultured for 7 and 4 d, respectively, in 35-mm dishes coated with 1.5% gelatin (Difco Laboratories Inc., Detroit, MI). Just before wounding, the medium was removed and replaced with fresh DME plus calf serum to give a 1× concentration of conditioned medium. Cultures were scraped, the cell suspension was centrifuged, and the supernatant was collected. Endothelial cell proliferation was assayed as an increase in cell numbers in subconfluent cultures as previously described (Klagsbrun and Shing, 1985). Briefly, DME containing 10% calf serum was added to the conditioned supernatant to give dilutions ranging from 5 to 100% of a 1× concentration of conditioned media, and 0.5 ml was placed onto cultures of BAEC or BCEC plated 1 d previously in 24-well gelatin-coated plates at 10⁴

cells/well. Control conditioned medium was taken from cultures treated similarly, but not wounded. The cells were incubated for 4 d at 37°C and were then counted using a Z_f counter (Coulter Electronics Inc., Hialeah, FL).

Heparin-Sepharose Affinity Chromatography

Sufficient cells (60-mm dishes) were wounded by scraping at 37°C in DB to give a 10× concentration of conditioned medium. The conditioned medium (2 ml) was added to 0.8 ml of wet-packed heparin-Sepharose (lot OC 05836; Pharmacia Fine Chemicals, Uppsala, Sweden), and the slurry was agitated at 4°C for 3 h before its addition to the column. Growth factor was eluted stepwise at 4°C with 0.1, 0.5, 1, and 2 M NaCl–0.01 M Tris, pH 7 (Schweigerer et al., 1987). Fractions (1 ml) were collected at a rate of 40 ml/h, dialyzed against PBS overnight and then against DME for 4 h, sterile filtered, and assayed for growth-promoting activity.

Detection of Cell Wounding during Endothelial Cell Trypsinization

FITC-labeled dextran (FDx) (9,000 mol wt, 20 mg/ml; Sigma Chemical Co.) in trypsin-EDTA was dialyzed for 48 h against Hanks' balanced salt solution plus 0.53 mM EDTA. FDx in PBS was dialyzed against PBS.

Experimental cultures received the trypsin-FDx until the cells rounded up, as much trypsin-FDx was removed as possible by aspiration, and any remaining adherent cells were freed by tapping the culturing flask on a solid object. The suspended cells were then washed twice in DME plus FBS by centrifugation. Part of the final suspension was plated onto coverslips and allowed to spread. The remainder was kept on ice in suspension for flow cytometry. Controls received FDx in PBS for the same length of time as the experimentals were in contact with trypsin-FDx. They were then washed five times with PBS, trypsinized, resuspended, washed, and treated as above.

Detection of Cell Wounding during Cell Migration

BAEC were plated onto coverslips for 7 d. Denuded zones were made on confluent cultures using a rubber policeman and the cells were allowed to recover in DME containing FBS for 1 h. FDx (20 mg/ml) in DME containing FBS, dialyzed against DME for 48 h, was added onto each wounded culture for 1, 4, or 24 h. Washing of cultures before microscopy was done with great care to avoid injuring cells in the presence of FDx, since this could cause artifactual, injury-induced labeling with FDx. Cultures were washed in three changes of PBS before observation of cells by fluorescence microscopy.

Fluorescence Microscopy

Cells on coverslips were washed thoroughly in warm PBS, observed in a "wet mount" by fluorescence microscopy using a photomicroscope (III; Carl Zeiss, Inc., Thornwood, NY) equipped with 16, 40, and 63× phase-contrast objectives (Neofluor; Carl Zeiss, Inc.), and photographed on T-Max 400 film (Eastman Kodak, NY).

Electron Microscopy and Staining for Horseradish Peroxidase (HRP)

Endothelial cells scraped from the substratum or frozen and thawed in the presence of exogenously added HRP (20 mg/ml; Boehringer Mannheim GmbH, Mannheim, FRG) were washed free of unincorporated HRP (three times by centrifugation in 37°C PBS), fixed (as a pellet), and processed for electron microscopy as previously described (McNeil and Ito, 1989). HRP staining was developed after the primary fixation in aldehyde as previously described (Adams, 1977). Sections were examined without lead or uranyl acetate staining to maximize visibility of staining due specifically to HRP.

Flow Cytometry

Flow cytometric measurements and analysis were performed in a flow cytometer (EPICS V; Coulter Electronics Inc.; Dana Farber Cancer Institute) as previously described (McNeil and Warder, 1987).

