Published May 1, 1990

Control of Intracellular pH and Growth by Fibronectin in Capillary Endothelial Cells

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Abstract. The aim of this work was to analyze the mechanism by which fibronectin (FN) regulates capillary endothelial cell proliferation. Endothelial cell growth can be controlled in chemically-defined medium by varying the density of FN coated on the substratum (Ingber, D. E., and J. Folkman. *J. Cell Biol.* 1989. 109:317–330). In this system, DNA synthetic rates are stimulated by FN in direct proportion to its effect on cell extension (projected cell areas) both in the presence and absence of saturating amounts of basic FGF. To investigate direct growth signaling by FN, we carried out microfluorometric measurements of intracellular pH (pH_i), a cytoplasmic signal that is commonly influenced by soluble mitogens. pH_i increased 0.18 pH units as FN coating densities were raised and cells progressed from round to spread. Intracellular alkalinization induced by attachment to FN was rapid and followed the time course of cell spreading. When measured in the presence and absence of FGF, the effects of FN and FGF on pH_i were found to be independent and additive. Furthermore, DNA synthesis correlated with pH_i for all combinations of FGF and FN. Ethylisopropylamiloride, a specific inhibitor of the plasma membrane Na^+/H^+ antiporter, completely suppressed the effects of FN on both pH_i and DNA synthesis. However, cytoplasmic pH per se did not appear to be a critical determinant of growth since DNA synthesis was not significantly inhibited when pH_i was lowered over the physiological range by varying the pH of the medium. We conclude that FN and FGF exert their growth-modulating effects in part through activation of the Na^+/H^+ exchanger, although they appear to trigger this system via separate pathways.

ANGIOGENESIS can be triggered by soluble mitogens such as basic FGF (Shing, 1985; Esch et al., 1985). However, insoluble extracellular matrix (ECM)[1] molecules appear to govern whether individual capillary endothelial (CE) cells will either grow or differentiate in response to FGF (Ingber and Folkman, 1989*b*). This regulatory mechanism is critical since successful morphogenesis does not result solely from repeated cell divisions, but also depends on establishment of local growth differentials and formation of differentiated tissue structures at selective sites (Ausprunk and Folkman, 1977; Folkman, 1982).

ECM molecules, such as fibronectin (FN), promote cell growth by binding to specific cell surface receptors (Hynes, 1987; Ruoslahti and Pierschbacher, 1987). However, FN differs from peptide growth factors in that it must be presented in an insoluble form (e.g., adsorbed to plastic or organized in a complex ECM) in order for it to influence CE cell proliferation (Ingber, 1990). The growth-promoting effects of insoluble FN also correlate with its ability to promote cell extension (Ingber et al., 1987; Ingber, 1990). This relation is consistent with results of previous studies which demonstrate that anchorage-dependent cells only respond to growth factors by traversing the cell cycle when attached to substrata that can resist cell-generated mechanical loads (Maragoudas, 1973*a*, *b*), and thus support cell spreading (Folkman and Moscona, 1978). Yet, little is known about the mechanism by which adhesion to FN and associated changes of cell shape might control cell growth.

In the present study, we investigated whether FN controls CE cell growth in a direct manner, i.e., by modulating intracellular signaling events. For several reasons, we chose to focus on cytoplasmic pH. Intracellular alkalinization, resulting from activation of a transmembrane Na^+/H^+ antiport on the cell surface, appears to be a common early effect of virtually every growth factor tested, including FGF (L'Allemain et al., 1984; Pouyssegur et al., 1984; Moolenaar, 1986; Moenner et al., 1987; Grinstein et al., 1989). A number of cytoplasmic oncogenes (Doppler et al., 1987; Schwartz et

1. *Abbreviations used in this paper*: BCECF-AM, 2', 7'bis-(2-carboxyethyl)-5(and-6)carboxyfluorescein acetoxymethyl ester; CE, capillary endothelial (cells); DM + FGF, defined medium including DME, transferrin (5 μg/ml), HDL (10 μg/ml), 1% BSA, 20 mM Hepes, and FGF (2 ng/ml); DM − FGF, defined medium without FGF; ECM, extracellular matrix; EIPA, ethylisopropylamiloride; FN, fibronectin; pH_i, intracellular pH.

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al., 1989*b*) and at least two important intracellular signaling pathways, one mediated by protein kinase C and the other involving calcium and calmodulin, also activate the Na^+/H^+ exchanger and raise intracellular pH (pH_i) (Ober and Pardee, 1987; Vicentini and Villereal, 1986). Furthermore, although individual cell types vary, almost all cells require that pH_i be maintained within a narrow range for optimal growth. Decreases of cytoplasmic pH as small as 0.2 pH units, when exerted within this range, can result in complete suppression of DNA synthesis (Grinstein et al., 1989). Finally, we recently found that when fibroblasts were prevented from spreading in serum-containing medium by coating tissue culture plastic with a nonadhesive polymer, cytoplasmic pH decreased by 0.15 pH units relative to maximally spread cells (Schwartz et al., 1989*a*). This correlation between cell extension and intracellular alkalinization led us to propose that decreases in pH_i may mediate some of the inhibition of growth that has been previously shown to accompany cell rounding (Folkman and Moscona, 1978).

In the present paper, we analyze the mechanism by which adhesion to a single type of ECM molecule, FN, can modulate CE cell growth in the presence and absence of a purified angiogenic growth factor, FGF. We focus on the role of the Na^+/H^+ exchanger and cytoplasmic pH in endothelial cell growth regulation.

