Microtubule assembly is regulated by externally applied strain in cultured smooth muscle cells

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SUMMARY

Mechanical forces clearly regulate the development and phenotype of a variety of tissues and cultured cells. However, it is not clear how mechanical information is transduced intracellularly to alter cellular function. Thermodynamic modeling predicts that mechanical forces influence microtubule assembly, and hence suggest microtubules as one potential cytoskeletal target for mechanical signals. In this study, the assembly of microtubules was analyzed in rat aortic smooth muscle cells cultured on silicon rubber substrates exposed to step increases in applied strain. Cytoskeletal and total cellular protein fractions were extracted from the cells following application of the external strain, and tubulin levels were quantified biochemically via a competitive ELISA and western blotting using bovine brain tubulin as a standard. In the first set of experiments, smooth muscle cells were subjected to a step-increase in strain and the distribution of tubulin between monomeric, polymeric, and total cellular pools was followed with time. Microtubule mass increased rapidly following application of the strain, with a statistically significant increase ($P<0.05$) in microtubule mass from 373 ± 32 pg/cell ($t=0$) to 514 ± 30 pg/cell ($t=15$ minutes). In parallel, the amount of soluble tubulin decreased approximately fivefold. The microtubule mass decreased after 1 hour to a value of 437 ± 24 pg/cell. In the second set of experiments, smooth muscle cells were subjected to increasing doses of externally applied strain using a custom-built strain device. Monomeric, polymeric, and total tubulin fractions were extracted after 15 minutes of applied strain and quantified as for the earlier experiments. Microtubule mass increased with increasing strain while total cellular tubulin levels remained essentially constant at all strain levels. These findings are consistent with a thermodynamic model which predicts that microtubule assembly is promoted as a cell is stretched and compressional loads on the microtubules are presumably relieved. Furthermore, these data suggest microtubules are a potential target for translating changes in externally applied mechanical stimuli to alterations in cellular phenotype.

INTRODUCTION

Mechanical signals are critical regulators in the development and function of a variety of tissues. Mechanical forces are known to influence organ morphogenesis (Beloussov et al., 1994), bone resorption and formation (Duncan and Turner, 1995), skeletal muscle differentiation and organization (Simpson et al., 1994), and development of the central nervous system (Van Essen, 1997). Increasing evidence suggests that cells mediate the response to such mechanical information, and they can respond by altering their rates of proliferation, phenotype, and extracellular matrix production in response to mechanical stress. For example, smooth muscle cells (SMCs) subjected to mechanical strain in vitro show increased proliferation (Sumpio and Banes, 1988; Smith et al., 1994; Birukov et al., 1995) and increased collagen production (Sumpio et al., 1988), while adherent endothelial cells exposed to shear stresses respond by reorganizing their cytoskeleton, activating ion channels, and altering gene expression (Davies and Tripathi, 1993). These cellular responses to mechanical information have important implications not only for normal development, but also in the pathogenesis of such diseases as atherosclerosis and hypertension.

While the mechanisms by which this mechanical information is transmitted intracellularly to alter gene expression remain unclear, a potential target for mechanical signals is the cytoskeleton. A direct linkage between the extracellular matrix (ECM) and the cytoskeleton mediated by integrins that recognize specific amino acids sequences in the ECM (Ruoshlati, 1991; Juliano and Haskill, 1993) may act as one route for the transmission of external mechanical information from the outside to the inside of a cell (Ingber et al., 1993). The nuclear matrix is also structurally interconnected with the cytoskeleton, and this structural

coupling may account for changes in gene expression associated with cytoskeletal reorganization (Sims et al., 1992).

Evidence that cytoskeletal mechanics and organization are altered in response to mechanical stimuli has been reported for cultured endothelial cells exposed to both shear stresses (Thoumine et al., 1995) and twisting torques (Wang et al., 1993; Wang and Ingber, 1994). An attractive model to explain these results is based on an interaction between tension-generating structures and compression-resistant elements (Ingber et al., 1994). Specifically, tension generated by actin-myosin interactions is balanced by the compression resistance of microtubules (MTs) and contacts with the ECM. External mechanical stimuli applied to cells are superimposed on this pre-existing force balance between cells and their adhesion sites (Ingber, 1993). Theoretical models of cytoskeletal filament assembly based on this model of tensional integrity suggest that externally applied physical forces transduced to the cytoskeleton would result in alterations in cytoskeletal filament assembly and organization (Buxbaum and Heidemann, 1988; Stamenovic et al., 1996; Coughlin and Stamenovic, 1997).

MTs are a likely cytoskeletal target for external mechanical stimuli that are transduced intracellularly, and could serve as a site where these mechanical signals could be converted into alterations in gene expression. MT rearrangements and organization have been extensively studied in the process of neuritogenesis and clearly contribute to neurite outgrowth (Drubin et al., 1985; Zheng et al., 1993; Tanaka et al., 1995). Thermodynamic modeling of MT assembly in developing neurites predicts that increased compressive loads on MTs will drive depolymerization unless the tubulin monomer concentration is also increased (Buxbaum and Heidemann, 1988). In vitro studies indicate that alterations in the loads placed on MTs during growth cone advance are responsible for integrating MT assembly with this process (Dennerll et al., 1988; Zheng et al., 1993; Tanaka et al., 1995), providing support for the predictions of this thermodynamic model. MTs have also been implicated in the control of cell shape and spreading in other cell types, including hepatocytes (Mooney et al., 1995) and macrophages (Rosania and Swanson, 1996), supporting the notion that loads can be transferred from the cytoskeleton to the ECM in cells with an intact MT network.