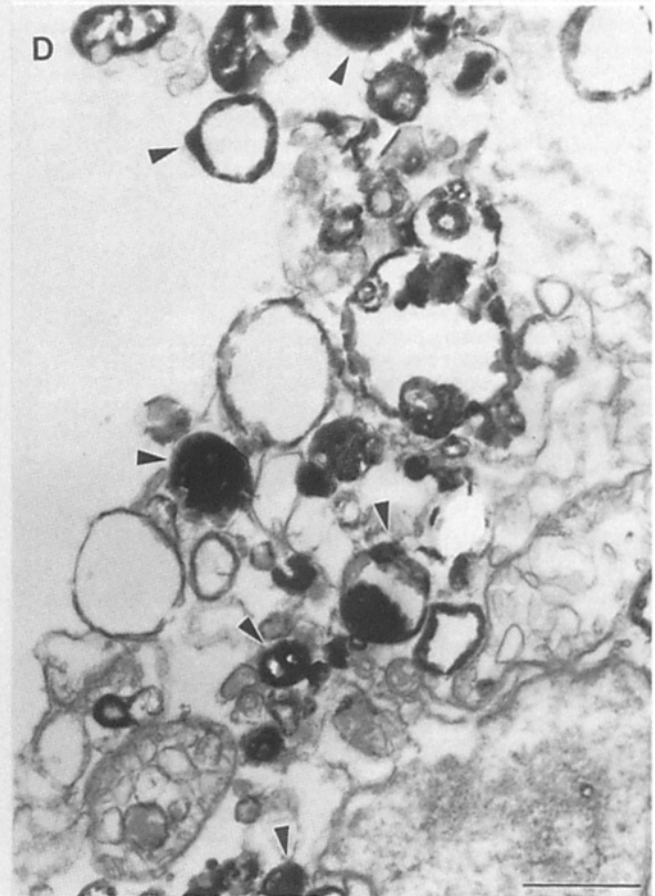
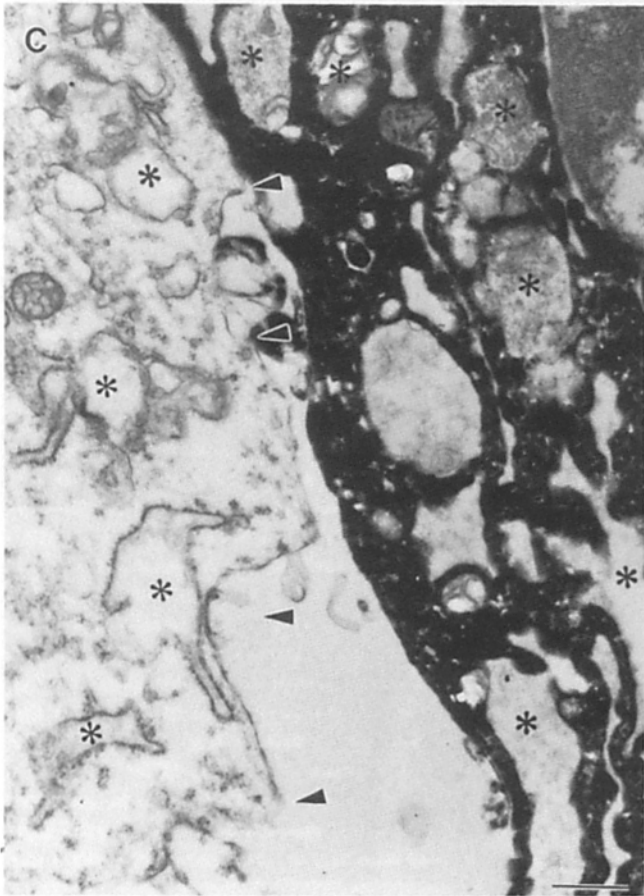
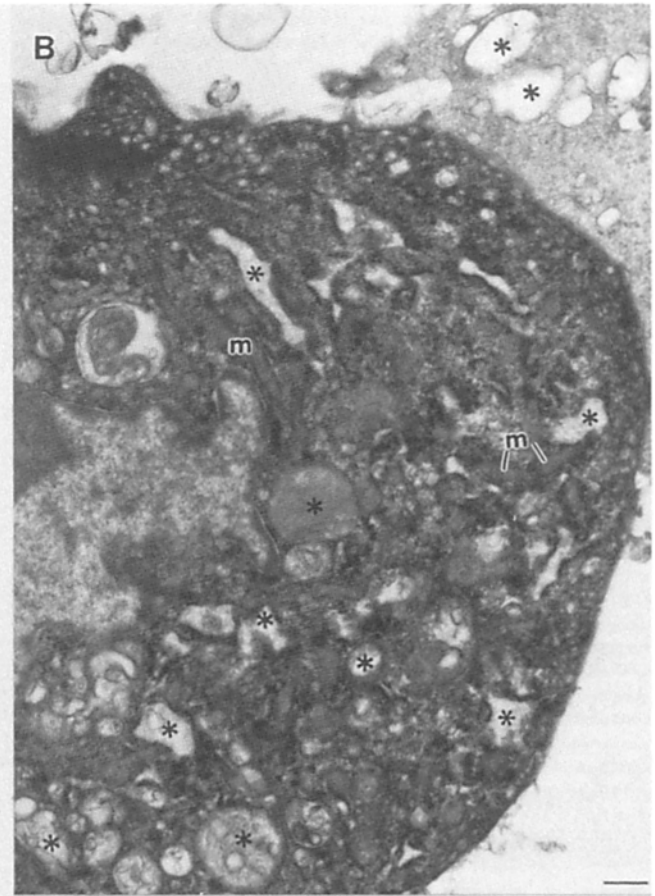
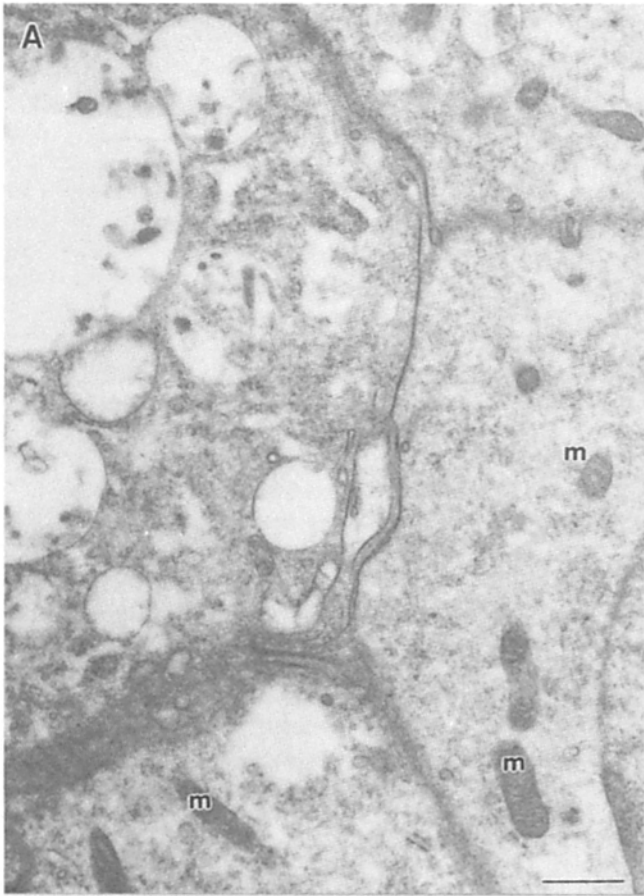
Results

The Scrape-loading Model for Cell Wounding

Mechanical trauma of tissues *in vivo* results both in irreversible and transient disruption of cell plasma membranes, while organellar membranes within dead or within wounded but surviving cells, appear not to be disrupted (McNeil and Ito, 1989; McNeil, P. L., S. Ito, and R. Chen, unpublished observations). We have based our selection of an *in vitro* model for studying growth factor release from mechanically wounded cells upon these *in vivo* observations which used HRP as a marker for cell wounding.

The technique of scrape-loading, in which cells growing *in vitro* are scraped off of their culturing substratum with a rubber policeman, appeared to be a good model for cell wounding *in vivo*. In contrast to cells trypsinized in the absence of HRP (Fig. 1 A), most endothelial cells scraped from the substratum in the presence of HRP were observed to be stained cytoplasmically with this electron-dense marker (Fig. 1 B). However, HRP staining was not observed within the organelles of scraped cells. Occasionally lysed cells were observed that were entirely unstained. When cells were scraped in cold medium, their organelles were similarly unstained, despite the fact that most cells were clearly irreversibly lysed by this treatment (Fig. 1 C). Scraping of cells in cold medium has been used to prepare irreversibly permeabilized cell models that retain organellar function (Beckers et al., 1987). In its effects on plasma and organellar membranes and on cell viability, scrape-loading therefore clearly resembles the effect of mechanical forces acting to wound cells *in vivo* (McNeil and Ito, 1989). This is perhaps not surprising: in both cases disruptive mechanical forces are exerted primarily on the plasma membrane.

Freezing and thawing, often followed by sonication and extraction in high salt (1 M NaCl), has been the most common biochemical technique used to release both PDGF and bFGF from endothelial cells (Vlodavsky et al., 1987; Schweigerer et al., 1987; DiCorleto et al., 1983). It was formally necessary, therefore, to ask whether freezing and thawing is a valid model for cell wounding *in vivo*. Cultures of endothelial cells were frozen and thawed three times in the presence of HRP and, 5 min after the last thaw at 37°C, they were prepared for electron microscopy. By comparison with trypsinization (Fig. 1 A), it was obvious that freezing and thawing (Fig. 1 D) killed or irreversibly permeabilized 100% of the endothelial cells. HRP was not trapped within what appeared to be the former cytoplasmic domain of frozen and thawed cells. Indeed, this was not surprising since it was often impossible to distinguish the plasma membrane from other membranes of these irreversibly permeabilized cells and no cytoplasmic matrix remained in association with the cellular remnants remaining after freezing and thawing. HRP was, however, present in numerous disrupted cellular vesicles, a clear indication that many membranous compartments had been permeabilized by the freezing and thawing but had resealed, trapping HRP in their lumen (Fig. 1 D). The membranes of those organelles not containing HRP were often clearly discontinuous. In line, therefore, with its accepted and widespread biochemical applications, our results indicate that freezing and thawing of cells indiscriminately disrupts plasma and organellar membranes and is unfailingly le-



thal. Thus freeze-thaw is a poor model with which to examine the consequences of cell wounding as it occurs in vivo at sites of traumatic injury or in undisturbed tissues, such as gut.