Materials and Methods

Experimental System

CE cells were isolated from bovine adrenal cortex as previously described (Folkman et al., 1979) and serially passaged on gelatin-coated 6-well tissue culture dishes in DME supplemented with 10% calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin (Gibco Laboratories, Grand Island, NY), and 10 μg/ml endothelial mitogen (Biomedical Technologies Inc., Stoughton, MA). Confluent endothelial monolayers were refed with DME containing 1% calf serum (without endothelial mitogen) 2 d before the experiment to ensure that the cells were quiescent and that exogenous growth factors were not accidentally carried over into the experimental medium. Quiescent monolayers were then dissociated into single cells by brief exposure (1–2 min) to trypsin-EDTA (Gibco Laboratories) and transferred to DME containing 1% BSA (Fraction V; Armour Pharmaceutical Co., Tarrytown, NY). Cell numbers were measured using a counter (Coulter Electronics Inc., Hialeah, FL). Cell aliquots were then pelleted, washed in DME containing 1% BSA, and resuspended in defined medium (DM + FGF) consisting of DME (with bicarbonate) supplemented with 5 μg/ml transferrin (Collaborative Research, Lexington, MA), 10 μg/ml high density lipoprotein (HDL; Bionetics Research, Rockville, MD), 1% BSA, and 2 ng/ml recombinant basic FGF (kindly supplied by Takeda Chemical Industries Limited, Osaka, Japan). Hepes buffer, pH 7.4 (Gibco Laboratories) was also included in the defined medium (20 mM, final concentration) in order to minimize variations of medium pH during the course of these experiments.

CE cells were plated in DM + FGF onto FN-coated 35-mm dishes (2×10^4 cells/dish) for determination of intracellular pH, morphometry, and [^{3}H]thymidine autoradiography and 96-well plates (5×10^3 cells/well) for quantitation of DNA synthesis on a per cell basis and morphometric analysis. All experiments used cells that were cultured for 18–24 h at 37°C. Similar studies were also carried out using DM without FGF (DM − FGF) as well as DME in the absence of any additional supplements (i.e., without HDL, transferrin, or BSA).

To analyze the role of the Na^+/H^+ exchanger, cells previously plated in DM + FGF were transferred to medium composed of 25 mM $NaHCO_3$, 135 mM choline chloride, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 4.0 mM KCl, MEM amino acids, and 10 mM glucose (Boyarsky et al., 1988), equilibrated with 5% CO_2. Ethylisopropylamiloride (EIPA) was added and pH_i was measured either 1 or 24 h later, as described below. To determine the effects of externally modulating pH_i, cells were cultured in DM + FGF minus bicarbonate. Medium pH was controlled using 20 mM Hepes buffer of varying pH.

Preparation of FN-coated Substrata

Human FN (Organon Teknika-Cappel, Malvern, PA) was coated onto bacteriological plastic dishes (i.e., not chemically treated for tissue culture) using a high pH adsorption technique as previously described (Ingber et al., 1987). In brief, FN was diluted in 0.1 M carbonate buffer, pH 9.4, plated on dishes, and allowed to incubate overnight at 4°C. For analysis of effects on cell shape and growth, FN was added to wells of 96-well plates (Immunolon II; Dynatech Laboratories Inc., Alexandria, VA) at 5, 25, 50, 100, 250, and 500 ng/well. For experimental determination of pH_i, droplets of carbonate buffer containing 10, 25, and 2,500 ng FN/50 μl were plated separately within the same 35-mm dish (Falcon 1008; Becton Dickinson & Co., Lincoln Park, NJ). In this manner, three different FN coating densities were obtained within each culture dish used for pH measurements. Immediately before use, the carbonate coating buffer was removed and dishes were washed sequentially with PBS, DME, and DME containing 1% BSA.

Cell Growth Determination

To determine CE cell DNA synthetic rates within CE cells, [^{3}H]thymidine (84 Ci/mmol, 5 μCi/ml final concentration; New England Nuclear, Boston, MA) was added to the defined medium, 90 min after cell plating. After 18-h incubation at 37°C, incorporation of [^{3}H]thymidine into TCA-precipitable material was measured directly within adherent cells on FN-coated 96-well plates as previously described (Ingber et al., 1987). To determine DNA synthesis on a per cell basis, attached CE cells cultured in parallel dishes were trypsinized, pooled, and counted using a counter (Coulter Electronics Inc.). We have previously shown that CE cell DNA synthetic rates correlate directly with increases in cell number (i.e., cell proliferation) using this experimental system (Ingber, 1990). In experiments with medium of varying pH, cells were allowed to attach to FN-coated 96-well plates (500 ng/well) in complete DM + FGF. After 4 h, the medium was removed and replaced with bicarbonate-free DM + FGF of varying pH, supplemented with [^{3}H]thymidine. DNA synthesis was measured 18 h later. Similar experiments were also performed by plating cells directly in different pH media; these experiments yielded identical results. Experiments using EIPA were carried out in a similar manner except that the low sodium medium was used. Control levels of DNA synthesis in CE cells cultured in low sodium medium were ~60% of those measured in normal culture medium.

Autoradiography of [^{3}H]thymidine-labeled cells was carried out using cells grown in 35-mm dishes in parallel with those used for pH measurements. After 24 h of culture in DME containing [^{3}H]thymidine (1 μCi/ml), excess nonradioactive thymidine (2 mM final concentration) was added and allowed to equilibrate with cytoplasmic pools for 15 min at 37°C. Cells were then briefly fixed by adding glutaraldehyde directly to the medium (2% final concentration), washed with PBS, and dehydrated in methanol. Autoradiographic grains were developed directly on the surface of the dish using Kodak NTB-2 nuclear track emulsion and D-19 developer. Photographs were taken on a Nikon Diaphot microscope using Hoffman Optics and Kodak Plus-X-Pan film.

