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Hyaluronic Acid Stimulates Human Fibroblast Proliferation Within a Collagen Matrix

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Human dermal fibroblasts suspended in a collagen matrix exhibit a 4-day delay in cell division, while the same cells in monolayer divided by day 1. The initial rates of ^{3}H-thymidine incorporation by cells in monolayer or suspended in collagen were not significantly different. When suspended in collagen, there was a threefold increase in the proportion of cells in a tetraploidal (4N) DNA state compared to the same cells in monolayer. Flow cytometry analysis and ^{3}H-thymidine incorporation studies identified the delay of cell division as a consequence of a block in the G2/M of the cell cycle and not an inhibition of DNA synthesis. The inclusion of 150 μg/ml of hyaluronic acid (HA) in the manufacture of fibroblast populated collagen lattices (FPCL) caused a stimulation of cell division, as determined by cell counting; increased the expression of tubulin, as determined by Western blot analysis; and reduced the proportion of cells in a 4N state, as determined by flow cytometry. HA added to the same cells growing in monolayer produced a minimal increase in the rate of cell division or DNA synthesis. HA supplementation of FPCLs stimulated cell division as well as tubulin concentrations, but it did not enhance lattice contraction. The introduction of tubulin isolated from pig brain or purchased tubulin into fibroblasts by electroporation prior to their transfer into collagen lattices promoted cell division in the first 24 hours and enhanced FPCL contraction. It is proposed that tubulin protein, the building blocks of microtubules, is limited in human fibroblasts residing within a collagen matrix. When human fibroblasts are suspended in collagen, one effect of added HA may be to stimulate the synthesis of tubulin which assists cells through the cell cycle. J. Cell. Physiol. 177:465–473, 1998.

The introduction of the fibroblast populated collagen lattice (FPCL) model by Bell et al. (1979), fibroblasts in a three-dimensional collagen matrix. When human dermal fibroblasts are cast in a collagen matrix, they rearrange the collagen fibrils causing a reduction in the size of the FPCL contraction. There are numerous alterations in fibroblasts incorporated within a collagen lattice such as variations in protein synthesis, cell morphology, and cell division (Bell et al., 1979; Nusgens et al., 1984; Gillery et al., 1996; Yoshizato et al., 1984). As an example, fibroblasts in monolayer tissue culture within 24 hours of plating divide. In contrast, human fibroblasts suspended in collagen do not divide until the fourth day following their incorporation into collagen (Nishiyama et al., 1989; Schor, 1980; Greco and Ehrlich, 1992). On the other hand, nonprimate fibroblasts such as rat dermal fibroblasts will complete a round of cell division by day 1 when suspended in collagen lattices (Steinberg et al., 1980; Buttle and Ehrlich, 1983; Greco and Ehrlich, 1992).

Human fibroblasts suspended in collagen actively synthesize DNA, indicating that retarding the S phase of the cell cycle apparently is not responsible for delayed cell division (Walter et al., 1985). Using flow cytometry analysis, human dermal fibroblasts suspended in collagen for 2 days show a high proportion of cells blocked at the G2/M premitotic interphase of the cell cycle. These human cells are in a 4N or tetraploidal DNA state. Other fibroblast species such as rat fibroblasts are predominantly found in a 2N diploid state when suspended in collagen (Greco and Ehrlich, 1992). These results suggest that the arrest of human fibroblasts in G2/M of the cell cycle causes the delay in their advancement through mitosis.

Hyaluronic acid (HA) is a negatively charged glycosaminoglycan composed of repeated disaccharides of D-glucuronic acid and N-acetylglucosamine (Goa and Benfield, 1994). Changes in HA concentrations within the extracellular matrix modulate a variety of cellular functions, such as cell migration (Melrose et al., 1996;

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Chen et al., 1989), adhesion (Klein et al., 1996; Hall et al., 1994), and proliferation (Wiig et al., 1996; Bernard et al., 1994; Matuoka et al., 1987). HA interactions with specific cell surface receptors can modulate morphogenesis (Toole, 1997). It influences a number of pathological processes, which include tumor metastasis (Bourguignon et al., 1997) and inflammation (McKee et al., 1996). Here we report added HA promotes cell division in human fibroblasts by their passage through the cell cycles when incorporated within a collagen matrix. It is suggested that promotion of tubulin synthesis by added HA may be involved in advancing fibroblasts through the cell cycles.