Endothelial Cells Are Wounded under Normal Culturing Conditions

Endothelial cells cultured in vitro condition their own culture medium with essential growth factors, including bFGF (Sato and Rifkin, 1988). We therefore asked whether endothelial cells are wounded under normal culturing conditions since this might be a mechanism for release of bFGF and possibly other growth factors by these cells in vitro. We looked first for endothelial cell wounding during trypsinization. Endothelial cells were trypsinized as usual but in the presence of FDx (9,000 mol wt), which could act as a marker for transient membrane wounding. Control cells received FDx for an equal interval of time before addition of trypsin, the FDx was washed off, and the control cells were then trypsinized in its absence. Among those endothelial cells trypsinized in the presence of FDx, many of those observed 1–24 h later after plating and spreading on a coverslip were lightly to intensely fluorescent. The latter could easily be photographed on 35-mm film, and it was clear from the uniform distribution of this fluorescence that its location was cytoplasmic (Fig. 2, *A* and *B*). The control cells were less frequently, but occasionally, labeled uniformly in the cytoplasm (Fig. 2, *C* and *D*). All cells in both groups also displayed the punctate fluorescence indicative of pinocytosis of the FDx. To determine the proportion of the cells wounded by trypsinization (i.e., uniformly fluorescent throughout their cytoplasm), we used flow cytometry to measure the fluorescent intensity of 20,000 cells from each population. A fluorescence intensity threshold was set below which 95% of the control cells fell. 23% of the trypsinized cells fell above this threshold, indicating that they had been wounded by trypsinization (Fig. 3). Thus, a large proportion of endothelial cells are wounded as a result of trypsinization, during which their cytoplasm becomes accessible to exogenous, membrane-impermeant probes and during which time cytoplasmic molecules would, presumably, be free to diffuse out.

Endothelial cells in vitro also apparently require, for migration into a zone of denuded substratum, that bFGF be continuously released into medium (Sato and Rifkin, 1988). We therefore asked whether the plasma membranes of endothelial cells are somehow transiently disrupted during such migratory activity. A confluent culture of endothelial cells was wounded with a rubber policeman to produce zones

of substratum denuded of cells. Membrane wounds are suffered by the majority of the cells remaining along the border of such monolayer wounds (Swanson and McNeil, 1987). We therefore waited 1 h before replacing normal culture medium with medium containing FDx (9,000 mol wt) so that such scraping-induced membrane wounds to individual cells would have ample time to reseal. At various intervals thereafter, the cultures were washed free of exogenous FDx and examined by fluorescence microscopy. Motile cells were reproducibly observed to be heavily labeled in their cytoplasm with FDx at 4–24 h after culture wounding (Fig. 4, *A* and *B*). However, the proportion of cells so labeled was very low. Because of the high background of cellular fluorescence imparted by the punctate, pinosomal fluorescence within all cells, it was not possible to identify those cells that were labeled lightly in their cytoplasm as well as heavily in their pinosomes or to quantitate the proportion of cytoplasmically labeled cells by flow cytometry. In conclusion, therefore, endothelial cell wounding during migration does occur, but is either a very rare event or one not readily detectable by our methods.

Scrape-loading Releases a Potent Growth-promoting Substance from Endothelial Cells

The medium conditioned by endothelial cells after scraping them from their substratum contained a potent growth-promoting substance for Swiss 3T3 fibroblasts (Fig. 5). Indeed, the activity derived from 1 ml of medium conditioned by one 35-mm dish of confluent endothelial cells was nearly as active as 10% calf serum in stimulating DNA synthesis and more active than optimal doses of purified bFGF. Release clearly depended on cell wounding by scraping, as shown by the low level of growth-promoting activity in medium incubated with control, undisturbed cultures for 5 min, 3 h, or 24 h (Fig. 6, *A*, *C*, and *E*, respectively). Release of growth-promoting activity by mechanically wounded cells occurred rapidly: it could be harvested at maximal levels within 5 min after scraping (Fig. 6 *B*). Growth-promoting activity appeared not to be degraded by the endothelial cells after wounding: there was little difference in the growth-promoting activity harvested from cultures 5 min, 3 h, or 24 h (Fig. 6, *B*, *D*, and *F*, respectively) after cell wounding.

Compared with cells scraped in the warm medium (Fig. 7 *A*), it was clear that scraping of cells in cold medium (4°C) also released a significant amount of growth-promoting activity (Fig. 7 *B*). The growth-promoting activity released was greatest if, after scraping in the cold, the cells were allowed to warm up to 37°C for 5 min (Fig. 7 *C*), but not if

Figure 1. Scraping of cells from the culturing substratum disrupts plasma but not organellar membranes. Endothelial cells were trypsinized from the substratum (*A*) or in the presence of exogenously added HRP were scraped substratum at 37°C (*B*), at 4°C (*C*), or frozen and thawed three times (*D*). *A* illustrates the electron microscopy appearance of endothelial cells not stained with HRP. Note for comparison that organelles such as mitochondria (*m*) are more darkly stained than surrounding cytomatrix. *B* and *C* illustrate the heavy cytoplasmic, but not intraorganellar, staining pattern of cells scraped in HRP. Organelles (*), even mitochondria (*m*), are less darkly stained than the surrounding cytomatrix of these cells. While cells scraped at 37°C often had normal electron microscopy morphologies (*B*), those scraped in the cold but returned for 20 min to 37°C before fixation invariably had highly abnormal morphologies (*C*), some even lacking a normal density of cytomatrix (region to the left of arrowheads). However, even in such cases HRP was not incorporated into the “naked” organelles. *D*, by contrast, illustrates the heavy labeling within many of the vesicular, membrane remnants of frozen and thawed cells (*arrowheads*). Those membranous remnants not labeled with HRP in frozen and thawed cells were often clearly discontinuous. Bar, 1 μm.