Previous studies suggest a role for MTs in transduction of mechanical signals, but there is little direct evidence that MT assembly is indeed regulated by external mechanical forces. In one previous study, this hypothesis was studied indirectly by quantifying MT assembly while varying the density of ECM ligands to which hepatocytes were attached (Mooney et al., 1994). At higher ligand densities, the cells were more spread, presumably resulting in a decreased load on MTs as more load is transferred from the cytoskeleton to the ECM via the increased number of cell-ECM contacts (Ingber and Jamieson, 1985; Joshi et al., 1985; Heidemann and Buxbaum, 1990). In agreement with the predictions of a thermodynamic model for MT assembly (Buxbaum and Heidemann, 1988), the net result was a decreased mass of monomeric tubulin (Mooney et al., 1994).

To more directly address the hypothesis that MT assembly can be directly regulated by mechanical stimuli, in the current study we have investigated MT assembly in cells cultured on substrates subjected to mechanical strain. Smooth muscle cells (SMCs) are used as a model cell system in this study as they are found in dynamic mechanical environments in vivo, and previous studies with SMCs have indicated that mechanical strain has a clear effect on gene expression (Wilson et al., 1995; Smith et al., 1995; Birukov et al., 1995; Sumpio and Banes, 1988). The results of the present study indicate that MT assembly is promoted in SMCs subjected to a positive strain in the surface to which these cells are adherent. This assembly occurs rapidly after the application of strain from the pool of monomeric tubulin present in the cytoplasm, and the size of this pool decreases 4- to 5-fold within 15 minutes. These findings are consistent with models predicting that stretching the surface to which a cell is adherent will reduce the compressive forces placed on the MTs, driving the net assembly of MTs from monomeric tubulin.

MATERIALS AND METHODS

Smooth muscle cell culture

SMCs were isolated from rat aortas using an adaptation of a previously published technique (Rothman et al., 1992). In brief, the descending aortas of 300-350 g adult male Lewis rats (Charles River Laboratories, Wilmington, MA) were dissected free and excised. Fat, adventitia, and connective tissue surrounding the arteries were removed by blunt dissection. Following a longitudinal cut to open the artery, the tissue was cut into multiple small pieces and incubated for 90 minutes at 37°C in a sterile spinner flask (100 ml, Bellco Glass Inc., Vineland, NJ) containing an enzymatic dissociation buffer. This buffer contains 0.125 mg/ml elastase (Sigma Chemical Co., St Louis, MO), 1.0 mg/ml collagenase (CLS type I, 204 units/mg, Worthington Biochemical Corp., Freehold, NJ), 0.250 mg/ml soybean trypsin inhibitor (type 1-S, Sigma), and 2.0 mg/ml crystallized bovine serum albumin (BSA, Gibco/Life Technologies, Gaithersburg, MD). The resultant tissue suspension was filtered through a 100 μm Nitex filter (Tetko, Inc., Briarcliff Manor, NY) and centrifuged at 200 *g* for 5 minutes. The pellet was resuspended in Medium 199 (Gibco) supplemented with 20% fetal bovine serum (FBS) (Gibco), 2 mM L-glutamine (Gibco), 100 units/ml penicillin (Gibco), and 0.1 mg/ml streptomycin (Gibco). A typical isolation yields approximately 6-8×10^5 cells, with greater than 90% viability. SMCs were maintained in growth media containing 20% FBS until the first passage, while all subsequent cultures were grown in the presence of 10% FBS. Cells were maintained and expanded in culture using standard methodologies. SMCs between passage 4 and 10 were used in all experiments.

Extraction of insoluble and total tubulin fractions from cultured cells

MTs and total tubulin were extracted from cultured cells using an adaptation of a previously published technique (Caron et al., 1985; Mooney et al., 1994). To isolate polymeric tubulin (referred to as the cytoskeletal fraction), cells were washed in a MT stabilization buffer (MTSB) and incubated twice for fifteen minutes with MTSB + 0.1% Triton X-100 to remove monomeric tubulin; the remaining cytoskeleton was subsequently solubilized in a lysis buffer. MTSB contains 0.1 M piperazine-N,N′-bis(2-ethanesulfonic acid) (Pipes), pH 6.75, 1 mM ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), 1 mM $MgSO_4$, 2 M glycerol, and protease inhibitors (10 mg/ml leupeptin, 10 mg/ml aprotinin, 5 mg/ml pepstatin A, and 0.5 mM phenylmethlsulfonyl fluoride). The lysis buffer contains 25 mM Tris (hydroxymethyl) aminomethane [Tris]-HCl pH 7.4, 0.4 M NaCl, and 0.5% sodium dodecyl sulfate (SDS). All components for both the MTSB and lysis buffer were from Sigma. Total tubulin was extracted by adding lysis buffer directly to intact

cells. All extraction steps were performed at 37°C with prewarmed reagents. Tubulin masses in the cytoskeletal and total cellular lysates were subsequently quantified using either a previously published competitive enzyme-linked immunosorbent assay (ELISA) (Thrower et al., 1991) or quantitative western blots. Monomeric tubulin mass was calculated by subtracting the MT mass from the total tubulin mass determined in cells maintained under the same experimental condition, as previously described (Caron et al., 1985; Mooney et al., 1994). Tubulin masses were normalized for cell number in these studies by determining the number of adherent cells attached in parallel plates using a Coulter counter. Triplicate wells for cell counts were subjected to the same number of washes as those wells used for protein extraction in order to get an accurate representation of the cell number from which the proteins were isolated.