Both cell division and FPCL contraction involve microtubules. The inclusion of either colchicine or vinblastine during the manufacture of FPCLs blocks lattice contraction (Bell et al., 1979; Ehrlich et al., 1989). Microtubules are also critical for cells passing through the cell cycle (Leslie, 1990). We find that added HA increases the expression of tubulin in primary human fibroblast lines and speculate that increasing intracellular tubulin pools advances cells through the cell cycle as proposed by John (1984). The possible association between HA promoting tubulin levels and cell division is explored in this article.

MATERIALS AND METHODS

Dermal fibroblasts

Human fibroblast line AG06103 was purchased from NIA Aging Cell Culture Depository (Camden, NJ) and line HF-136 was derived from the outgrowth of fibroblasts from newborn foreskin explants. The fibroblasts were studied between their 5th and 12th passages. Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 15 μg/ml gentamycin (complete DMEM) was used in both FPCL and monolayer tissue culture experiments.

Collagen

Native collagen was extracted from isolated rat tail tendons. Tendons were stirred in ice-cold 0.5 M acetic acid for 4 days. The extract was cleared by centrifugation, NaCl was added to the supernatant at 10% w/v, the mixture was stirred, and the precipitated collagen was collected by centrifugation. The precipitate was resolubilized in 0.1 M acetic acid, dialyzed against 1 mM HCl, frozen, lyophilized, weighed, redissolved in sterile 1 mM HCl at 5 mg/ml, and stored at 4°C. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the preparation was shown to contain only collagen by the Coomassie Brilliant Blue staining pattern of protein bands.

FPCL

Two milliliter FPCLs were cast containing 25,000, 80,000 or 100,000 cells according to the specific experiment. Human dermal fibroblasts maintained in monolayer culture were trypsinized, resuspended in complete DMEM, counted, and the appropriate cell number contained in 0.5 ml was combined with 1.0 ml of complete DMEM which included any supplement. Finally, a 0.5 ml aliquot of collagen solution was added, the three components were rapidly mixed together, and then poured into a 35 mm Petri dish. The dish was transferred to an incubator set at 37°C with 5% CO_2 and 95% air in an H_2O saturated atmosphere. The collagen polymerizes under these conditions, usually in less than 90 sec, trapping the cells in the rapidly forming matrix. FPCLs were measured with a ruler at 24-h intervals. The area of the FPCLs was calculated by taking the average of two diameter measurements to determine the radius of each FPCL.

Cell counting

Enzymatically released cells from collagen lattices or monolayer culture were counted. An FPCL was placed in 1 ml of 50 mM Tris HCl, pH 7.6, 100 mM NaCl, 0.1 mM $CaCl_2$, and 4 μg/ml bacterial collagenase IV (Sigma Chemical Co., St. Louis, MO). The mixture was incubated for 30 min at 37°C with gentle shaking. Both cells from monolayer culture harvested by trypsinization and the collagenase released cells were collected by centrifugation, resuspended in 1 ml of complete DMEM, and counted with a hemocytometer.

Thymidine labeling

Cell uptake of [^{3}H]-thymidine was determined in both FPCLs and monolayer cultures by adding 40 μl of 0.5 mCi [^{3}H]-thymidine (Amersham Corp., Arlington Heights, IL) in 9.5 ml of complete DMEM with 0.3 mM thymidine. After a 24-h incubation period in the [^{3}H]-thymidine medium, the cells were released from their respective culture environments by limited proteinase digest, collected by centrifugation, resuspended in complete DMEM, and the radioactivity determined in 0.1 ml aliquots with a β-scintillation counter.

Flow cytometry

Fibroblasts were released from FPCLs by collagenase digest (see above), washed twice with phosphate-buffered saline (PBS), pelleted, and resuspended in 100 μl of sterile PBS with 0.1% Triton X-100 detergent. The cell-detergent suspension was incubated with 20 μg ribonuclease (Sigma) for 30 min at 37°C to remove RNA. The nuclei were stained for 10 min at 4°C with 50 μg/ml propidium iodide (PI; Sigma). The nuclei were analyzed by a Coulter Model EPICS-725 flow cytometer and their chromosomal 2N or 4N state determined by the incidence of relative PI staining.