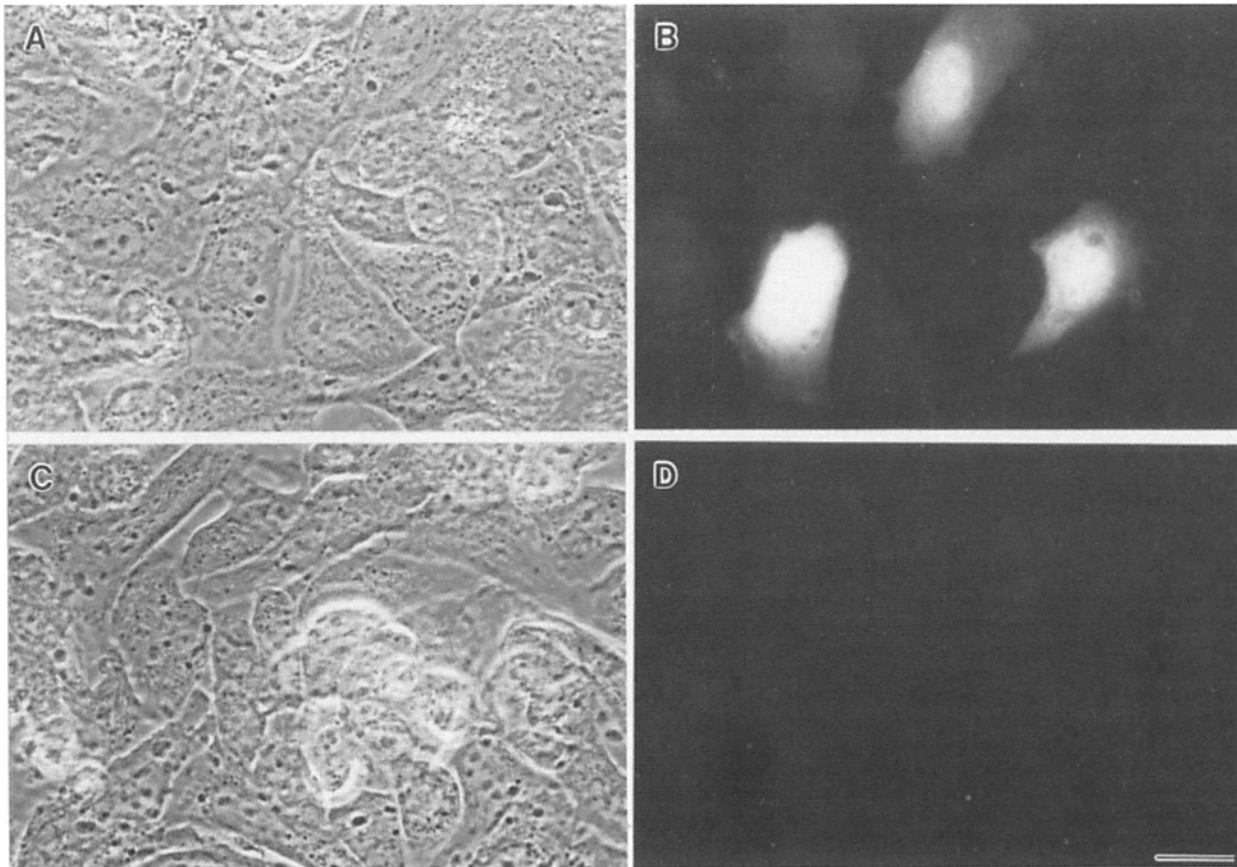


Figure 2. Trypsinization wounds plasma membranes of endothelial cells. (A and B) Paired phase and fluorescence micrographs showing that endothelial cells trypsinized in the presence of FDx were frequently labeled cytoplasmically with fluorescence. (C and D) Paired phase and fluorescence micrographs showing that control endothelial cells incubated with FDx for an interval equal to that of trypsinized cells but then trypsinized in its absence were less frequently stained cytoplasmically with the FDx. Bar, 10 μ m.

the supernatant was removed from the endothelial cells before it was warmed (Fig. 7 D). These results are consistent with release of part of the growth-promoting activity through membrane disruptions, whose scraping-induced formation would be independent of temperature. An additional temperature- and cell-dependent process, either coupled to or separate from diffusion through membrane disruptions, may also

play a role in release of growth factors from cells scraped in the cold and then warmed to 37°C.

We also assayed the conditioned media of a variety of cell types other than endothelial cells for growth-promoting activity (Fig. 8). None significantly stimulated DNA synthesis in Swiss 3T3 cells, implying that the growth-promoting activity is not a general cellular constituent released from

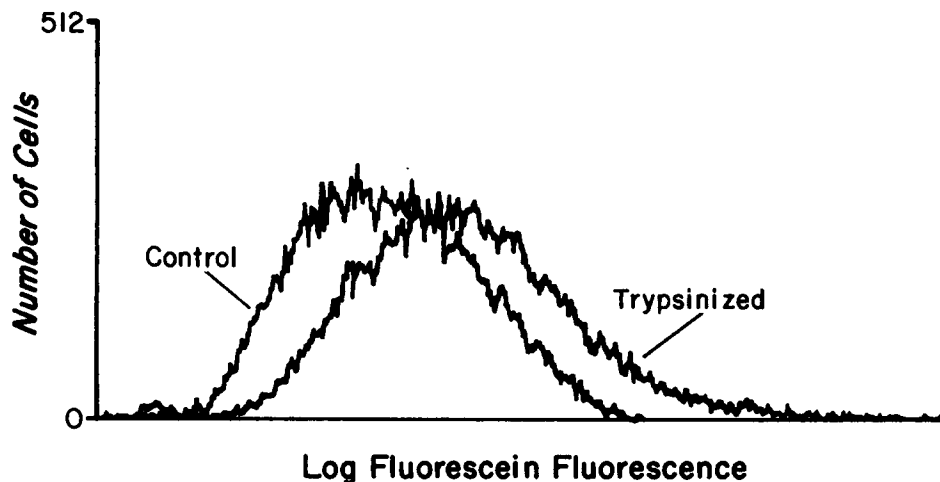


Figure 3. Flow cytofluorometric analysis of cells wounded during trypsinization. Control and experimental cells were trypsinized in the absence and presence of FDx, respectively (see Fig. 2 legend and Materials and Methods for details). Shown are the frequency distributions of fluorescence intensities within these two populations as measured by flow cytofluorometry (20,000 cells each sample). The experimental cell profile is shifted to the right of the control, indicative of a subpopulation of experimental cells of higher fluorescence intensity than controls.