Quantification of tubulin mass by a competitive ELISA

The ELISA technique (adapted from Thrower et al., 1991) was performed by loading serial dilutions of cellular extracts and bovine brain tubulin standard (Molecular Probes, Eugene, OR) into a 96-well microtiter assay plate (Corning 96-well flat bottom ELISA plate, Corning Costar Corp., Cambridge, MA), followed by the addition of a mouse monoclonal anti-β-tubulin antibody. Two different monoclonal antibodies (one from Boehringer-Mannheim, Indianapolis, IN, and the other from Amersham Life Science, Arlington Heights, IL) were used in this assay, yielding similar results. After a one hour incubation, a fixed volume of this pool of antibody and tubulin was transferred to a fresh microtiter plate that had been previously coated with a tubulin standard solution of 6 µg/ml. Residual unbound antibody can now bind to the standard on the surface of the 96-well plate. An enzyme-coupled 2° antibody (either horseradish peroxidase-conjugated goat anti-mouse immunoglobulin or alkaline-phosphatase conjugated goat anti-mouse immunoglobulin, both from Bio-Rad Laboratories, Hercules, CA) was then added, followed by the substrate for the enzyme (Bio-Rad), resulting in the development of a colored reaction product which is inversely proportional to the amount of tubulin in the original sample. A standard curve was generated by plotting absorbance values as a function of tubulin standard concentration. Experimental samples were analyzed by plotting absorbance values as a function of cell concentration. For a given absorbance value, the corresponding tubulin concentration was obtained from the linear portion of the tubulin standard curve, and the cell concentration was obtained from the linear portion of the experimental sample plots. Tubulin levels on a per cell basis in experimental samples were then determined by dividing the tubulin concentration by the cell concentration. Using the absorbance values over a range of dilutions to determine the tubulin concentration in the experimental samples ensures that the absorbance values are in the linear region, and that the signal is not saturated. The 1° antibodies used to quantify tubulin in these studies were raised against bovine brain tubulin, the standard used in these experiments. The affinity of these 1° antibodies for rat smooth muscle tubulin may differ relative to the brain standard; hence, the values for tubulin mass reported here should be considered relative values.

Quantification of tubulin mass by western blotting

Cytoskeletal and total protein fractions were also analyzed using standard western blotting techniques. Extracted protein was loaded on a per cell basis (5,000 cells worth of protein/well) and electrophoresed in 10% SDS polyacrylamide gels according to the method of Laemmli (1970). After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad) for 1 hour using a Bio-Rad MiniBlot apparatus (Bio-Rad). Membranes were subsequently blocked using a 5% dried milk blocking solution, washed in a Tris buffered saline solution containing 0.1% Triton X-100 (TBS-T), and then exposed to a monoclonal anti-β-tubulin antibody diluted 1:500 in TBS-T for 1 hour. The blots were then exposed to an HRP-conjugated goat anti-mouse immunoglobulin (Bio-Rad) for 1 hour.

Proteins were detected by means of a chemiluminescence detection kit (Amersham) and visualized by capturing the emitted light on Hyperfilm ECL (Amersham). Blots were exposed for variable lengths of time to ensure that the films gave a linear response within the range of tubulin concentrations examined. Blots were scanned and quantified from densitometric analysis using NIH Image software (National Institutes of Health).

Validation of quantitative tubulin assays using nocodazole and paclitaxel

In order to validate the assays utilized to quantify tubulin pools within cells, control experiments were performed in which SMCs were exposed to either paclitaxel (15 µM, Sigma) or nocodazole (10 µg/ml, Sigma) to alter the distribution of tubulin between the cytoskeletal and total protein fractions. Nocodazole induces disassembly of MTs (Joshi et al., 1985; Middleton et al., 1988) while paclitaxel stabilizes, and in some cases promotes, MT assembly (Schiff and Horwitz, 1980). Screening experiments were performed in order to verify the action of these agents in SMCs. Cells cultured in Lab-Tek chamber slides (Nalge-Nunc Intl, Naperville, IL) in the presence of either nocodazole or paclitaxel were subsequently permeabilized, fixed, and their MTs stained using indirect immunofluorescence methods. These screening experiments allowed determination of the optimal amount of time required for paclitaxel to stabilize MTs and for nocodazole to induce significant MT disassembly. SMCs were subsequently plated at a density of 20,000-25,000 cells/cm^2 in 6-well tissue culture polystyrene dishes and allowed to adhere for 24-48 hours. Cells were then refed with medium containing either nocodazole or paclitaxel and incubated for the times determined in the initial screening experiments. Protein extracts were prepared in triplicate and the distribution of tubulin quantified using both the ELISA and western blotting assays as described above.

Application of step-increase in strain over time to the cultured SMCs

In the first set of mechanical strain experiments, cells were plated at densities between 40,000-60,000 cells/cm^2 on 6-well culture dishes made of silicon rubber precoated with type I collagen (Flex I plates, Flexcell Intl Corp., Hillsborough, NC) and then allowed to attach and grow for approximately 48 hours. These dishes were then exposed to a single step-increase in strain with a maximal strain of 20% using the Flexercell FX-2000 Mechanical Strain Unit (Flexcell Intl Corp.) and held in the strained position over the course of the experiment. Cytoskeletal and total protein fractions were extracted from SMCs held in the strained position using the methods described above. Samples were extracted in triplicate at $t=0$ (static), $t=1$ minute (after application of strain), $t=15$ minutes, $t=60$ minutes, $t=24$ hours, and $t=72$ hours.

A device to apply a uniform strain to cultured cells

A cellular strain device was designed and fabricated to subject cells to increasing doses of uniform strain. The device consisted of a set of 6 cylindrical Teflon rods embedded into a polycarbonate sheet, with two additional bolts extending upwards out of the polycarbonate sheet (Fig. 1). A 6-well culture dish (Bio-Flex plate, Flexcell Intl Corp.) with a silicon rubber substrate fit snugly into an aluminum collar, which then fit over the two bolts extending upwards from the polycarbonate sheet. The culture surface was subsequently strained by loading the 6-well dish over the Teflon cylinders, and the dish was secured in place by two wing nuts that clamp the aluminum collar to the bolts on the polycarbonate sheet. The amount of strain was manipulated by controlling the distance between the polycarbonate sheet and the culture dish. Strain in the silicon rubber membrane and cellular strain were determined by performing a two-dimensional finite strain analysis as previously described (Barbee et al., 1994; Lee et al., 1996). In brief, fluorescent microspheres (1 µm, Yellow-Green Fluorospheres, Molecular

