Extracellular matrix and pulmonary hypertension: control of vascular smooth muscle cell contractility

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Lee, Kyung-Mi, Kenneth Y. Tsai, Ning Wang, and Donald E. Ingber. Extracellular matrix and pulmonary hypertension: control of vascular smooth muscle cell contractility. Am J Physiol 274 (Heart Circ Physiol 43): H76–H82, 1998.—Pulmonary hypertension is characterized by increased vascular resistance due to smooth muscle cell hyperactivity and excess deposition of extracellular matrix (ECM) in the vessel wall. We investigated the possibility that changes in cell-ECM interactions may play an active role in this process by modifying the contractile response of pulmonary vascular smooth muscle cells (PVSM cells). Contractility was measured within individual cultured PVSM cells, when resting or stimulated with vasoactive agents, by quantitating changes in stiffness of the cytoskeleton (CSK) using magnetic twisting cytometry (N. Wang, J. P. Butler, and D. E. Ingber. Science 260: 1124–1127, 1993). Control studies confirmed that changes in CSK stiffness closely paralleled alterations in cell contraction and relaxation as measured in response to endoethelin-1 (ET-1) and dibutyryl guanosine 3',5'-cyclic monophosphate (cGMP), respectively, in a collagen gel contraction assay. CSK stiffness and contractile tone in cultured PVSM cells increased in direct proportion as the density of fibronectin (FN) coating was raised from 10 to 500 ng/well in 96-well plates. Dibutyryl cGMP had no effect in cells on low FN, although it completely inhibited the FN-dependent increase in CSK stiffness on higher ECM densities. In contrast, ET-1 induced the greatest increase in CSK stiffness on the intermediate FN density (100 ng/well). The reduced sensitivity to ET-1 on high FN was not due to dysfunction of the contractile apparatus nor to changes in protein tyrosine phosphorylation. Taken together, these results show that ECM can modulate PVSM cell contractility and suggest that the changes in ECM observed in hypertensive vessels could play an important role in the etiology of this disease.

fibronecin; gel contraction; cytoskeleton; cell mechanics; magnetic twisting cytometry

PERSISTENCE OF ELEVATED pulmonary vascular resistance after birth is the leading cause of death due to respiratory failure in the neonatal period. This condition is a common neonatal response to injury (e.g., hypoxia, high shear stress) and a key feature in newborns with persistent pulmonary hypertension. Patients with pulmonary hypertension exhibit pulmonary vascular smooth muscle (PVSM) cell hyperplasia, enhanced vascular tone, and increased accumulation of extracellular matrix (ECM) in their pulmonary vessels (5, 19). The cause of persistent pulmonary hypertension remains uncertain. Past studies have focused on the role of soluble growth factors that are upregulated in hypertensive conditions. However, the diseased smooth muscle cells appear to be generally hyperreactive at the level of signal transduction, regardless of the level of soluble growth factors (4, 22, 26). We have previously shown that ECM controls the set point of multiple intracellular signaling pathways, including Na+/H+ exchange, tyrosine phosphorylation, and inositol lipid turnover in cells of the vascular wall (16, 17, 25). As demonstrated in past studies with other cell types (9, 28) and in recent work with PVSM cells (H. Liley and D. E. Ingber, unpublished observations), ECM also regulates cell growth in response to mitogenic stimulation. However, the role of ECM in the control of PVSM cell contractility remains unclear.

Contraction of smooth muscle cells is achieved by actomyosin filament sliding. This process is facilitated by myosin light-chain phosphorylation regulated by myosin light-chain kinase, a Ca2+/calmodulin-dependent enzyme (13, 27). Recent studies also suggest that the small-molecular weight G protein, Rho, and downstream signaling molecules (e.g., Rho-kinase and phosphatase) may provide a secondary means to modulate myosin light-chain phosphorylation (1, 3, 11). This is important because cell adhesion to ECM results in Rho activation (7, 21).