HA

The HA used had a molecular weight of 1.1×10^6 daltons (lot C8019) and was a gift from Genzyme Corporation (Cambridge, MA). A sterile 3 mg of HA/ml of PBS solution was prepared as a stock solution. In one experimental series, the predigestion of HA, prior to its addition to FPCL, combined 300 μg HA with 50 U hyaluronidase (Sigma) and the mixture incubated at 37°C for 1 h. The entire digest was added during the manufacture of FPCL.

Tubulin purification

Porcine brains were purchased from Pel-Freeze Biologicals (Rogers, AR). Tubulin was purified from brain homogenates using the method of Weisenberg and coworkers (1968). All purification procedures were performed at 4°C. Briefly, the brain homogenate was subjected to successive ammonium sulfate fractionations and ion exchange chromatography. The authenticity of the purified pig brain tubulin was verified by SDS-

PAGE under reducing conditions, protein transfer to a membrane and tubulin antibody probing, and Western blot analysis. Bovine brain tubulin protein was purchased from Cytoskeleton (Denver, CO) at 0.25 mg per vial.

Western immunoblotting

The protein concentrations for all SDS-PAGE experiments were determined by the Bradford method (1976). SDS-PAGE used the Tris-HCl/glycine buffer system on a Bio-Rad Minigel system (Hercules, CA) following the supplier's instructions. Protein was transferred to a PVDF membrane (Millipore Corp., Medford, MA). The detection of protein bands by immunoblotting used Amersham's ECL system according to the manufacturer's instructions.

Tubulin introduction by electroporation

Fibroblasts were harvested from monolayer culture, counted, and resuspended in complete DMEM. The cells were pelleted through a 2 ml cushion of 0.35 M sucrose at 400 × g for 2 min. The sucrose layer was aspirated away and the cells resuspended at 400,000 cells per 0.05 ml of 0.35 M sucrose. The 0.05 ml cell suspensions were mixed with 0.05 ml aliquots of pig brain purified tubulin at 2.5, 5, 10, or 15 ng of protein in 0.35 M sucrose. The combined volumes of tubulin and cells were dispensed to a cell Electroporation chamber (DEP System Inc., Troy, MI) and electroporation performed at settings of 800 V for 30 μsec (Ehrlich et al., 1991). After electroporation, cells were resuspended in complete DMEM, recounted, and immediately incorporated in FPCLs.

The incorporation of isolated tubulin into microtubules was examined by introducing fluorescent-tagged tubulin into fibroblasts. Rhodamine (Rh) isothiocynate celite, 1 mg (Sigma), was added to 0.4 ml of isolated tubulin (392 μg/ml in 0.35 M sucrose) in a 1.5 ml minicentrifuge tube at room temperature. After briefly mixing, the tube was immediately centrifuged for 1 min, and the supernatant containing the tagged tubulin was transferred to a light shielded tube. The tubulin was passed through a Sephadex G-25 (Pharmacia, Inc., Piscataway, NJ) desalting column packed in 0.35 M sucrose and filter sterilized. The Rh-tubulin was introduced into cells by electroporation as described above. The inoculated cells from the electroporation chamber were suspended in complete DMEM. The cell suspension (6 ml) was placed in a 10 cm tissue culture dish containing four 22 × 22 mm glass coverslips and the dishes placed in the incubator. At 6, 18, 24, and 48 h, the coverslips were viewed by fluorescent microscopy (Zeis IM-35 microscope with a 63 X objective) to see autofluorescence.

To confirm that the effect of tubulin and not some contaminate contained in the pig brain tubulin preparation was promoting FPCL contraction as well as releasing cells from the block in the cell cycle, purified bovine tubulin (stock T237, Cytoskeleton) was purchased. The commercial tubulin was a bovine brain preparation determined to be 99% pure by SDS gel electrophoresis. In this experiment, the purchased tubulin was compared to bovine serum albumin (Sigma) used at a concentration of 10 μg/ml of 0.35 M sucrose. HF 136 fibroblasts were processed by electroporation as described above and two FPCLs with each containing 52,000 cells inoculated with tubulin and two FPCLs with each containing 57,000 cells inoculated with bovine albumin were manufactured. The size of the FPCLs was measured over a 2-day period. The cells were counted with a hemocytometer after their release by collagenase digestion.