Cell Shape Determination

Cell shape changes were quantitated by measuring projected cell areas using a modified version of our previously published technique (Ingber et al., 1987). In brief, cells attached to dishes coated with varying concentrations of FN were fixed with glutaraldehyde, dehydrated in methanol, and stained with Coomassie brilliant blue. Stained cells were visualized on a video monitor using a Zeiss photomicroscope in conjunction with a Sony XC57 video camera. Average projected cell areas were determined by computerized image analysis using an Image Technology image processor in conjunction with an IBM PC2 Model 30 computer. For each cell shape determination, >30 cells were chosen from six randomly selected areas in three different wells. At least 15 randomly selected cells were used for measurements of the shape of cells used for pH measurements.

Determination of Intracellular pH

The cytoplasmic pH of single CE cells was measured by microfluorimetry as previously described (Schwartz et al., 1989*a*, 1990). The pH-sensitive fluorochrome, 2′,7′-bis-(2-carboxyethyl)-5(and-6)carboxyfluorescein, ace-

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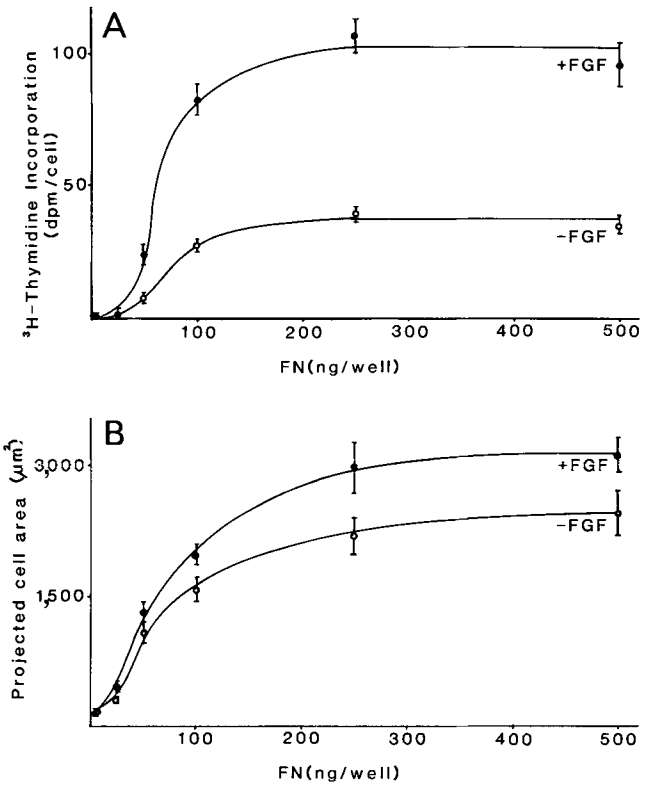

Figure 1. Effects of surface-adsorbed FN on DNA synthesis (*A*) and cell spreading (*B*). CE cells were grown on bacteriological plastic coated with the indicated amount of FN in defined medium in the absence (*open circles*) or presence (*closed circles*) of a saturating amount of FGF (2 ng/ml) as described in Materials and Methods. Error bars indicate standard error of the mean.

toxymethyl ester (BCECF-AM), was obtained from Molecular Probes Inc. (Eugene, OR) and was stored as a solution in dimethyl sulfoxide at −20°C. Briefly, cells in FN-coated 35-mm dishes were incubated with 1.5 μM BCECF-AM for 15–30 min and then transferred to a microscope stage where they were maintained at 37°C under 5% CO_2 (i.e., except in experiments that used bicarbonate-free media). Fluorescence intensity at >530 nm was measured from a 12-μm-diam circular region in the field, using excitation light at 450 and 490 nm. Background fluorescence from nearby cell-free regions was subtracted, and the ratio of intensity at 490/450 was calculated. Absolute pH was determined by comparison with a calibration curve using cells in Hepes-buffered medium of known pH containing high potassium and nigericin (Thomas et al., 1979).

To determine the effects of FGF, cell shape, and varying medium pH on pH_i, CE cells were plated in 35-mm dishes that contained regions coated with different densities of FN. Cytoplasmic pH was measured in ~15 individual cells within each area from two or three different dishes for each condition. Cells were then fixed with glutaraldehyde and their average projected cell areas were determined using computerized image analysis as described above. Thus, values for pH_i, projected cell areas, and [^{3}H]thymidine incorporation all represent data averaged from samplings of large populations of cells, although they are presented on a "per cell" basis.

To determine the time course of intracellular alkalinization after cell adhesion to FN, trypsinized cells were suspended in DM − FGF and incubated at least 30 min at 37°C before plating. The cells were then plated in DM − FGF in 35-mm dishes containing circles that were precoated with FN at high (2,500 ng/circle) or low (10 ng/circle) densities, as described above. pH_i was measured in 10–15 cells in each area at different times over the course of the first hour after cell plating. For each time point, ~10 min was needed to measure the 20–30 cells; cells from the two populations were sampled so that both were evenly distributed around the reported time. For measurements with EIPA, cells were transferred to the low sodium buffer (described above) containing 50 μM EIPA, and pH_i was measured 10 min after transfer.