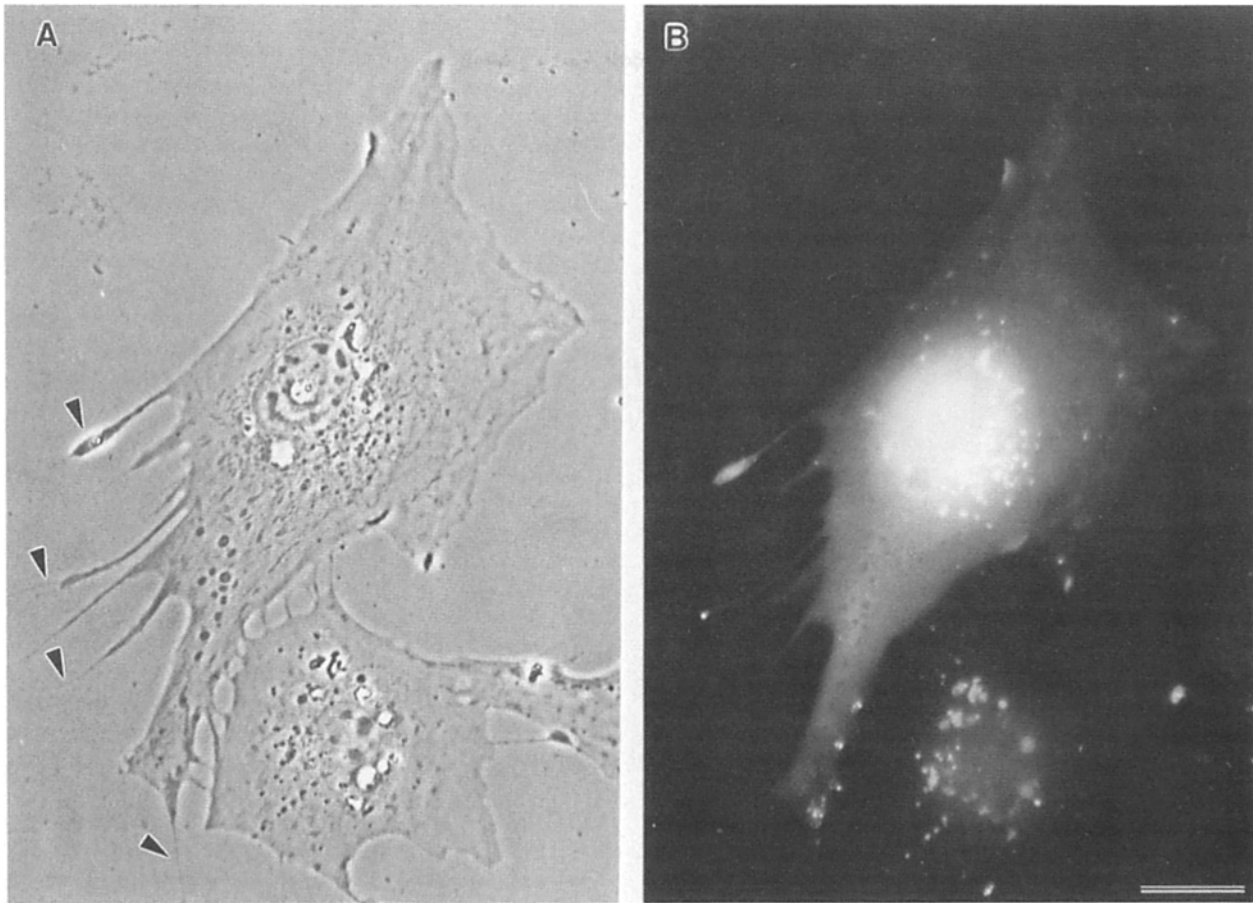


Figure 4. Wounding of endothelial cell plasma membranes can occur during migration. (*A* and *B*) Paired phase (*A*) and fluorescence (*B*) micrographs show that endothelial cells moving into a denuded zone are sometimes labeled cytoplasmically while migrating in the presence of FDx. Note in the phase-contrast micrograph (*A*) the retraction fibers at the presumptive trailing end of this locomoting cell (*arrowheads*). Bar, 10 μm .

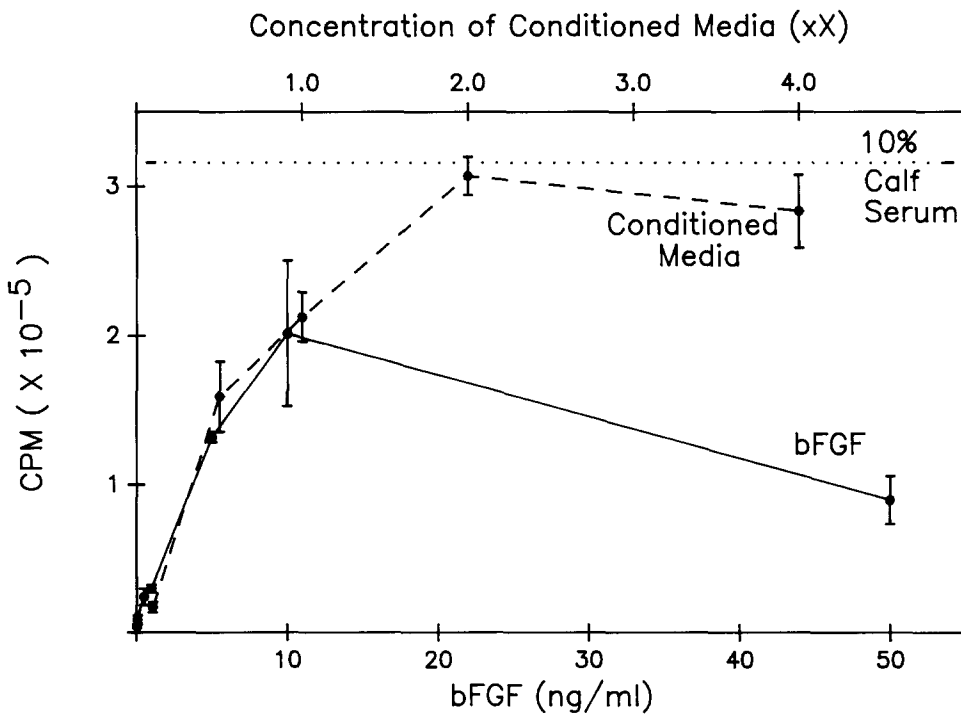


Figure 5. Wounding of endothelial cell plasma membranes releases growth factors. (○---○) BAEC were wounded by scraping from the substratum at 37°C; the conditioned medium was diluted to the indicated concentrations (as described in Materials and Methods) and assayed for growth-promoting activity. (●—●) Purified bFGF was assayed for growth-promoting activity at the indicated concentrations. (.....) DME plus 10% calf serum served as a positive control ($n = 2$; values are mean \pm SD).

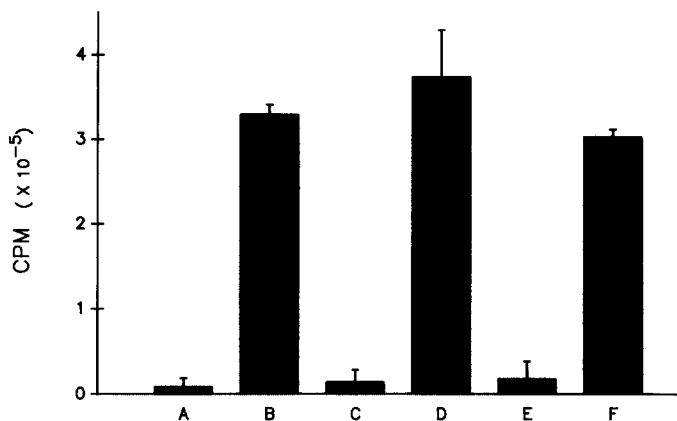


Figure 6. Time course of release of growth-promoting factors by wounded endothelial cells. BAEC were wounded by scraping at 37°C and were allowed to condition the media for 5 min (B), 3 h (D), or 24 h (F) before the media were collected and assayed for growth-promoting activity. Media in contact with undisturbed cultures for 5 min (A), 3 h (C), or 24 h (E) served as controls ($n = 2$; values are mean \pm SD).

mechanically wounded cells, but rather is specific to the endothelial cell.

Growth Factor Release by Wounded Cells Is Not Due to Scraping-induced Disruption of Extracellular Matrix

Endothelial cells deposit bFGF and possibly other growth factors in extracellular matrix, which they elaborate beneath themselves (Vlodavsky et al., 1987; Folkman et al., 1988). Therefore it was important to test whether scraping, which might disrupt extracellular matrix, could somehow release growth factors present within this matrix. Endothelial cells were scraped from the substratum 4 h, 4 d, or 7 d after plating, and the medium, conditioned by each culture of wounded cells, was assayed for growth-promoting activity. The activity released per unit cell protein was threefold higher from 4-h-old cultures (Fig. 9 A) than from either the 4- or 7-d-old cultures (Fig. 9, B and C). This result strongly indicates that the growth-promoting factor is not being released from disrupted extracellular matrix, since extracellular matrix deposition is a time-dependent and continuing process initiated at plating (for reviews see Gospodarowicz and Greenberg, 1981; Gospodarowicz and Vlodavsky, 1982). Moreover, plating time and scraping can be maintained as constants, while cell adherence to the substratum is varied, and the result is variable release of growth activity by wounded cells (see below).