Probes) were allowed to non-specifically adhere to the surface of the membrane. Triads of spheres in various locations were selected for measurement. Radial and circumferential components of the strain were calculated based on the displacement of the spheres in each triad. In order to visualize the boundaries of individual cells, 10% of the cells were labeled with a fluorescent dye prior to plating the cells on the flexible substrate. A subpopulation of adherent cells was stained for 2 hours at 37°C with the membrane-specific lipophilic dye DiI (Molecular Probes), at a concentration of 0.025 mg/ml in Medium 199. Both stained and nonstained cells were trypsinized, combined at a ratio of 1:10, and plated at a density of 45,000-55,000 cells/cm^2 onto the flexible 6-well culture dish coated with type-I collagen. Cells were allowed to adhere and spread for 36-48 hours. Fluorescent microspheres were non-specifically attached to cells by incubating for 15 minutes at a dilution of 1:333 in PBS supplemented with 1 mM CaCl$_2$ and 5 mM dextrose. The cells were thoroughly washed immediately before strain measurements to remove non-adherent microspheres. Triads of spheres on single cells were selected for measurement, and the radial and circumferential components of the strain were calculated based on the displacement of the spheres in each triad. The strain components were averaged to yield an average cellular strain. Statistical analysis of data was performed using the nonparametric unpaired Mann-Whitney test with a one-tailed P value on InStat Software (GraphPad Software).

Application of increasing doses of strain to the cultured SMCs

For this set of experiments, cells were plated at densities between 10,000-50,000 cells/ cm^2 on 6-well culture dishes made of an ultra-thin silicon rubber membrane precoated with type I collagen (BioFlex plates, Flexcell Intl Corp.). After 48 hours of static culture, these dishes were exposed to single step-increases in applied strain using the custom-built cellular strain device. Cytoskeletal and total protein fractions were extracted from SMCs held in the strained position using the methods described above. Samples were extracted in triplicate 15 minutes after initiation of the strain and analyzed for polymeric and total tubulin content.

RESULTS

Confirmation of ELISA methodologies to measure tubulin masses

In the first experiment, the accuracy of the ELISA method for quantifying tubulin in cell extracts was compared to results obtained using western blotting. Cells were exposed to chemicals which are known to promote MT assembly (paclitaxel) or disassembly (nocodazole) for this study. Cytoskeletal and total protein fractions were extracted from SMCs cultured in standard 6-well tissue culture polystyrene dishes after exposure to either nocodazole (10 µg/ml, 1 hour) or paclitaxel (15 µM, 4 hours). Quantification of the distribution of tubulin in these samples using the competitive ELISA showed the expected decrease in MT mass following exposure to nocodazole and the increase in MT mass following exposure to paclitaxel (Fig. 2A). Similar results were obtained with western blotting (Fig. 2B). The slight differences in the MT masses determined by these two methods were not statistically significant ($P>0.05$). To visually confirm the action of these chemical agents, cells were permeabilized, fixed, and the MT arrays stained using standard immunofluorescence methodologies. Control cells showed intact MTs (Fig. 2C, panel 1). SMCs exposed to nocodazole showed disassembled MTs (Fig. 2C, panel 2),
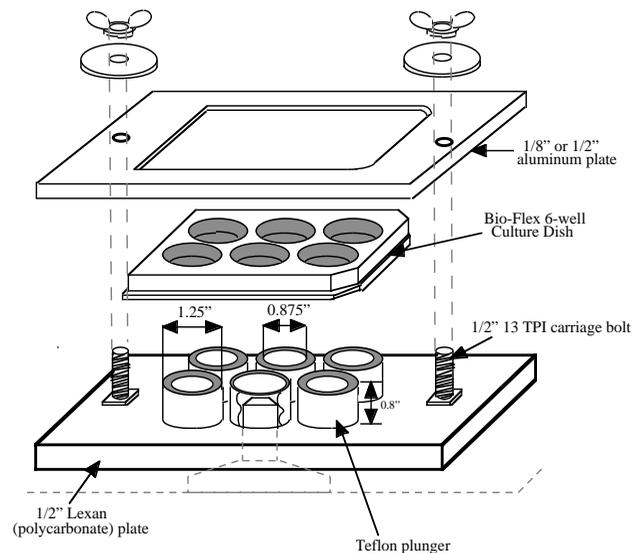
Fig. 1. Schematic of the cell stretching device. The device consists of a set of 6 Teflon rods embedded in a polycarbonate sheet. A 6-well culture dish with a flexible substrate (Bio-Flex plate, Flexcell Intl Corp.) is placed in an aluminum collar, loaded over the Teflon rods, and clamped into place with a wing nut/carriage blot assembly. The degree of strain is controlled by clamping the plate down to specific distances, with the distance controlled by aluminum spacers between the polycarbonate sheet and the aluminum collar.

while those exposed to paclitaxel showed intact MTs with unusual MT bundles characteristic of taxol-treated cells after a few hours (Fig. 2C, panel 3).