Results

Control of CE Cell Growth and Form by FN

CE cells cannot attach to bacteriological dishes in the absence of serum or preadsorbed ECM proteins and therefore are completely dependent upon exogenous FN for attachment and spreading. Under these conditions, CE cell spreading (projected cell areas) and DNA synthetic levels increased in parallel when the density of adsorbed FN attachment sites was raised (Fig. 1, *A* and *B*). DNA synthetic rates (normalized for cell number) increased ~100-fold as CE cells progressed from round to maximally spread in chemically defined medium supplemented with a constant, saturating amount of soluble FGF (2 ng/ml).

Control of Cytoplasmic pH by FGF and FN

The effects of FN on pH_i were determined in cells grown on small areas coated with different FN densities within the same 35-mm culture dish. This protocol was used to be certain that observed alterations of pH_i were due to differences in FN-coating density rather than to possible variations in the medium between dishes (e.g., due to differences in cell density, cell death, secretion, or depletion of growth factors, etc.). These studies demonstrated that cytoplasmic pH increased as FN-coating densities were raised, even in the presence of a saturating concentration of FGF (Fig. 2). CE cells grown on the highest FN density were 0.18 units more alkaline than round cells on the low FN concentration. These differences were not due to optical artifacts since spread and round cells had the same cytoplasmic pH when nigericin was used to equilibrate pH_i with medium pH, and since the pH_i difference was abolished by EIPA (see below).

The next step was to determine whether FN modulated the effects of FGF on pH_i, or if it regulated pH_i independently. Therefore, cytoplasmic pH was measured within cells grown on different FN-coating densities in the absence of FGF. Cells without FGF had a lower pH_i on all FN concentrations, however, they displayed a similar FN-dependent increase of cytoplasmic pH (Fig. 2). When FGF was removed from the defined medium, cell spreading was also less exten-

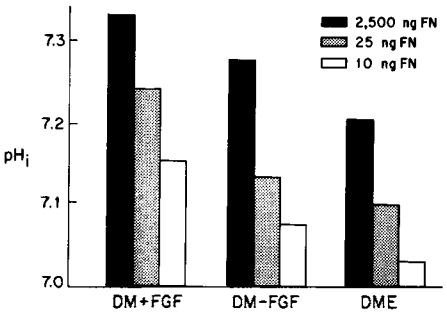

Figure 2. Effects of FN and soluble growth factors on pH_i. Cytoplasmic pH was measured within cells grown in areas coated with different FN densities within the same 35-mm dish in DM + FGF, DM − FGF, or DME without any additional supplements. pH measurements were carried out as described in Materials and Methods. The solid, stippled, and open bars represent FN-coating concentrations of 2,500, 25, and 10 ng/50 μl droplet, respectively. Standard error was <10% of the mean for all values presented.

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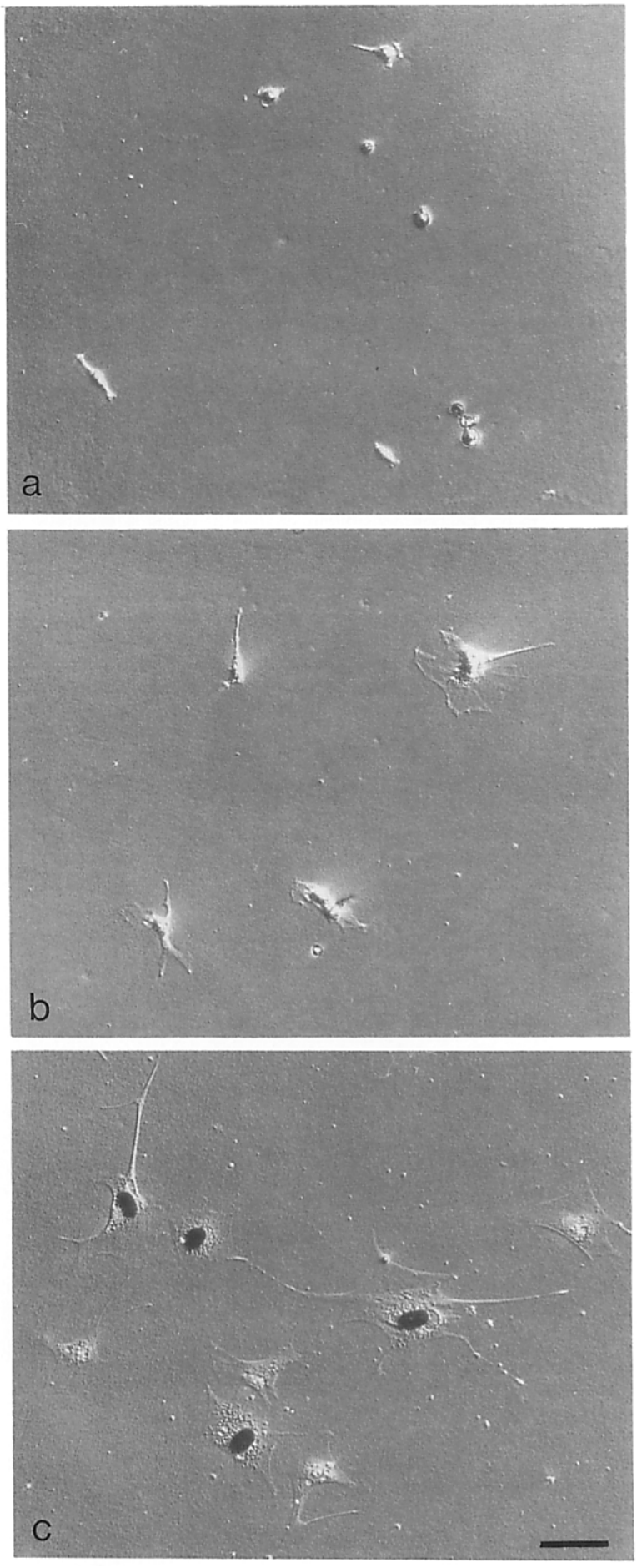

Figure 3. Light microscopic [^{3}H]thymidine autoradiographs of CE cells grown on different FN densities in DME, in the absence of exogenous mitogens. Separate regions of the same 35-mm culture dish were coated with 10 (*a*), 25 (*b*), or 2,500 ng (*c*) FN as described in Materials and Methods. Note the wide range of cell shape control that can be obtained by varying FN coating densities. All photographs were taken under Hoffman optics at the same magnification. Bar, 60 μm.