TABLE 1. Fibroblast number in FPCL[1]

	Initial cell number			
	100,000		25,000	
	HA	PBS	HA	PBS
Day 2	16.5 (1.6)	9.58 (0)	3.5 (1.6)	2.38 (0)
Day 3	34.8 (3.5)	18.8 (1.9)	12.0 (4.8)	6.1 (2.4)
Day 7	45.2 (4.5)	30.7 (3.1)	22.4 (9.0)	6.8 (2.7)

[1]At times indicated, cells were freed from collagen matrix and counted. The addition of HA at 300 μg (150 μg/ml) on human dermal fibroblast proliferation was compared to unsupplemented FPCLs. The number of cells is reported as cell number $\times 10^4$ per FPCL. The numbers in parentheses show the fold increases in cell number, compared to the initial cell number seeded. Initial cell numbers incorporated into collagen matrices were either 25,000 or 100,000.

RESULTS

Cell counts

Fibroblasts were released from the FPCL by collagenase digestion and counted. The cell number increased on day 2 when 150 μg/ml of HA was included in the manufacture of FPCL. In the absence of added HA, fibroblast numbers did not increase by day 2 (Table 1). Experiments with 100,000 cells, FPCL containing 150 μg/ml of HA, demonstrated an increase in cell number of 65%, 249%, and 482% at 2, 4, and 7 days, respectively. Identical lattices without added HA showed cell number increases of 0%, 88%, and 206% measured on days 2, 4, and 7, respectively (Table 1). FPCLs made with only 25,000 fibroblasts and 150 μg/ml of HA demonstrated a 96% increase in cell number compared to lattices made without HA that showed only a 9% increase on day 2. At day 4, it was 380% for the HA-treated group compared to 144% for the untreated group. At day 7, it was 794% for the HA-treated group and 170% in the HA-free group (Table 1). The lower the initial number of fibroblasts seeded in the FPCL, the greater the response to added HA.

To demonstrate that HA and not a contaminate promotes cell division in FPCLs, 36 two milliliter FPCLs were made containing 100,000 cells. These FPCLs were divided into four treatment groups of nine lattices each. Group I had 300 μg HA added (150 μg/ml); group II had 300 μg of HA preincubated with 50 U of hyaluronidase added; group III had 50 U of hyaluronidase added (25 U/ml); and group IV had buffer alone added. Three FPCLs from each treatment group were harvested daily and cell numbers determined. At day 1, only group I (HA alone) had an increase in cells (74%), while no change in cell number was recorded in the other three treatment groups (Table 2). At day 2, the fibroblasts in group I continued to proliferate and had increased by 358%. Again, no change in cell numbers was recorded in the other treatment groups. By day 4, group I showed an increase of 410%, group II (HA digest) had an increase of 220%, group III (hyaluronidase alone) had an increase of 171%, and group IV (buffer) had a 163% increase. FPCLs with buffer alone or with hyal-

TABLE 2. Effects of HA and hyaluronidase[1]

	Cell numbers ($\times 10^4$)			
	HA alone	Preincubated HA + hyaluronidase	Hyaluronidase alone	Control PBS alone
Day 2	17.4 (1.7)	9.8 (0)	9.25 (0)	9.65 (0)
Day 3	35.8 (3.6)	9.6 (0)	8.8 (0)	9.0 (0)
Day 4	41.0 (4.1)	22.0 (2.2)	17.1 (1.7)	16.3 (1.6)

[1]Numbers of fibroblasts released from 2 ml FPCLs initially seeded with 100,000 cells are reported. The FPCLs were divided into four experimental treatment groups. Treatment groups were 1) 300 μg of HA (150 μg/ml); 2) 300 μg of HA predigested with 50 U hyaluronidase; 3) 50 U (25 U/ml) of hyaluronidase alone; 4) PBS alone (control). Three FPCLs from each treatment group were collagenase digested and cells counted on days 1, 2, or 4. The proportional increase in cell number compared to the initial seeding number is presented in parentheses.