MT assembly in SMCs exposed to a step-increase in externally applied strain

In the first set of external strain experiments, SMCs were exposed to a step-increase in strain and held in the strained position using a commercially available strain unit (Flexcell Intl Corp.) as described in Materials and Methods. Cells attached and spread on the type I collagen-coated flexible substrates with a similar plating efficiency as cells on standard tissue culture polystyrene (data not shown). After 48 hours of static culture, SMCs cultured on these substrates were subjected to a step-increase in strain. A competitive ELISA was used to quantify changes in the mass of monomeric, polymeric, and total tubulin levels over time. Values reported here represent the mean and standard error from 3 separate experiments in which both the polymeric and total protein fractions were extracted in triplicate. The initial MT mass of SMCs in static culture (i.e. in unstretched cells) was determined to be 373±32 pg/cell. The total amount of cellular tubulin in static SMCs was determined to be 521±15 pg/cell. Approximately 25% of the total cellular tubulin in control cells existed in unpolymerized form (130±60 pg/cell). The assembly of MTs in SMCs following the application of a step-increase in strain to the culture surface was monitored over time. The mass of total cellular tubulin did not change significantly up to 1 hour after the application of strain (Fig. 3A). Measurements of total cellular tubulin at later time points (24 and 72 hours) also revealed no significant changes in tubulin mass (data not shown). The MT mass, however,

increased almost immediately following application of the strain, with a small but reproducible change in mass from $t=0$ (373±32 pg/cell) to $t=1$ minute (409±38 pg/cell) (Fig. 3B). A continued increase in MT mass was observed 15 minutes after the initial exposure to the applied strain, with a statistically significant increase ($P<0.05$) to 514±30 pg/cell at $t=15$ minutes (Fig. 3B). In parallel with the findings of increased MT assembly, the mass of monomer decreased approximately 5-fold after 15 minutes exposure to the strain from a value of 130±60 pg/cell at $t=0$ to 17±22 pg/cell at $t=15$ minutes (Fig. 3C). Longer exposures to the applied strain resulted in a decreased MT mass, with a value of 437±24 pg/cell after 1 hour (Fig. 3B). In parallel, the results revealed a subsequent increase in the amount of monomeric tubulin within the cytosol, with a value at 1 hour (63±1 pg/cell) similar to that of unstretched cells (Fig. 3C). Samples analyzed by quantitative western blotting verified the results obtained with the competitive ELISA (data not shown).

## Characterization of a device to apply a uniform strain to cultured cells

A device was designed and fabricated for these studies in order to subject the cells to a uniform, equibiaxial strain (Fig. 1). The commercial unit used in the time-dependent step-increase experiments subjects cells to an uneven strain across the culture surface, with increasing strain values at increasing radial distances from the center of each culture