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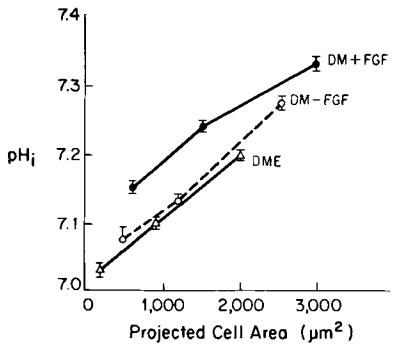

Figure 4. Relation between pH_i and cell spreading. Cells used for Fig. 2 were analyzed by computerized morphometry and the data were replotted as pH_i vs. average projected cell area. *Closed circles*, cells grown in DM + FGF; *open circles*, cells in DM − FGF; *open triangles*, cells in DME without any additions. pH_i values represent means ± SEM. The standard error of the projected cell areas was consistently <12% of the mean.

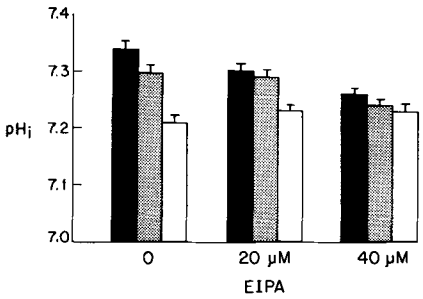

Figure 6. Effects of EIPA on cytoplasmic pH. CE cells were plated in DM + FGF on areas coated with different FN concentrations within the same 35-mm dish, as described and labeled in Fig. 2. After plating, cells were transferred to bicarbonate-buffered medium containing 25 mM sodium, choline chloride (to keep ionic strength constant), and EIPA. pH_i was determined after 18 h. Values are means ± SEM.

sive and fewer cells were able to synthesize DNA (Fig. 1, *A* and *B*). Although we can not exclude the possibility that some endogenous production of FGF occurs (Schweigerer et al., 1987), it is clear that removal of exogenous FGF results in a substantial decrease of DNA synthesis. Nevertheless, the number of cells able to synthesize DNA continued to increase in response to increasing FN densities.

To rule out the possibility that FN altered pH_i by modulating the effects of some other soluble regulator of growth within the defined medium (i.e., HDL or transferrin), we repeated this experiment in DME without any additions. Under these conditions, cells were slightly more acidic than cells in defined medium, but continued to exhibit similar FN-dependent changes in pH_i (Fig. 2). Autoradiography of [^{3}H]thymidine-labeled cells cultured in parallel dishes confirmed that increasing FN coating densities promoted both cell spreading and entry into S phase in the absence of any exogenous growth factor (Fig. 3, *a–c*).

Role of Cell Shape

Morphometric analysis of cells fixed and stained after completion of pH measurements revealed that pH_i increased as a linear function of projected cell area when averaged for the entire population, for cells in all three media (Fig. 4). It appears that the dependence of pH_i on cell extension is similar for all conditions, i.e., the three lines have similar slopes (although some saturation may occur at the highest levels of cell spreading or the highest pH_i values). This analysis shows that the cytoplasmic pH increase due to addition of FGF can be attributed in part to a small increase in cell spreading, but that FGF also produced a significant increase of pH_i (~0.06 pH units) separate from its effects on cell shape. Note that this is the first demonstration that a potent angiogenic factor, FGF, raises pH_i in capillary cells. The finding that FGF stimulated similar increases of pH_i in cells on low and high FN densities indicates that functional FGF receptors exist on both round and spread cells. This type of analysis also suggests that the small decrease of pH_i produced by removal of the defined medium supplements, HDL and transferrin, was primarily due to a decrease in cell extension.

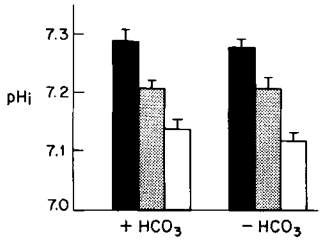

Figure 5. Role of bicarbonate. CE cells were plated in DM + FGF on areas coated with different FN concentrations within the same 35-mm dish, as described and labeled in Fig. 2. pH_i was determined before or 1 h after transfer to bicarbonate-free medium buffered with Hepes. Values represent means ± SEM.

Role of the Na^+/H^+ Exchanger

In bicarbonate-containing medium, cells can regulate their pH_i via HCO_3^-/Cl^- exchangers as well as by the Na^+/H^+ antiporter (Bierman et al., 1988). Therefore, it was necessary to determine which transporter was responsible for the differences in cytoplasmic pH induced by changes of FN coating densities. To inactivate HCO_3^-/Cl^- exchangers, cells were transferred to bicarbonate-free medium buffered with Hepes, pH 7.4. Cells underwent rapid alkalinization due to loss of membrane-permeable CO_2, followed by a slower return to steady state pH_i within 5–10 min (not shown, but see Bierman et al., 1988). Measurements made 1 h after transfer demonstrated that cells on high and low FN densities returned to their original pH_i (Fig. 5). Thus, bicarbonate was not required to maintain steady-state cytoplasmic pH or to maintain a pH_i difference between round and spread CE cells. Also, bicarbonate was not required to maintain differences in CE cell DNA synthetic levels on the different FN densities (not shown).