TABLE 3. Comparison of fibroblast proliferation within an FPCL and on a monolayer surface[1]

	Cell numbers ($\times 10^4$)			
	Group I	Group II	Group III	Group IV
Day 1				
FPCL	13.9 (1.7)	8.90 (0.1)	7.4 (0)	8.1 (0.1)
Monolayer	18.5 (2.3)	17.0 (2.1)	17.5 (2.2)	16.4 (2.0)
Day 2				
FPCL	17.4 (2.2)	8.5 (0.1)	7.7 (0)	9.2 (0.1)
Monolayer	40.8 (5.1)	39.2 (4.9)	38.6 (4.8)	35.2 (4.4)

[1]The ability of HA to promote fibroblast proliferation is compared between FPCLs and monolayer cultures prepared with an initial 80,000 cells. FPCLs and monolayer cultures were studied in four experimental groups: Group I, 300 μg (150 μg/ml) HA alone; group II, 300 μg of HA predigested with 50 U of hyaluronidase; group III, 50 U (25 U) hyaluronidase alone; group IV, PBS alone (control). The corresponding fold increases in cell numbers from the initial cell number are presented in parentheses. Cells from FPCLs were harvested by collagenase and cells in monolayer harvested by trypsinization.

TABLE 4. Thymidine incorporation in FPCL and monolayer[1]

	300 μg (150 μg/ml) HA	PBS control
FPCL	4,902 cpm	3,731 cpm
Monolayer	9,184 cpm	8,661 cpm

[1]Effect of HA on [^{3}H]-thymidine incorporation by 100,000 human fibroblasts cultured for 1 day either within FPCL or in monolayer culture. The level of radioactivity in cpm per FPCL or cpm per monolayer culture is given. Each number is the average of three counts for each sample.

uronidase alone showed equivalent cell numbers at day 4. FPCLs containing hyaluronidase-treated HA showed more cell division at 4 days but almost half of that seen with intact HA in group I.

Added HA to fibroblasts maintained in monolayer culture did not enhance cell division. In parallel experiments with FPCLs containing 80,000 cells, the inclusion of 150 μg/ml of HA caused a 124% increase in cell number at 24 h, compared to only a 13% increase over untreated control cells in monolayer (Table 3). At day 2, the fibroblast number in treated FPCLs had increased to 218% over untreated controls and only an 18% increase over untreated cultures in monolayer culture. Fibroblast response to HA was much greater in FPCLs than in monolayer cultures.

FPCL contraction

HA addition at 150 μg/ml did not stimulate FPCL contraction. Likewise, no significant differences were demonstrated in FPCL contraction among the other treatment groups (figure not shown). The promotion of cell division by added HA neither increased the rate nor the degree of lattice contraction.

Thymidine incorporation

When tritiated thymidine was added to FPCLs for 24 h, there was a 31% increase in counts with added HA compared to unsupplemented FPCLs (Table 4). There was only a modest increase in DNA synthesis when added to fibroblasts in FPCLs. In monolayer culture, the addition of 150 μg/ml HA produced only a 6% increase in tritiated thymidine incorporation compared to controls. Fibroblast response to added HA was greater with cells suspended in collagen compared to cells in monolayer.

Flow cytometry

Flow cytometry analysis of untreated fibroblasts released from FPCLs showed 47% of the cells in a 4N state (Fig. 1B). Fibroblasts in FPCLs revealed only 13% of those cells were in a 4N state when supplemented with HA (Fig. 1A). Incubating HA with hyaluronidase first and adding that digest during the manufacture of FPCL showed that 46% of the nuclei were in a 4N state (data not shown). The digestion of HA with added hyaluronidase prevented the advancement of cells through G2/M of the cell cycle.

Tubulin

Western blot analysis revealed that the addition of HA at 150 μg/ml in the manufacture of an FPCL increased the levels of α tubulin at day 2. As shown in Figure 2, which is typical of three separate experiments, there was an increase in the expression of tubulin as demonstrated by an optical density increase in tubulin expression of 55%. There were equal concentrations of cell protein loaded as demonstrated by equal amounts of β actin. This suggested that HA promotion of cell proliferation may be linked to increased levels of tubulin, the protein unit of microtubules. To test that possibility, isolated tubulin was introduced into fibroblasts by electroporation prior to their incorporation into FPCLs. Tubulin was isolated from the pig brain and the Coomassie Brilliant Blue stained protein profile of the isolated tubulin fraction run on SDS-PAGE is shown in Figure 3a. Lane 1 shows protein staining of the DEAE ion exchange chromatography fraction of isolated pig brain tubulin protein. There are six distinct bands with molecular weights greater than 50,000 daltons. Tubulin protein bands were identified by Western blotting (Fig. 3b). There are other bands within the preparation that are not tubulin proteins. The possibility that the contaminating proteins would