well (Gilbert et al., 1994). The details of the design and characterization of the new custom-built device utilized in these studies are provided in Materials and Methods. The strain imparted to the silicon rubber substrate was linear with respect to the vertical displacement of the membrane in the range tested (Fig. 4A). Strain measurements of the plates confirmed a true biaxial strain (i.e. the mean circumferential strain (E_{cc}) and the mean radial strain (E_{rr}) were approximately equal) (Fig. 4a). The shear component of strain (E_{rc}) is a measure of the twisting force in the flexible membrane, and was determined to be negligible in this device. SMCs attached to the type I collagen-coated BioFlex plates with a similar efficiency as cells on standard tissue culture polystyrene, and subsequently spread to take on a similar morphology to cells on polystyrene (data not shown). Fluorescent microbeads were nonspecifically attached to SMCs to verify that strain in the surface to which the cells are adherent actually imparts a strain to the cells. The strain imparted to the cells was subsequently determined by measuring the displacement of triads of these fluorescent microbeads as described in Materials and Methods. Cellular strain increased in a dose-dependent manner with increases in substrate strain, with a statistically significant difference in mean cellular strain between 5% and 20% applied strain ($P<0.05$) (Fig. 4B). It is important to note that the strain in the flexible substrates was significantly higher than the strain imparted to the cells.
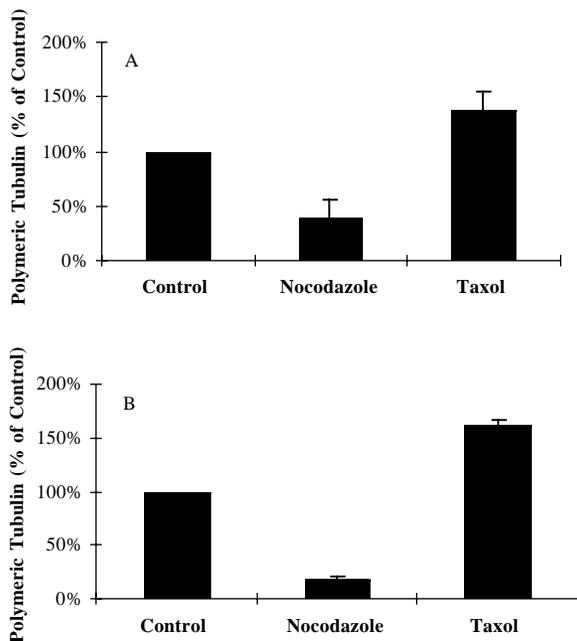
Fig. 2. The quantification (A,B) of MT mass and visualization of MTs (C, panels 1-3) in SMCs exposed to paclitaxel or nocodazole. Protein samples were extracted from cells cultured in static conditions exposed to either nocodazole (10 µg/ml, 1 hour) or paclitaxel (15 µM, 4 hours), and the distribution of tubulin quantified using both a competitive ELISA (A) and western blotting (B). Both analysis methods verify the depolymerization of MTs by nocodazole and the assembly of MTs by paclitaxel. Values represent the amount of polymeric tubulin as a percentage of that in control (untreated) cells. Samples were analyzed in triplicate with the mean and standard deviation plotted. Cells exposed to these agents were permeabilized, fixed, and visualized by immunofluorescence localization of the microtubules as described in Materials and Methods. Control cells with intact MTs (C, panel 1) are compared to cells exposed to nocodazole (C, panel 2) for 30 minutes or paclitaxel for 4 hours (C, panel 3).
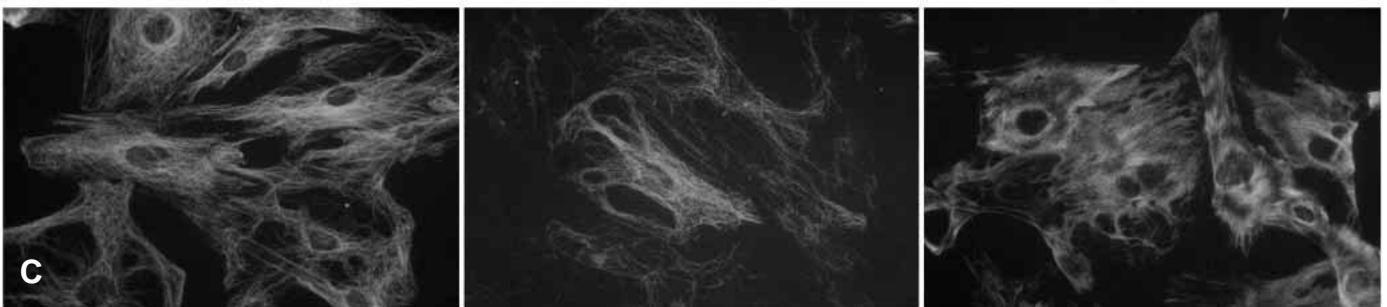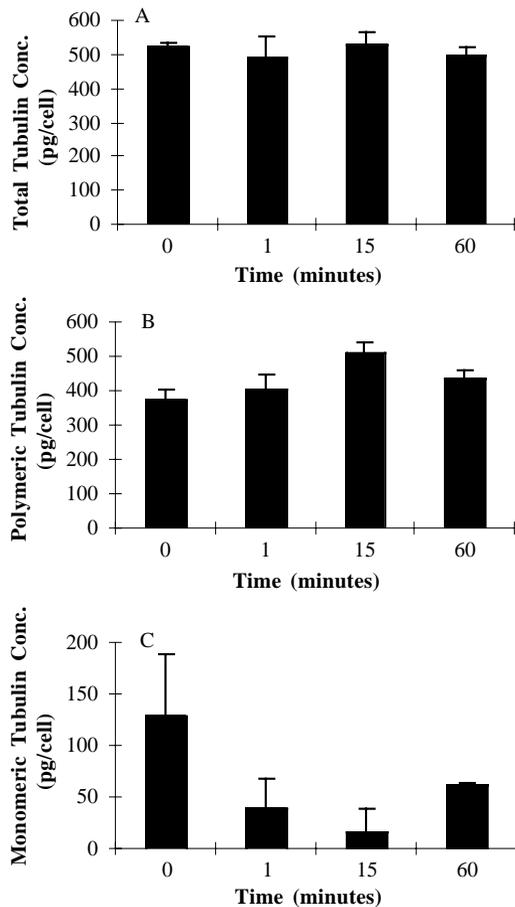
Fig. 3. The mass of total (A), polymeric (B), and monomeric (C) tubulin in SMCs over time following the application of external strain. SMCs were cultured on flexible 6-well culture dishes (Flex I plates, Flexcell Intl Corp.) and allowed to attach and spread for 48 hours before initiating strain. Protein samples were extracted from the cells as described in Materials and Methods. Values were determined by comparison to a bovine brain tubulin standard using a competitive ELISA and normalized for cell number by counting adherent cells in parallel wells. The change in total tubulin mass over time (A) was not significant. In contrast, the change in both polymeric (B) and monomeric (C) tubulin mass between t=0 and t=15 minutes were significant (P<0.05). Values represent the mean from triplicate samples in 3 different experiments. Error bars represent s.e.m.