To test whether the Na^+/H^+ exchanger is involved in control of pH_i, cells in medium with bicarbonate were treated with EIPA, an amiloride analogue that is a more specific inhibitor of the Na^+/H^+ exchanger (Vigne et al., 1983; Boyarsky et al., 1988). EIPA competes with Na^+ for binding to the extracellular site of the antiporter. However, because inhibition is competitive, rather high concentrations are required to completely inhibit proton transport in normal culture medium. We therefore used the approach of L'Allemain et al. (1984) and used medium with reduced sodium (choline chloride was added to keep osmotic strength constant). CE

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cells in medium with 25 mM sodium had the same pH_i as cells in normal medium (not shown). When EIPA was added, cytoplasmic pH decreased over 10–15 min and reached a new, lower steady state within 20 min that remained constant for at least an additional 24 h. The difference in cytoplasmic pH between round and spread cells was almost completely eliminated when cells were exposed to 40 μM EIPA (Fig. 6). The effect of EIPA appeared to be specific since it was antagonized by sodium. In culture medium with 140 mM sodium, approximately three times higher concentrations of EIPA were required to reduce the difference in pH_i between round and spread cells. The ability of EIPA to induce a rapid and persistent decrease of pH_i in spread cells, in conjunction with the absence of a requirement for bicarbonate, strongly suggests that activation of the Na^+/H^+ exchanger is responsible for the effect of FN on pH_i.

Finally, to confirm that there is a link between FN receptor occupancy and Na^+/H^+ exchanger activation, pH measurements were made during the first hour after cell plating. We found that cells plated on high FN rapidly increased their pH_i relative to cells on low FN and that this intracellular alkalinization could be inhibited by EIPA (Fig. 7). Interestingly, cell spreading on the high FN density also progressed over a similar time course whereas cells on low FN remained entirely round throughout the experiment.

Cytoplasmic pH and Growth

To determine the relation between DNA synthesis and pH_i, we estimated DNA synthetic levels within CE cells used for pH measurements based upon their projected cell areas (as shown in Fig. 4). This was done using the relation between DNA synthesis and cell areas described in Fig. 1. This analysis revealed that DNA synthesis and pH_i correlate for all combinations of FGF and FN (Fig. 8).

Previous studies suggest that cells require an alkaline pH_i to proliferate (Grinstein, 1988). The decrease in pH_i observed in cells on low FN densities might therefore, by itself, account for growth suppression in round cells. To test this hypothesis, cells were plated on a high FN density in Hepes-buffered medium (without bicarbonate) in which the medium pH was varied from 7.9 to 6.5. Measurements of pH_i showed that cytoplasmic pH decreased from 7.3 to 6.9 under these conditions (Fig. 9 *A*). Measurements of [^{3}H]thymidine incorporation within cells cultured in parallel under similar conditions (and normalized for cell number) showed that lower pH_i was associated with significant inhibition of DNA synthesis (Fig. 9 *B*), as previously reported with other cell lines (Musgrove et al., 1987). However, when the relation between pH_i and DNA synthesis obtained by this method is compared with results from cells grown in normal medium on varying FN coating densities (Fig. 8), it is clear

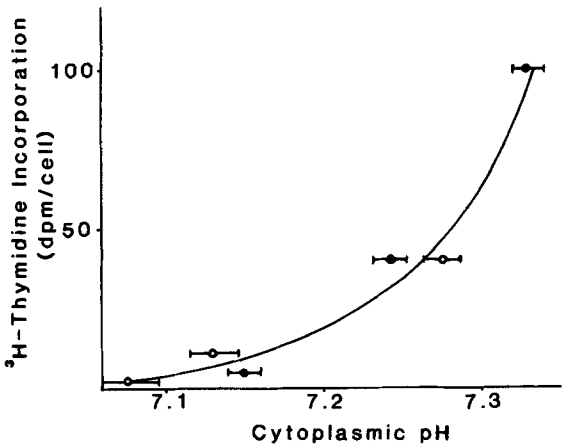

Figure 8. Relation between DNA synthesis and pH_i. Data for cells grown in defined medium in the presence and absence of FGF on different FN-coating densities (from Figs. 1 and 4) are replotted to show DNA synthesis (normalized for cell number) in cells with different cytoplasmic pH. *Solid circles*, DM + FGF; *open circles*, DM − FGF. pH_i and DNA synthesis correlate closely both in the presence and absence of FGF. Error bars indicate SEM.

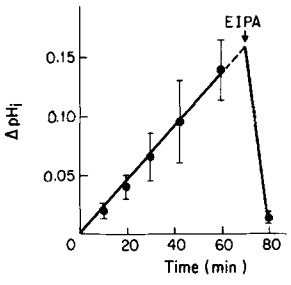

Figure 7. Time course of intracellular alkalinization after cell adhesion to FN. Cells were plated in DM − FGF onto 35-mm dishes containing circles coated with high and low densities of FN, as described in Fig. 2. pH_i was measured within cells on different FN-coating densities at the indicated times. The difference between the two populations, ΔpH_i, was calculated for each time point (ΔpH_i = pH_i on high FN − pH_i on low FN). After 70 min, cells were transferred to the low sodium buffer (described in Fig. 6) containing 50 μM EIPA and pH_i was measured 10 min later. Values represent means ± SEM. Data were averaged from four experiments; each data point was averaged from at least two experiments.