Release of Growth Factors Is Not Due to Cell Death

Scraping of cells from the substratum irreversibly permeabilizes and, therefore, kills some cells, but only transiently permeabilizes others (McNeil et al., 1984; McNeil and Warder, 1987). Growth factors present in cytoplasm might be released in either case, since both provide a route outwards through disrupted plasma membrane. If cell death after mechanical wounding is somehow necessary for release of growth factors, then this release should correlate strongly with the viability of cells after wounding. We therefore designed conditions that, by varying strength of cell adherence

to substratum, resulted in variable cell viability after scraping (Fig. 10). The correlation obtained between cell viability and growth factor release was weak (coefficient of determination, $r^2 = 0.46$), indicating that cell death, induced by mechanical wounding, is probably not the primary determinant of growth factor release by endothelial cells. Release through transient membrane disruptions of cells surviving wounding is a possibility compatible with these findings.

To determine whether cell injury had to occur via a mechanical disruption of plasma membrane for growth factors to be released, we treated cells with the metabolic poisons, sodium arsenite and iodoacetate, under conditions that resulted in the death of 95% of the population (as assessed by trypan blue exclusion). This strategy has previously been used to study cell death by "hypoxia" (Lemasters et al., 1987). We then collected the medium conditioned by these dead cells and, after removal of poison by dialysis, assayed its growth-promoting activity. Release of growth-promoting activity from cells scraped as usual in the warm medium is shown in Fig. 11 A. No release of growth-promoting activity was measurable from the lethally poisoned populations (Fig. 11 B), unless such cells were scraped from the substratum before collection of conditioned medium (Fig. 11 C). Cell death, defined by permeability to a 1,000-mol wt molecule such as trypan blue, is not therefore sufficient for growth factor release but must be coupled with mechanical disruption of the plasma membrane. This disruption can apparently be either transient (resealable and survivable) or permanent (irreversible and lethal).

Medium Conditioned by Wounded Endothelial Cells Has an Autocrine Growth-promoting Activity with bFGF-like Properties

A likely target for the growth factors released by wounded endothelial cells would be neighboring endothelial cells or perhaps the wounded cell itself. We therefore asked whether

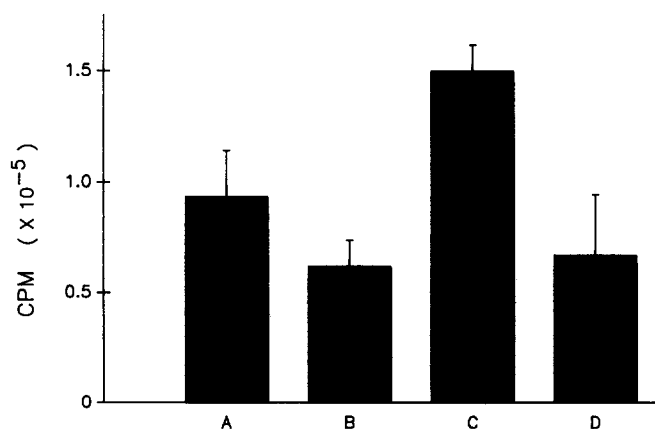


Figure 7. Wounding of endothelial cells in the cold releases growth-promoting activity. BAEC were (A) wounded by scraping at 37°C; (B) wounded at 4°C and then allowed to condition the medium at 4°C; (C) wounded at 4°C and then warmed to 37°C before removing the pellet; or (D) wounded and maintained at 4°C during conditioning after which the pellet was removed and the supernatant was warmed to 37°C. Control values (no wounding) were subtracted ($n = 3$; values are mean \pm SD).

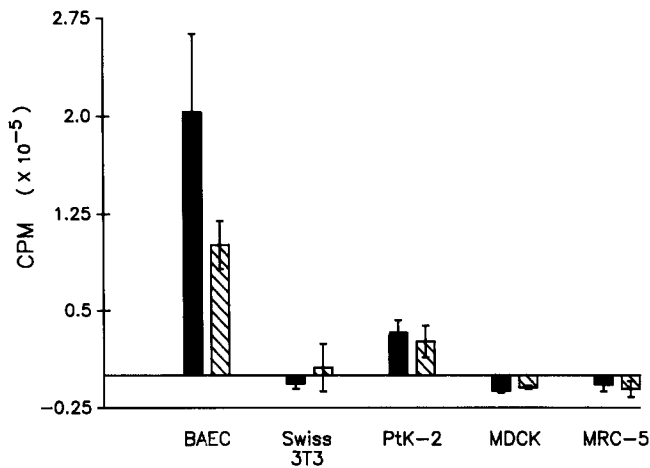


Figure 8. Not all cell types release growth factors upon plasma membrane wounding. Various cell lines (BAEC, Swiss 3T3, PtK-2, MDCK, and MRC-5) were wounded at 37°C (▨) or at 4°C (■). Control values (no wounding) were subtracted ($n = 2$; values are mean \pm SD). Conditioned media were assayed for growth-promoting activity for Swiss 3T3 cells.

the medium conditioned by endothelial cells could support endothelial cell growth, which we measured as increase in cell numbers. Both BAEC and BCEC wounded by scraping produced conditioned medium capable of supporting growth of their own kind (Fig. 12).

Two well-characterized growth factors known to be synthesized by endothelial cells are bFGF and PDGF (for review see DiCorletto and Fox, 1988). A first line of evidence that medium conditioned by wounded endothelial cells contained bFGF was our finding (above) that such medium had an autocrine activity for endothelial cells, which are mitogenically stimulated by bFGF but not PDGF (Vlodavsky et al., 1987). Second, 98% of the growth-promoting activity was destroyed by heat (data not shown). bFGF is heat sensitive but PDGF is not (Vlodavsky et al., 1987). Third, >95% of growth-promoting activity of wound-conditioned medium could be adsorbed by heparin-Sepharose, and the majority eluted with 2 M but not with 0.5 M NaCl (Fig. 13). This kind of behavior in the presence of heparin-Sepharose is specific for bFGF (Schweigerer et al., 1987; for review see Lobb et al., 1986). Since considerable growth-promoting activity also eluted at 1 M NaCl, it is possible that aFGF is also released from wounded endothelial cells.

Discussion

This paper is the first to experimentally address the question: are growth factors released from endothelial cells wounded at their plasma membranes by mechanical forces? We have used the technique of scrape-loading as an *in vitro* model for endothelial cell wounding. Scrape-loading was developed as a method for introducing impermeant molecules into cytoplasm of living cells (McNeil et al., 1984) and, as indicated by the electron microscopic data of this paper, appears to closely mimic the predominantly plasma membrane wounds we have observed in cells *in vivo* (McNeil and Ito, 1989). Using this technique, we show that growth factors are released from endothelial cells wounded at their plasma membranes by mechanical forces.