Thus, to investigate the role of ECM in pulmonary hypertension, we set out to examine whether the contractile responses induced by soluble vasoagonists and vasoantagonists, such as endoethelin (ET)-1 and guanosine 3',5'-cyclic monophosphate (cGMP), can be modulated by altering the number of cell-ECM contacts in PVSM cell cultures. Unlike isolated muscle strips, individual cultured cells do not visibly shorten when stimulated with contractile agonists because they are adherent to a rigid culture dish. One way to assess this isometric contraction is by quantitating upstream signaling events such as calcium release. However, this pathway can be uncoupled from the actual actomyosin filament sliding response that generates tension (19, 34). An alternative method to measure contractility in single cells is to measure mechanical changes in the actin cytoskeleton (CSK), such as an increase in mechanical stiffness, that should accompany the rise in isometric tension induced by contractile agonists. We recently developed a noninvasive method that utilizes magnetic twisting cytometry (MTC) to directly measure CSK stiffness in living cells (31–33). We also showed that MTC provides a rapid and effective measure of the vasoconstrictor and vasodilator responses in cultured human airway smooth muscle cells (8). Thus, the purpose of this study was, first, to explore whether MTC can be applied to PVSM cells and, second, to determine whether altering cell-ECM contacts can modulate cell sensitivity to soluble vasoregulators and thereby control contractility within individual PVSM cells.
MATERIALS AND METHODS

Cell culture. PVSM cells were prepared from explants of the main pulmonary artery of newborn calves (2). Cells were used between passages 2-5 after harvest and passage in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, and 10% fetal bovine serum (Hyclone, Logan, UT). All experiments were carried out in defined medium, as described below.

Magnetic twisting cytometry. CSK stiffness was measured as described previously (31–33). In brief, PVSM cells were cultured to confluence, serum deprived, trypsinized, and plated in defined medium [1% bovine serum albumin (BSA), 5 µg/ml transferrin (Collaborative Research, Lexington, MA), and 20 µg/ml high-density lipoprotein (Bionetics Research, Rockville, MD)] on 96-well plates (Removawells, Immulon II; Dynatech, Chantilly, VA) that were coated with various densities (10–1,000 ng/well) of human serum fibronec- tin (FN; Cappel); these coating concentrations result in different specific avidities for FN-coated wells for 4–6 h were allowed to bind to spherical ferromagnetic beads (4.5-µm diameter; Spherotech) that were precoated with a synthetic Arg-Gly-Asp (RGD)-containing peptide (Peptide 2000; Telios), which is a specific ligand for integrin receptors. After 20 min, unbound beads were washed away with 1% BSA-DMEM and the wells were individually placed into the magnetic twisting device and maintained at 37°C. A brief (10 µs) but strong (1,000 gauss) homogeneous magnetic pulse was then applied to magnetize all surface-bound beads to the horizontal direction. After 20 s, twisting torque was produced by applying a weaker magnetic field (30 gauss) in the vertical direction for 1 min. Because this field was small, it did not realign the bead magnetic moments; rather, the beads rotated to realign with the new field. The extent of bead rotation was measured by an in-line magnetometer that continuously detected the magnitude of the bead magnetic vector in the horizontal direction. CSK stiffness was determined as described previously (32).

Collagen gel contraction assay. Collagen gels for the contraction assay were prepared using bovine epidermal collagen (Vitrogen 100, Collagen). Concentrated DMEM and bicarbonate buffer solutions were mixed 1:1 with the Vitrogen 100 (3.1 mg/ml) on ice to yield the final isotonic collagen gel solution (pH 7.4) according to the manufacturer’s instructions. Serum-deprived cells were suspended in this solution, plated on 6-well plates (1.2 ml/well) precoated with 2% BSA to minimize sticking, and allowed to solidify at room temperature for 30 min. DMEM containing 0.2% calf serum was then added (1.5 ml/well), and the gels were lifted off the bottom of the wells and allowed to float freely. Cultures were incubated at 37°C for 48 h before the addition of ET-1 or dibutyryl cGMP. Gel diameters and projected areas of the circular gels were measured at subsequent times. Vasoactive agents were added at 48 h after release.