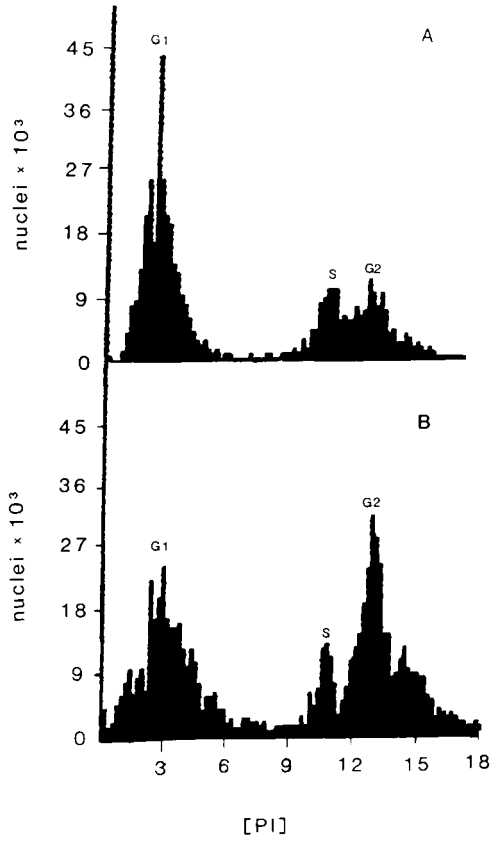


Fig. 1. Flow cytometry of propidium iodide (PI) stained nuclei released from fibroblasts derived from control and HA (150 μg/ml)-treated FPCLs by limited enzymatic digestion. **A:** The HA-treated group shows a major portion of the nuclei in G1 of the cell cycle. **B:** The control group shows a major portion of the nuclei in G2 of the cell cycle.

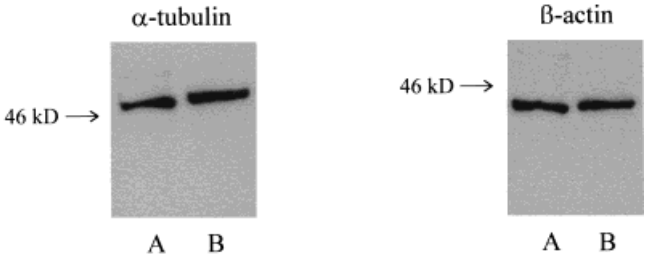


Fig. 2. Western blot analysis of α tubulin and β actin from homogenates of fibroblasts grown in monolayer culture. **Right:** α tubulin blot results. Well A is from HF 136 fibroblasts incubated with PBS, the control treatment group, and well B is from the same cells incubated with HA at 150 μg/ml. The optical density (OD) of the band in well A is 1.29 OD units and that of well B is 2.0 OD units. **Left:** β actin Western blot results, where the OD for well A, the control treatment group, is 2.10 OD units. For well B, the HA-treated cells had 1.94 OD units, indicating equal amounts of cell protein applied to each well. The arrow points to the site where a 46,000 MW standard runs.

interfere with the incorporation of tubulins into microtubules was examined in two ways.

The pig brain tubulin protein was conjugated with a fluorescent tag and introduced into cells by electroporation. In Figure 4, autofluorescent cytoplasmic microtubules were identified in fibroblasts 24 h after plating. A control, electroporated, cell preparation on coverslips that was neither fixed nor stained demonstrated autofluorescent microtubules (Fig. 4A). Including colchicine at 10 μg/ml for 1 h disrupted the autofluorescent microtubules and a diffuse autofluorescence staining pattern appeared (Fig. 4B). To eliminate the possibility that the autofluorescent cytoplasmic structures were microfilaments, cytochalasin B that disrupts microfilaments was added. Cytochalasin B at 10 μg/ml for 1 h did not disrupt the autofluorescent microtubular structures (Fig. 4C). Though the cells had rounded up as a consequence of added cytochalasin B, polymerized tubulin microtubule structures were present. It was established that the isolated pig tubulin protein fraction was incorporated into microtubules.