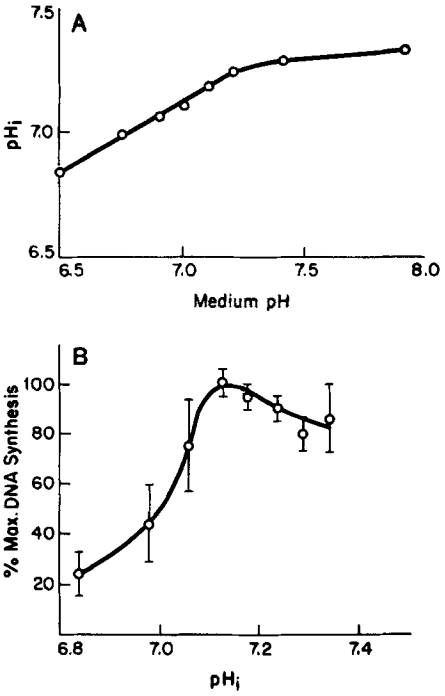

Figure 9. (*A*) Control of pH_i by varying medium pH. Data were pooled from two different experiments. (*B*) Effects of varying medium pH on pH_i and DNA synthesis. Data were pooled from three different experiments. Error bars indicate SEM; SEM was consistently <10% of the mean within each individual experiment.

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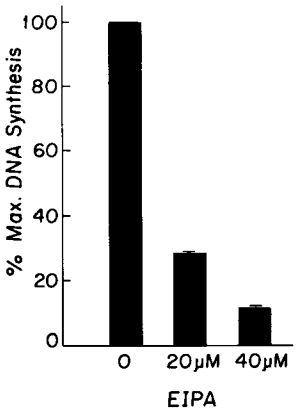

Figure 10. Effects of EIPA on DNA synthesis. CE cells were handled as described in Fig. 6 except that they were plated in 96-well plates and only a high FN-coating density (500 ng/well) was used. DNA synthesis was measured after 18 h as described in Materials and Methods. Data is presented as percent maximal DNA synthesis ± SEM (i.e., percent [^{3}H]thymidine incorporation exhibited by cells cultured in low sodium medium in the absence of EIPA). Control levels of DNA synthesis in low sodium medium were ~60% of those measured in CE cells grown in normal culture medium.

that a small decrease of pH_i in the physiological range (i.e., from 7.3 to 7.1) can not alone account for the major reduction of DNA synthesis observed in round cells. In contrast, inhibition of Na^+/H^+ exchange using EIPA resulted in suppression of both intracellular alkalinization (Figs. 6 and 7) and DNA synthesis (Fig. 10). The inhibitory effects of EIPA on DNA synthesis also appeared to be specific since EIPA was much less inhibitory when added in normal, high sodium–containing medium (e.g., 4.1% inhibition at 20 μM EIPA vs. >70% inhibition at the same dose in low sodium medium). Separate experiments showed that lowering pH_i by changing the pH of the medium gave similar results in both normal and low sodium medium (not shown). These data suggest that activation of the Na^+/H^+ exchanger may play a role in CE cell growth control distinct from the subsequent changes of steady-state pH_i measured by microfluorimetry.

Discussion

We recently showed that CE cells can be switched between modes of growth, differentiation, and involution by altering ECM integrity in vitro (Ingber and Folkman, 1989*b*; Ingber, 1990) and in vivo (Ingber et al., 1986; Ingber and Folkman, 1988). In these studies, the growth-modulating effects of different ECM configurations appeared to be based on their ability to produce alterations of CE cell shape. In the present study, we set out to determine whether adhesion to FN and associated changes of cell shape modulate CE cell growth by altering chemical signaling pathways. We chose to examine pH_i because it is a consequence of activation of a variety of growth signaling pathways and has been suggested to act as a direct regulator of cell growth.

FN Acts Separately from FGF

In past studies using fibroblasts, we demonstrated that pH_i increases induced by cell attachment to standard tissue culture plastic correlate with cell shape changes, reverse rapidly during cell detachment, and result from activation of the Na^+/H^+ exchanger (Schwartz et al., 1989*a*, 1990). These studies, however, did not determine the relative importance of different serum mitogens or ECM molecules. To address this question more directly in the present study, CE cells were grown in chemically defined medium that was supplemented with a single type of peptide growth factor, FGF, and cell shape was perturbed in a specific manner by varying FN-coating densities on nonadhesive, bacteriological dishes.

The results of these experiments demonstrate that adhesion to FN acts separately from FGF to activate the Na^+/H^+ exchanger and thereby regulate CE cell growth. This notion is based on data showing that FN activates the Na^+/H^+ exchanger to a similar degree with or without FGF in CE cells (Figs. 2 and 7). These data also clearly demonstrate that the pH-modulating effects of FGF and adhesion to FN are additive. Thus, FGF apparently activates the Na^+/H^+ exchanger through a separate signaling pathway. However, it is important to note that with respect to both growth and pH_i, FN appears to be the more potent regulator.