Western blots. Whole cell lysates were prepared by extracting cells with modified RIPA (50 mM tris(hydroxymethyl)amino- methane (Tris), pH 7.4, 150 mM NaCl, 1% deoxycholate, 0.1% sodium lauryl sulfate, 1% Triton X-100, 2 µg/ml apro- tinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.2 mM sodium orthovanadate, 20 mM β-glycerophosphate, and 30 mM sodium pyrophosphate). Twenty micrograms of proteins were loaded onto 7.5% minigels and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Resolved proteins were electrophoretically transferred to nitrocellulose. The membrane was then blocked with 5% BSA in TBS-T (10 mM Tris, pH 7.2, 50 mM NaCl, 0.2% Tween 20) for 1 h and incubated with monoclonal antiphosphotyrosine antibodies (Upstate Biotechnology) for 3 h at room temperature. Blots were washed extensively with TBS-T and incubated for 1 h with 2 µg/ml affinity-purified goat anti-mouse horseradish peroxi- dase-conjugated antibody (Upstate Biotechnology). Labeled proteins were visualized with enhanced chemiluminescence (Amersham).

Measurement of [3H]thymidine incorporation. Quiescent PVSM cells were trypsinized and replated onto FN-coated 96-well plates (500 ng/well) in defined medium. Cells were incubated with ET-1 or platelet-derived growth factor (PDGF) at the time of plating and pulsed with 1 µCi [3H]thymidine (84 Ci/mmol; New England Nuclear, Boston, MA) for 24 h. Incorporation of [3H]thymidine into trichloroacetic acid-precipitable material was measured directly as previously described (10).

RESULTS

Measuring changes in isometric tension within individual cultured PVSM cells. We first examined whether CSK stiffness measured by MTC can be used to determine the contractile state of individual cultured PVSM cells. The MTC technique involves application of controlled mechanical stresses directly to cell-surface integrins (transmembrane ECM receptors) and, hence, to the CSK using ferromagnetic microbeads that are coated with a specific integrin ligand (synthetic RGD-containing peptide; Refs. 31–33). The cellular response to applied stress is simultaneously measured by quantitatively changing the rate and degree of bead rotation using an in-line magnetometer. When PVSM cells plated on FN-coated dishes were treated with a saturating dose of ET-1 (20 nM), CSK stiffness increased approximately twofold within 3 min and remained elevated for the duration of the 10-min experiment.
(Fig. 1). In contrast, treatment with a vasodilator, 8-bromoguanosine 3', 5'-cyclic monophosphate (8-BrcGMP) (6), induced a time-dependent decrease in CSK stiffness, with levels decreasing by >50% relative to controls within 5 min after drug addition (Fig. 1). Similar results were obtained with dibutyryl cGMP, another cGMP analog (data not shown). These data suggest that the changes in CSK stiffness measured by MTC in response to ET-1 and 8-BrcGMP reflect effects on PVSM cell contraction and relaxation, respectively.

To confirm that the changes in CSK stiffness were representative of the contractile state of these cells, we quantitated the ability of ET-1 and dibutyryl cGMP to modify PVSM cell tension in a conventional collagen gel retraction assay (14). Circular native collagen gels containing PVSM cells were detached from the culture dish surface and allowed to float for 48 h before addition of vasoactive agents. During this time, the cells actively contracted the gel until a mechanical steady state was obtained. When ET-1 was added to PVSM cells in this prestressed configuration, an additional 20% decrease in gel area (i.e., increased contraction) was observed within 30 min after drug addition (Fig. 2, A and B). These results confirm that ET-1 induces contractility in cultured PVSM cells. In contrast, when dibutyryl cGMP was added to these precontracted gels, a time-dependent increase in collagen gel area up to 40% above control levels was observed within 90 min (Fig. 2B). Although this relaxing effect of dibutyryl cGMP could be suppressed by simultaneous addition of ET-1, only partial reversal was observed (Fig. 2B), suggesting that the relaxing effect of dibutyryl cGMP on the CSK may be dominant under these conditions. Similar results were obtained when the gels were pretreated with dibutyryl cGMP for 15 min before addition of ET-1 (data not shown). Thus cultured PVSM cells can generate active contractile forces in response to stimulation by the vasoconstrictor ET-1 and be induced to mechanically relax by treatment with cGMP. These findings confirm that MTC provides a valid measure of vasomotor tone within individual cultured PVSM cells.