MT assembly in SMCs exposed to increasing doses of externally applied strain

The second set of external strain experiments involved the application of increasing doses of applied strain to the cultured SMCs using this custom-built strain device. The distribution of tubulin between monomeric, polymeric, and total cellular fractions in SMCs exposed to increasing doses of applied strain was determined using both the competitive ELISA and western blotting. Values reported represent the mean and standard deviation from triplicate samples in a representative experiment as determined by the ELISA. For control cells (i.e. not strained), the masses of monomeric, polymeric, and total cellular tubulin were determined to be 124±60 pg/cell, 391±21 pg/cell, and 515±43 pg/cell, respectively. All of these values agree well with the values obtained from control cells in the previous experiments
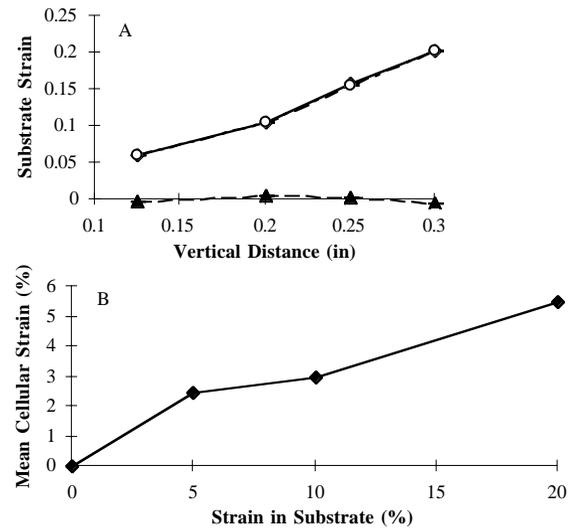
Fig. 4. Substrate strain as a function of vertical displacement (A) and cellular strain as a function of substrate strain (B). Radial (E_{rr}, ■), circumferential (E_{cc}, ○), and shear (E_{rc}, ▲) components of strain were measured as a function of the vertical displacement of a flexible 6-well dish (BioFlex plate, Flexcell Intl Corp.) using the custom-built device described in Materials and Methods (A). Radial and circumferential components of strain are superimposed on each other with no shear component of strain, indicating an equibiaxial strain in the substrate. Cellular strain was measured as a function of applied strain using a two-dimensional finite strain analysis (B). The difference in mean cellular strain between 5% and 20% applied strain is statistically significant (P<0.05). Displacements of fluorescent microbeads on the flexible substrate and on attached cells were measured optically to determine the strain as previously described (Barbee et al., 1994; Lee et al., 1996).
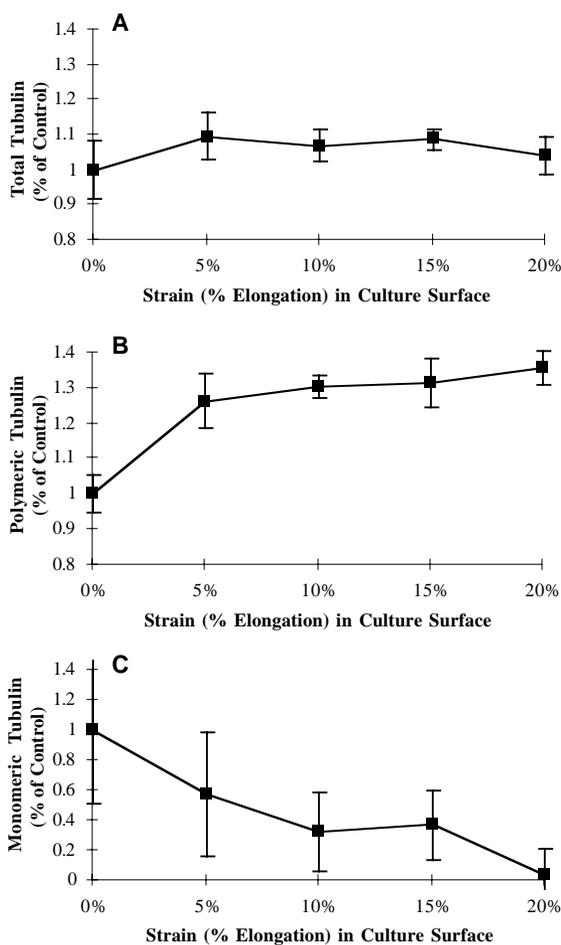
monitoring MT assembly over time. The amount of total cellular tubulin did not change significantly with increasing strain between 0% and 20% (Fig. 5A). However, the MT mass increased in a dose-dependent fashion with increasing strain. The MT mass increased monotonically between values of 391±21 pg/cell in control cells and 530±19 pg/cell in cells cultured on substrates that were strained 20% (Fig. 5B). MT mass values in all strained conditions were statistically significant (P<0.05) compared to unstrained controls, but not compared to each other (e.g. the MT mass at 5% is not statistically different from that at 20%). This increase in MT mass was paralleled by a corresponding decrease in monomer mass with increasing strain values. The monomeric tubulin within the cytosol decreased monotonically from 124±60 pg/cell in control cells to 5±20 pg/cell in cells cultured on substrates strained to 20% (Fig. 5C). At these high degrees of external strain, almost all of the tubulin monomer within the cytosol was assembled into MTs with only approximately 1% remaining in the soluble form.

DISCUSSION

The results from the present study reveal that the assembly of MTs in cultured SMCs is regulated by externally applied mechanical signals. Cells cultured on flexible substrates exposed to step-increases in strain responded by increasing the assembly of MTs a short time after the application of the strain.

In addition, SMCs cultured on surfaces exposed to increasing doses of strain displayed increasing assembly of MTs. Taken together, these results suggest that the process of MT assembly is dependent on external forces in both time and dose-dependent fashions, providing support for a thermodynamic model for MT assembly in which a decreased compressive force on MTs induces assembly of MTs from the cellular pool of monomeric tubulin (Buxbaum and Heidemann, 1988).
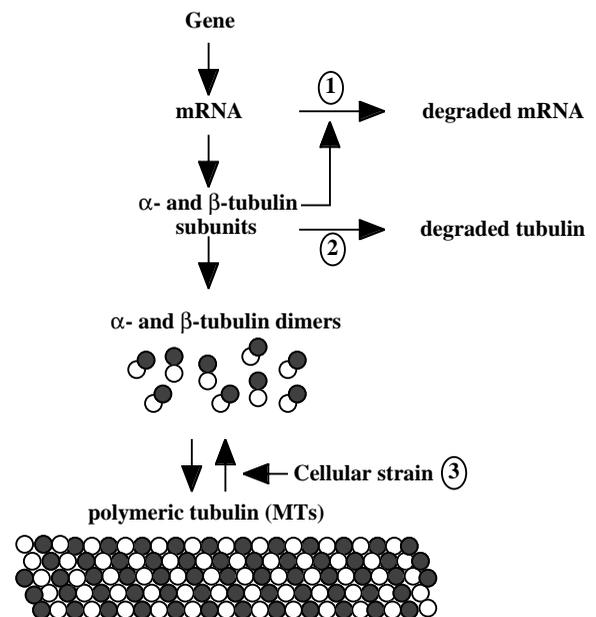
A new, simple device was developed in this study to apply mechanical strain to cultured cells. A number of commercially available devices suffer from limitations, the most serious of which is the nonuniform strain across the culture surface. The device described here is relatively simple to design and fabricate, and characterization of the device demonstrates that the strain is both uniform and equibiaxial across the majority of the culture surface. This device allows manipulation of cells in numerous culture wells at the same time, allowing for high sample numbers from the same experimental condition. In

addition, the cell stretching device fits easily into a humidified incubator. Analysis of cellular strain indicates that increasing strain in the substrate corresponds to increasing strain imparted to the cells, although the magnitude of that strain is diminished.