Rapid Integration of Signals

Cells in culture commonly require multiple stimuli, for example, two or more specific growth factors, in order to progress through the cell cycle (Rudland and Jimenez de Asua, 1979). Although little is known about how the cell processes separate signals, it is generally assumed that integration occurs at the level of gene expression (Denhardt et al., 1986; Dynan, 1989). We have found, however, that intracellular alkalinization is induced within minutes after cell attachment to FN and that pH_i shows a strong correlation with DNA synthesis for cells stimulated with all combinations of FN and FGF. The fact that pH_i is a quantitative marker for DNA synthesis under physiological conditions suggests that these separate growth stimuli may in fact converge at an early step during signal transduction. It has also been observed that EGF and insulin, which have synergistic effects on cell growth (Rudland and Jimenez de Asua, 1979), have synergistic effects on pH_i (Moolenaar et al., 1983).

Growth Control: Role of the Na^+/H^+ Exchanger versus pH_i

Past studies have demonstrated inhibition of growth by low pH medium (Musgrove et al., 1987) and by inhibitors of Na^+/H^+ exchange (L'Allemain et al., 1984), but to our knowledge no comparison has been made between these two methods within the same experimental system. We found that when pH_i was controlled externally that the cytoplasmic pH of spread cells could be lowered to the level of round cells without substantially inhibiting DNA synthesis. On the other hand, studies using EIPA showed that inhibition of Na^+/H^+ exchange strongly inhibited growth at the same concentrations at which it inhibited cytoplasmic alkalinization, suggesting that activation of the Na^+/H^+ exchanger itself may be critical for growth control. These paradoxical results would appear to indicate that Na^+/H^+ exchange plays a role in cell growth control beyond that of regulating steady-state pH_i. While no direct evidence to support this idea has been published, several authors have made similar proposals. For example, Williams suggested that activation of the Na^+/H^+ antiporter might trigger growth as a result of local changes of proton concentration directly beneath the cell surface (Williams, 1988). It has also been proposed that Na^+/H^+ exchange may regulate growth via influx of sodium and stimulation of the Na^+/K^+ ATPase (Szwergold et al., 1989), and evidence has been presented that Na^+ can be critical for

growth apart from its effects on pH_i (Burns and Rozengurt, 1984; Panet et al., 1989).

Transmembrane Signaling by FN

It is likely that FN alters cytoplasmic pH as a result of specific binding interactions with a member of the integrin family of receptors. Although this point has not been directly addressed in the present study, we have recently shown that the growth and shape-modulating effects of insoluble FN can be inhibited by addition of low concentrations of soluble RGD-containing peptides to adherent CE cells (Ingber, 1990) and that cell rounding induced by RGD-peptides produces a rapid decrease in pH_i (Schwartz et al., 1989*a*). Interestingly, fibrinoger -integrin interactions appear to similarly modulate the runction of the plasma membrane Na^+/H^+ exchanger in platelets (Banga et al., 1986), although resultant intracellular alkalinization results in cell aggregation rather than growth in these cells. It also may be relevant that integrin clustering has recently been shown to mediate the effects of FN peptides on gene expression in fibroblasts (Werb et al., 1989), however, no effects on growth were reported in that study.

The pH and growth-modulating effects of FN also may be based, in part, upon its ability to support changes of CE cell shape. Our results clearly demonstrate that changing cell–FN contacts produces parallel effects on cell shape, cytoplasmic pH, and growth. This possibility is also supported by the observation that removal of HDL and transferrin from our defined medium resulted in parallel decreases of cell spreading and pH_i, regardless of the FN density. The biochemical pathway by which cell shape perturbation might alter pH_i is completely unknown. However, cell spreading is a mechanochemical process which exerts mechanical tension on various cytoskeletal and membrane components (Ingber and Jamieson, 1985; Ingber and Folkman, 1989*a*). Tension-sensitive membrane channels have been identified (Guharay and Sachs, 1984) and stretch-activated calcium channels are known to exist on endothelial cell surfaces (Lansman et al., 1987). Local tension-dependent changes of membrane curvature in cell–matrix contact zones could also affect the chemical composition of the plasmalemma in these regions (Williams, 1988) or modulate receptor clustering. However, the role of mechanical tension in the control of pH_i remains to be determined.

Conclusion

Many past studies on the effects of ECM and cell shape on growth have focused on regulation of cell sensitivity to soluble growth factors (Folkman and Moscona, 1978; Gospodarowicz et al., 1978; Salomon et al., 1981; Ingber et al., 1987). Thus, one of the most important findings of this study is that FN appears to control growth by activating chemical signaling pathways directly. The growth-modulating effects of FN correlate with its ability to promote cell spreading, activate the cell surface Na^+/H^+ exchanger, and raise pH_i. FGF, a potent endothelial mitogen, also produces intracellular alkalinization during growth stimulation, although apparently through a separate transduction pathway. While physiological alterations of cytoplasmic pH correlate directly with the effects of FGF and FN on DNA synthesis, externally modulated changes of pH_i do not. Thus, activation of the Na^+/H^+ exchanger may represent a common signaling pathway that ECM molecules and soluble peptide mitogens use to regulate growth. However, in capillary cells, resultant changes of pH_i alone do not appear to be sufficient to regulate progression through the cell cycle.

We would like to thank Lynn Jennings and John Mckenna for their expert technical assistance and Dr. Judah Folkman and Dr. Susan Doctrow for their helpful suggestions during the course of this project.

This work was supported by National Science Foundation/National Aeronautics and Space Administration grant DCB-8711281/NAGW-1125 (D. E. Ingber) and United States Public Health Service grants P01-CA-45548 (D. E. Ingber; subproject II), GM-32377 (M. A. Schwartz), and 5P441RR02604 (C. Lechene).

Received for publication 4 August 1989 and in revised form 7 February 1990.

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