**Fig. 2. Effects of ET-1 and dibutyryl-cGMP (DBcGMP) on the ability of PVSM cells to contract floating collagen gels.**

**A**: photographs of floating gels. **B**: quantitation of data obtained in gel contraction assays. Serum-deprived cells were incorporated within collagen gels and allowed to float free in Dulbecco’s modified Eagle’s medium containing 0.2% calf serum for 48 h to precontract the gel (time 0). ET-1 (20 nM), 1 DBcGMP, or ET-1 + DBcGMP was then added, and changes in the area of the circular gels were measured over a 90-min time course. Drug-induced changes in gel area were normalized by the gel area measured at 0 h time point (100%); SE was <1% of mean for all conditions (error bars are obscured by symbols).
Control of vasoregulator action by modulating cell-ECM contacts. Previous studies in other cell types, including smooth muscle cells from lung, have demonstrated that CSK stiffness increases as the number of cell-ECM contacts are raised and cell spreading is promoted, regardless of the type of ECM molecule used for cell attachment (e.g., FN vs. collagen; Refs. 8, 32, 33). We found that PVSM cells exhibit a similar ECM density-dependent rise in CSK stiffness when they were plated on dishes coated with increasing densities of FN (Fig. 3). Furthermore, although the basal level of CSK stiffness immediately decreased by >50% on addition of dibutyryl cGMP in cells plated on the high density of FN (500 ng/well; 96-well plates), dibutyryl cGMP had progressively less effect on CSK mechanics as the FN density was lowered to 100 and 10 ng/well (Fig. 3). This reduction in CSK stiffness in response to treatment with dibutyryl cGMP was not due to cell detachment or death because CSK stiffness quickly returned to the control level after cells were washed with fresh medium (data not shown). These results suggest that cells bound to a high ECM density develop more isometric tension and thus are more sensitive to the relaxing effects of dibutyryl cGMP.

Varying ECM densities also altered the contractile response induced by ET-1. ET-1 induced stiffening in cells plated on all three FN densities. However, unlike the cGMP-induced relaxation, which showed maximal effect on high FN, the maximal stiffening response elicited by ET-1 was observed in cells plated on the moderate FN density (Fig. 3). Taken together, these data suggest that the number or density of cell-ECM contacts can modulate basal contractile tone within individual PVSM cells, in addition to regulating their sensitivity to soluble vasoregulators.

One potential mechanism for the preferential increase in CSK stiffness induced by ET-1 on the moderate FN density could be the specific induction of a greater increase in intracellular calcium on this substrate. For example, in certain cells, binding of FN to one particular integrin subtype (αv) has been shown to mediate a rise in intracellular calcium (24). To explore the possibility that different ECM densities altered CSK stiffness by changing intracellular calcium, we treated cells plated on different ECM densities with the calcium ionophore ionomycin (1 μM). Increasing intracellular calcium in this manner causes activation of Ca2+/calmodulin-dependent kinases, which in turn activate myosin light-chain kinases, leading to actomyosin sliding and contraction. Addition of ionomycin for 3 min resulted in more than a twofold increase in CSK stiffness in all cells, regardless of the ECM density (Fig. 3). Thus the suboptimal contractile response observed with addition of ET-1 in cells on high FN (Fig. 3) is not due to generalized dysfunction of the contractile apparatus, although it could be caused by a defect in calcium signaling in cells on this particular substrate. These data also suggest that the number of cell-ECM contacts and/or the degree of cell extension determines the absolute maximal level of isometric tension and CSK stiffness that may be obtained in these cells.