Our findings are consistent with models of cytoskeletal assembly in which the assembly of a cell's MTs is regulated by the mechanical load imposed on the MTs (Buxbaum and Heidemann, 1988). Previous efforts have suggested indirectly that MT assembly is controlled by a force balance between the cytoskeleton and the extracellular environment, and that net MT assembly occurs when compressive forces are transferred from the MTs to the ECM (Ingber and Jamieson, 1985; Joshi et al., 1985; Dennerll et al., 1988; Zheng et al., 1993; Mooney et al., 1994). Important findings in the current study are that MT assembly occurs within minutes, or perhaps seconds, in response to a step-increase in strain, and that the extent of MT assembly is dependent on the degree to which the cells are strained. Thermodynamic modeling of MT assembly predicts a proportionality between tubulin monomer and tubulin polymer that is a function of the compressive forces on the MTs (Buxbaum and Heidemann, 1988). Our results support this prediction by showing that the assembly process is regulated by externally applied strain, which may alter the compressive forces on the MTs. The rapidity of the response in our studies is consistent with previously measured rates of MT assembly as well (Cassimeris et al., 1988). Recent experimental evidence in acellular studies of MT assembly also demonstrates that the rate of MT elongation is diminished as increasing compressional

Fig. 5. The mass of total (A), polymeric (B), and monomeric (C) tubulin in SMCs exposed to increasing doses of externally applied strain. SMCs were cultured on flexible 6-well culture dishes (Flex I plates, Flexcell Intl) and allowed to attach and spread for 48 hours before initiating strain. Protein samples were extracted from the cells as described in Materials and Methods. Values were determined by comparison to a bovine brain tubulin standard using a competitive ELISA and normalized for cell number by counting adherent cells in parallel wells. Plotted values represent the mean from triplicate samples in a single experiment.

Fig. 6. Schematic representation of a model for tubulin gene expression as it relates to control of MT assembly. Previous studies have identified control points (1) and (2) in tubulin gene expression. (1) Represents the autoregulatory control of tubulin monomer levels by a feedback mechanism which controls the mRNA stability (Ben-Ze'ev et al., 1979; Cleveland et al., 1981; Caron et al., 1985). (2) Represents the dependence of tubulin levels on the degradation of the protein half-life, which is controlled by the extent of cell-ECM contacts (Mooney et al., 1994). The findings in the present study suggest a third control mechanism (3) for tubulin assembly, in which MT assembly is regulated by externally applied mechanical stimuli.

forces are applied to the advancing MT (Dogterom and Yurke, 1997). Interestingly, in the first set of experiments in the current study, the state of MT assembly returned to conditions similar to the control (unstrained) conditions after longer times (≥60 minutes). We hypothesize that this is due to a rearrangement of the focal adhesion sites with the ECM (Davies et al., 1994; Smith et al., 1997) over time to return to a favored basal stress state (Ingber et al., 1994). Although not directly addressed in this study, these types of cytoskeletal rearrangements are presumably reversible when the strain is removed.

Our findings coupled with recent studies with cardiac myocytes indicate that mechanical signals are responsible for both immediate and delayed cytoskeletal reorganization. Increased MT density correlates with contractile dysfunction in cardiac myocytes subjected to pressure hypertrophy (Tagawa et al., 1996; Watson et al., 1996; Tagawa et al., 1997). However, increasing MT densities are not the immediate result of stress loading in these studies. Instead, increased pressure results in an increased cardiac mass, and increased MT mass parallels the increased cardiac mass (Tagawa et al., 1997). These changes were observed on the time scale of hours to days, suggesting that chronic, cyclic mechanical signals can induce longer term effects in addition to the shorter term effects noted in the present study. These effects of chronic, cyclic mechanical stimuli are likely mediated not solely by direct changes in MT assembly, but by intracellular chemical signals which alter gene expression.

Alterations in MT assembly due to mechanical stimuli may trigger chemical signaling events that are responsible for altering gene expression and further feedback control of MT assembly. Previous studies indicate that disruption of MTs can enhance signal transduction by GTP-binding proteins (Leiber et al., 1993) and activate cellular protein kinases (Shinohara-Gotoh et al., 1991). In addition, an intact MT network can modulate the activity of myosin by influencing the phosphorylation of the myosin regulatory light chain, suggesting that disruptions in the MT cytoskeleton can trigger chemical pathways which control the force balance between actin microfilaments and MTs (Kolodney and Elson, 1995). Alternatively, it has been suggested that depolymerization of MTs may release associated proteins that can interact with actin filaments, controlling the contractility and the state of organization of the actin network (Danowski, 1989). Disruptions in the MT network have been shown to influence the expression of nerve growth factor (Baudet et al., 1995), activate the transcription factor NF-κB (Rosette and Karin, 1995), and influence the synthesis and processing of extracellular matrix molecules in fibroblasts (Evangelisti et al., 1995). Combined with the fact that MTs function as tracks for protein trafficking and mRNA localization (Suprenant, 1993; Cole and Lippincott-Schwartz, 1995), alterations in MT assembly and organization could directly alter cellular gene expression at the levels of transcription, translation, and protein transport. Although our findings support a tensional integrity model for cellular architecture, other explanations for our results based on soluble chemical mediators cannot be excluded by the data presented here. For example, mechanical signals are known to trigger tyrosine phosphorylation of focal adhesion proteins (Shyy and Chien, 1997; Schmidt et al., 1988) that may induce downstream chemical signaling events that ultimately induce changes in cytoskeletal assembly.

Mechanical-based control of tubulin assembly must operate in conjunction with the established autoregulation of tubulin gene expression. Tubulin autoregulation functions to control the amount of tubulin within a cell by a feedback mechanism that regulates the level and stability of tubulin mRNA (Ben-Ze'ev et al., 1979; Cleveland et al., 1981; Caron et al., 1985). Autoregulation functions normally in hepatocytes in which the tubulin set-point is independently regulated by the extent of cell spreading (Mooney et al., 1994). In this situation, the stability of tubulin protein is also altered to balance the effects of autoregulation on monomer availability for assembly. It is likely that a similar integration between the mechanical and autoregulatory control of tubulin assembly occurs in cells subjected to mechanical stimuli.

In summary, the results presented here indicate that MT assembly is regulated by mechanical strain applied to the cell's adhesion substrate and suggest a new control point for the process of MT assembly (Fig. 6). These results are consistent with the predictions of a thermodynamic model for MT assembly based on the tensegrity concept for cellular architecture.

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