In evaluating the biological significance of this observation, we must address two questions. First, what is the evidence that plasma membrane wounding occurs *in vitro* and *in vivo* and under what conditions does the wounding occur? Second, how consistent is our hypothesis of growth factor release with what is already known of growth factor biology at the molecular, cellular, and organismal levels?

Occurrence of Membrane Wounding *In Vivo* and *In Vitro*

In a previous study, we used HRP and FDx to label cells in gut that suffered membrane disruptions but were able to reseal themselves (McNeil and Ito, 1989). We observed labeling within many cell types of the stomach mucosa, including endothelial cells, after light abrasion. Even after a light massage of the stomach mucosa, that apparently did not damage tissue integrity, many epithelial cells were labeled. Most surprising, however, was our observation of labeled epithelial cells in the stomach, small intestine, and colon of experimentally undisturbed animals that were simply allowed to ingest the FDx or HRP labels. Thus even the mechanical forces generated by normal gut mobility may be sufficient to wound epithelial cells: these forces would cause cells to be pressed against other cells or would cause cells to be pressed against gut contents. We have suggested that cell wounding, such as we have observed in gut, may be more common than is usually appreciated. Many other highly vascularized sites in the body, such as skin (Bertsch et al., 1976) and muscle (Schwane and Armstrong, 1983; Warhol et al., 1985), are also normally subject to mechanical wear and tear, as well as traumatic injuries. Furthermore, hemodynamic forces might also be capable of mechanically wounding endothelial cell plasma membranes and have been suspect as a possible causative agent leading to atherosclerosis (for review see Davies, 1988). Interestingly, in this regard, endothelial cell mi-

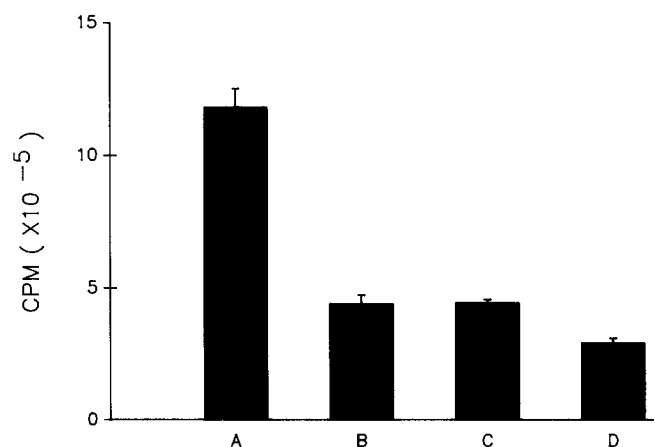


Figure 9. Role of extracellular matrix disruption in growth factor release. BAEC were plated for 4 h (A), 7 d (B), 4 d (C), and 7 d with fresh addition of DME plus FBS after 3 d (D) before wounding by scraping at 37°C, collecting of conditioned media, and assaying of growth-promoting activity. Control values (endothelial cells not wounded) were subtracted and counts per minute per dish of responding fibroblasts were then normalized for the protein content of the endothelial cell cultures used for conditioning. Protein was determined using a bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL) ($n = 2$; values represented are mean \pm SD).

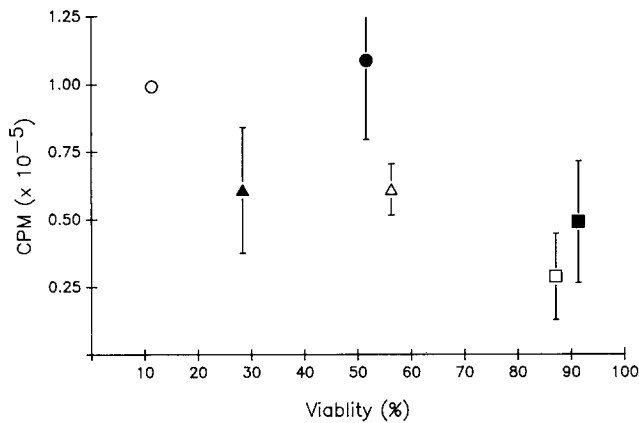


Figure 10. Cell viability after wounding correlates weakly with the amount of growth factor release by endothelial cells. The following methods for wounding cells by scraping were designed to result in variable cell viability. (\blacktriangle) BAEC were wounded by scraping at 37°C. (\bullet) Cells were wounded as above but 4 h after plating. (\circ) Cells were wounded as above, but in DB at pH 8.9. (\triangle) Cells were soaked for 14 min in DB, 1 min in calcium- and magnesium-free PBS (CMF PBS), and then wounded by scraping at 37°C in CMF PBS. Cells were soaked for 15 min in CMF PBS (\square) or CMF PBS plus EDTA (\blacksquare) and then wounded by scraping at 37°C in the presence of CMF PBS or CMF PBS plus EDTA, respectively. The conditioned media were assayed for growth-promoting activity and cell viability was determined using fluorescein diacetate as described (Materials and Methods). Counts per minute per dish of responding fibroblasts were normalized for the protein content of the endothelial cell cultures used for conditioning ($n = 2$; values are mean \pm SD).

toses are more frequently found at bifurcations, where turbulence of flow is greatest, than in unbranched regions of aorta (Wright, 1968).

Using FDx as a microscopic and flow cytometric marker, we have shown here that endothelial cells suffer repairable wounds to their plasma membrane during trypsinization and, at a much lower or less readily detectable frequency, while attached and locomoting. Direct evidence is lacking that such wounded cells release growth factors, but if dextran (10,000 mol wt) can enter cytoplasm through the transiently disrupted plasma membranes of these cells, then smaller cytoplasmic molecules, such as bFGF, might leave via the same diffusional route.

A simple mechanism can explain how trypsinization and, perhaps, cell locomotion wound endothelial cell plasma membranes. During trypsin-induced cell rounding, retraction fibers link the rounded cell body to the substratum (Revel, 1974). Similar structures link the substratum to the trailing "tail" of cytoplasm in locomoting cells. If these "retraction" fibers are severed, as the cell is released completely from the substratum during trypsinization or as the trailing tail of cytoplasm is drawn forward, then transient membrane disruptions would result.

Release of Growth Factors through Disrupted Plasma Membrane: Cellular and Molecular Aspects

We propose that growth factors are released from the cytoplasm of mechanically wounded endothelial cells by diffusion through disruptions in plasma membrane. Patterns of

staining with HRP indicate that scraping of endothelial cells from the substratum disrupts plasma membranes, but not organellar membranes. This conclusion is supported by the data of previous studies which used cell scraping and similar mechanical methods for introducing macromolecules into cytoplasm of living cells (for review see McNeil, 1989). Furthermore, release of growth factors by endothelial cells occurred upon scraping in the cold, a result incompatible with an exclusively exocytic mechanism. Cell death alone was not sufficient for release. Cytotoxic doses of metabolic poisons failed to release any growth-promoting activity unless the membranes of the dead cells were subsequently disrupted by scraping. Also, growth factor release did not correlate well with the extent of cell death induced by scraping. It is possible that a significant amount of growth factor was released through transient membrane disruptions of cells that survived the mechanical injury of scraping.