Role of protein tyrosine phosphorylation. Another possible explanation for the enhanced reactivity of PVSM cells to ET-1 when plated on the moderate ECM density is that different signaling mechanisms (i.e., other than calcium) may be preferentially activated under these conditions. To explore this possibility, we examined the effect of ET-1 on protein tyrosine phosphorylation. In the absence of ET-1, a dramatic increase in tyrosine phosphorylation was observed as FN density was raised from 10 to 100 ng/well (Fig. 4). However, no significant difference was detected in cells plated on 100 vs. 500 ng FN/well, that is, no gross change in protein tyrosine phosphorylation under conditions in which CSK stiffness was significantly altered. These results also suggest that the level of tyrosine phosphorylation induced by ECM attachment alone may saturate at 100 ng FN/well in these cells. On ET-1 addition, no significant changes in phosphorylation levels were observed in cells plated on low FN density (Fig. 4). However, a significant increase in tyrosine phosphorylation was detected in bands migrating with molecular weights of ~120 and 60 kDa in cells on both moderate and high FN densities compared with their controls. The degree of phosphorylation induced by ET-1 was similar in both conditions, with increases in the 60- and 120-kDa regions of 1.5- and 2-fold, respectively. These results suggest that ET-1 apparently is capable of activating intracellular signaling systems to a similar level in cells plated on moderate or high FN density. Yet, ET-1 produces a greater effect on cell contractility on the moderate FN density. It is also important to note that, regardless of its effects on tyrosine phosphorylation, ET-1 did not stimulate growth in cells plated on any of the FN coating densities, as determined by quantitating incorporation of [3H]thymidine into DNA (Fig. 5). In contrast, PDGF, a potent PVSM cell mitogen, induced a large increase in DNA synthesis under similar culture conditions. These results indicate that the effects of ET-1 on tyrosine
DISCUSSION

PVSM cell hyperplasia, enhanced contractility, and increased ECM deposition are classic features of pulmonary vessels in patients with persistent pulmonary hypertension of the newborn. Although aberrant production of growth factors and vasoconstrictors has been implicated in the development of this disease, the deposition of ECM molecules in the arterial wall is commonly considered to be a consequence of cell overgrowth and stress-induced tissue remodeling. In this study, we explored the possibility that ECM may be actively involved in the development of the hypercontractile state of PVSM cells that characterizes this disease. Specifically, we asked whether the contractile response of PVSM cells can be altered by modulation of cell-ECM contacts in vitro. MTC was used to quantitate changes in contractility within PVSM cells cultured on rigid plastic dishes coated with different ECM densities. This approach offers the advantage over other in vitro contractility assays in that thousands of individual cells can be analyzed simultaneously while cell-ECM binding interactions are varied in a controlled manner.

Using this approach, we found that known vasoconstrictors, such as ET-1, induced a time-dependent increase in CSK stiffness within individual adherent cells whereas vasorelaxants (e.g., cGMP analogs) induced a rapid increase in CSK flexibility. Studies carried out in parallel to measure tension generation directly within PVSM cells cultured within floating collagen gels demonstrated a direct correlation between changes in CSK stiffness measured by MTC and changes in cell tension (i.e., gel contraction). However, the CSK stiffness values we measured represent the total integrated stiffness of the entire CSK network of which actomyosin filaments are only one part. CSK stiffness therefore can be influenced by a number of factors (e.g., changes in cell spreading, the number or location of ECM attachment sites, cytoskeletal reorganization or remodeling, changes in composition or chemical state of the focal adhesion complex, etc.). Therefore, the specific molecular mechanism by which ECM modulates CSK stiffness and cell tension remain unclear. However, regardless of the mechanism, our results show that CSK stiffness correlates directly with PVSM cell shortening and thus provides a valid measure of contractility in these cultured cells. MTC may therefore provide an effective and quantitative way to record contractility within cultured cells as well as a tool to screen for potential soluble vasomodulators.

The finding that PVSM cells responded to cGMP analogs by both decreasing their CSK stiffness and relaxing precontracted collagen gels confirms that cultured cells maintain some degree of basal tone or internal prestress in their CSK. Prestress also has been demonstrated by MTC in other types of cells, including capillary endothelial cells and arterial smooth muscle cells (32, 33). In those studies, prestress increased with the degree of cell spreading and was greater in contractile cells than in noncontractile cells. Similarly, PVSM cells also exhibited higher stiffness when attached and spread on a high ECM density, which could then be reduced on treatment with vasorelaxants. The fact that the cGMP-induced relaxation was the greatest in cells on high FN reveals that the higher level of CSK stiffness measured in these cells is, in part, due to ECM or cell shape-dependent activation of the contractile apparatus. The component of CSK stiffness that remained after cGMP treatment may be due to the phosphorylation events may be uncoupled from its effects on both cell growth and contractility in PVSM cells.

Fig. 4. Effect of ET-1 on protein tyrosine phosphorylation. Cells were plated on low (10), moderate (100), or high (500) FN concentrations (ng/well) for 4 h and either left alone (lanes 1-3) or stimulated with 20 nM ET-1 for 1 min (lanes 4-6). Whole cell lysates were prepared and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins transferred to nitrocellulose membrane were analyzed in Western blots using monoclonal anti-phosphotyrosine antibodies and visualized using chemiluminescence. Ordinate shows molecular mass in kDa.

Fig. 5. Effects of ET-1 on DNA synthesis in PVSM cells. Serum-deprived cells were plated on high FN (500 ng/well) in the defined medium containing either ET-1 (20 nM) or platelet-derived growth factor (PDGF; 5 ng/ml) and 1 µCi/ml [3H]thymidine. Radioactivity incorporated into trichloroacetic acid-precipitable material was measured 24 h after drug addition and normalized for cell number as described in MATERIALS AND METHODS, cpm, Counts/min.
passive mechanical properties of CSK, which, in turn, appear to be governed by cell shape and CSK organization (32). These results are consistent with a mathematical description of CSK mechanics based on tensegrity architecture recently reported by Stamenovic et al. (30) in which prestress and CSK architecture were found to be the key determinants of CSK stiffness. In this context, vasoregulators modulate prestress whereas ECM modulates both basal stiffness and the contractile response to soluble vasoregulators.

Interestingly, we observed that the contractile effect induced by ET-1 was highest in cells plated on a moderate FN density (Fig. 3). Currently, we do not know the reason for this selective response to ET-1. One possibility is that the number or arrangement of available contractile elements is optimal in moderately spread cells adherent to this ECM density. Alternatively, this difference in responsiveness could be due to ECM density-dependent differences in signal transduction (e.g., Rho-dependent changes in myosin light-chain phosphorylation, changes in intracellular calcium). The finding that cells on high FN could still increase CSK stiffness when treated with ionomycin to increase intracellular calcium demonstrates that the contractile elements are available and capable of generating a higher level of CSK stiffness (isometric tension) than in cells on the moderate ECM density. One possible explanation for the relative decrease in responsiveness to ET-1 in cells on high FN therefore may be that calcium is limited or uncoupled from the contractile cascade in these cells; however, this remains to be demonstrated experimentally.

Interestingly, the effects of ET-1 on contractility did not correlate with any change in protein tyrosine phosphorylation in the present study and were not accompanied by an increase in cell growth. Similar results were also obtained in studies with rat aortic smooth muscle cells (12). The lack of mitogenicity is most likely due to the fact that we used serum-free, defined medium in the present study; past work has demonstrated that ET-1 requires serum factors to stimulate cell growth in vitro as well as in vivo (20). The identity of proteins phosphorylated by ET-1 was not investigated in this study. However, given findings in other cell types (29, 35), focal adhesion kinase and paxillin, signaling proteins localized to focal adhesions, are likely to represent at least a subset of those proteins undergoing tyrosine phosphorylation. It is also possible that phosphorylation of certain proteins that are not detectable in our assay could mediate the differential response we observed.

Regardless of the molecular mechanism, the results presented here show that ECM can modulate basal tone in PVSM cells as well as their response to soluble vasoregulators. ECM may exert these effects by modulating signal transduction or altering resting mechanical tension that is transmitted across integrins (23, 31) and thereby increasing isometric tension directly. Importantly, the finding that PVSM cell contractility can be significantly altered depending on the degree of cell binding to ECM, in the presence or absence of soluble vasoregulators, raises an intriguing possibility regarding the etiology of hypertensive disease. We postulate that increased deposition of ECM molecules (e.g., FN, collagen, tenasin, osteonectin) in the vascular wall would produce a higher level of prestress in PVSM cells, in addition to promoting cell extension and growth. Increased cell-ECM contacts also might enhance PVSM cell sensitivity to soluble vasoconstrictors and hence initiate to a positive feedback loop that could facilitate the development of pulmonary vascular hypertension.

This work was supported by National Heart, Lung, and Blood Institute Grants HL-56398 and HL-33009. Present address of K. M. Lee: The Ben May Institute for Cancer Research, University of Chicago, Chicago, IL 60637. Address for reprint requests: D. Ingber, Enders 1007 Children’s Hospital, 300 Longwood Ave., Boston, MA 02115. Received 29 April 1997; accepted in final form 18 September 1997.

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