We show here that part of the growth-promoting activity released from wounded endothelial cells can be accounted for by a bFGF-like molecule. A particularly intriguing question concerning bFGF has been how, lacking a signal peptide sequence (Abraham et al., 1986), bFGF is released by endothelial and other cells that are known to produce it. Our data suggest that release of bFGF may be through membrane disruptions in endothelial cells, rather than via the typical, membrane-bound secretory route. Such a mechanism would clearly insure that release was accurately localized to sites requiring the repair processes initiated by growth factor: dead, dying, or surviving cells that were transiently wounded at their plasma membrane by mechanical force would all rapidly release growth factors like bFGF at sites of traumatic injury or at sites requiring routine cell renewal due to normal processes of mechanical wear and tear. The ubiquity of the endothelial cell, which lines all vasculature, makes it a near-

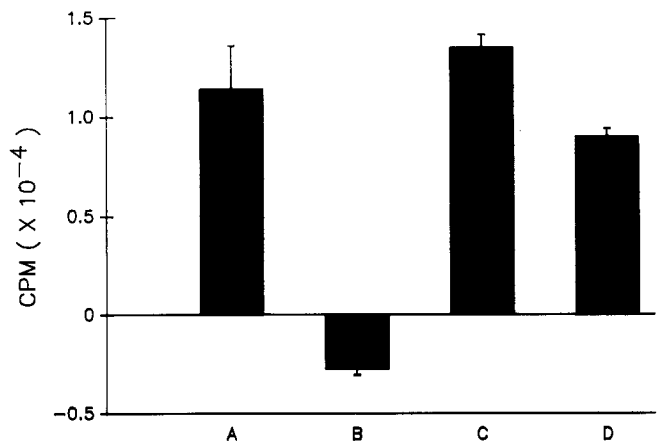


Figure 11. Chemically-induced cell death does not release growth factors unless accompanied by mechanical wounding. Conditioned media from the following BAEC cultures were collected, dialyzed, sterile filtered, and assayed for growth-promoting activity. (A) Cells were wounded in the presence of DB by scraping at 37°C. (B) Cells were soaked for 1.5 h in DB plus poison (3 mM sodium arsenite plus 10 mM iodoacetate). (C) Cells were soaked 1.5 h in the poison and then scraped at 37°C in its presence. (D) Cells were scraped at 37°C as usual, but in the presence of poison ($n = 2$; values are mean \pm SD).

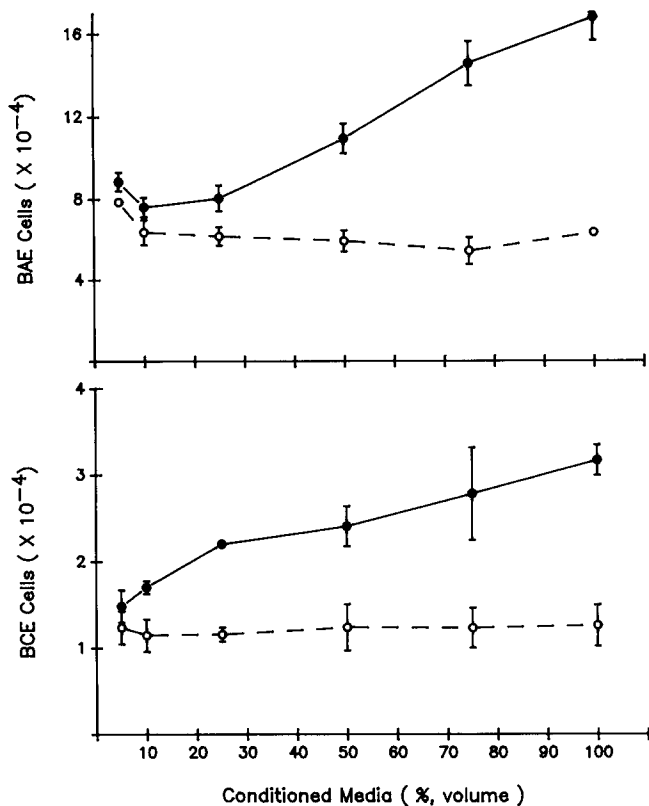


Figure 12. Effect of medium conditioned by wounded endothelial cells on endothelial cell growth. (●—●) BAE and BCE were wounded by scraping at 37°C, conditioned media were collected, and growth-promoting activity measured on like cell types. (○---○) Activity from control medium which had been in contact with undisturbed cells ($n = 2$; values are mean \pm SD).

ly ideal wound-related “endocrine” organ for locally releasing growth factors where needed.

Heparin-Sepharose chromatography also indicated that an aFGF-like molecule was released by wounded endothelial cells. Two interesting points may be made regarding this ob-

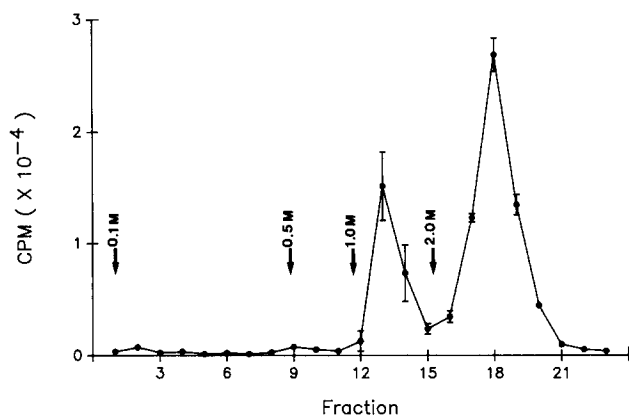


Figure 13. Heparin-Sepharose chromatography of media conditioned by wounded endothelial cells. Conditioned medium was eluted stepwise from a 0.8-ml heparin-Sepharose column with 0.1, 0.5, 1, and 2 M NaCl-0.01 M Tris, pH 7. Arrows indicate changes in the molarity of the eluting solution. The fractions collected were dialyzed against DME and assayed for growth-promoting activity.

ervation. First, aFGF, like bFGF, also lacks a signal sequence (Jaye et al., 1986). Second, the association of aFGF with endothelial cells has not previously been detected by heparin-Sepharose chromatography (Vlodavsky et al., 1987) or, to our knowledge, by other methods. Additional work will clearly be required for clarification of this point and in establishing the identity of the bFGF- and aFGF-like molecules released from scraped endothelial cells.

Endothelial cells in vitro apparently release bFGF which is apparently necessary both for growth and motility (Schweigerer et al., 1987; Sato and Rifkin, 1988). This could explain why they do not grow at clonal densities in the absence of added bFGF (Gospodarowicz et al., 1976) and why addition of a bFGF-neutralizing antibody to dense endothelial cultures inhibits cell growth and motility (Sato and Rifkin, 1988). The mechanism of such bFGF release by endothelial cells in vitro has not been explained. However, if, as suggested by this study, bFGF is released upon trypsinization, then this would explain why dilution of cells and medium containing recently released bFGF to achieve clonal densities would inhibit endothelial cell growth. Interestingly, fibroblasts transfected with the cDNA for IL-1, which also lacks the signal sequence, released this molecule when trypsinized but not when left undisturbed in culture (Young et al., 1988).

As an overlooked cellular phenomenon with possibly important biological and pathological implications, plasma membrane wounding deserves greater experimental attention. Plasma membrane wounds may occur frequently and at numerous sites in vivo (McNeil and Ito, 1989) and, as we have shown here, under normal culture conditions in vitro. The emphasis of this paper has been to show that such plasma membrane wounds provide a molecular route out of cytoplasm for molecules like bFGF which lack a signal peptide sequence.

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