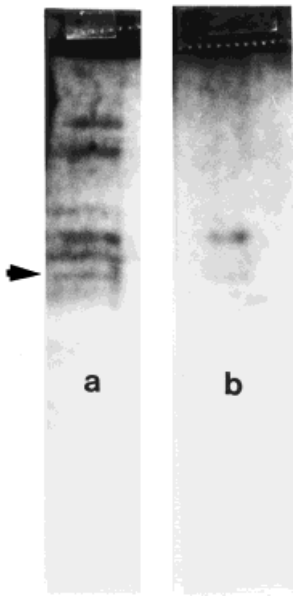
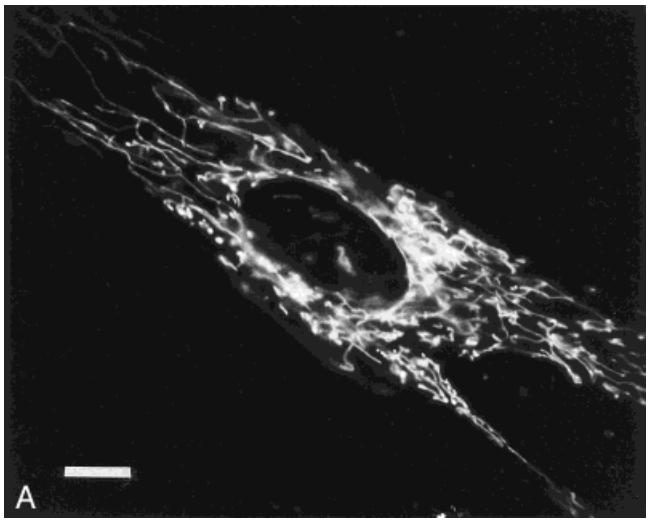
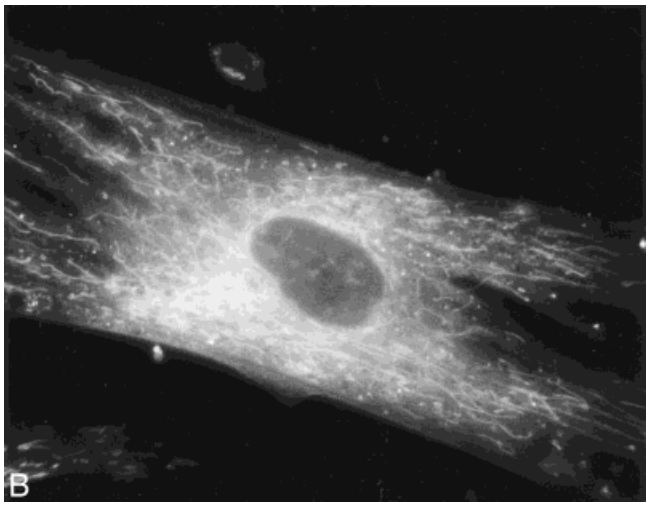


Fig. 3. The pig brain tubulin fraction, isolated by ion exchange chromatography, was analyzed by SDS-PAGE. **a:** Protein bands stained by Coomassie Brilliant Blue. **b:** The protein separated by electrophoresis was transferred to a PVDF membrane, probed with antibody directed to tubulin, and processed by Western blotting. The arrowhead points to the expected location of α tubulin.

Fibroblasts inoculated with purified tubulin protein from the pig brain were incorporated into FPCLs and lattice contraction and cell proliferation monitored over a 4-day period. By electroporation, either pig brain purified tubulin in 0.35 M sucrose or 0.35 M sucrose alone were introduced into fibroblasts before they were incorporated into FPCLs. Three FPCLs containing tubulin supplemented cells had an average decrease in area from 960 mm^2 to 221 ± 31 mm^2 at day 2. The controls, cells receiving only sucrose, showed a smaller decrease of 453 ± 77 mm^2 (Fig. 5a). Pig brain tubulin supplemented cells in FPCLs continued to show enhanced lattice contraction through day 3. By day 4, however, both tubulin and sucrose supplemented FPCLs were equivalent in size. The inoculation of tubulin into fibroblasts enhanced the initial rate of lattice contraction, but not the final degree of lattice contraction.





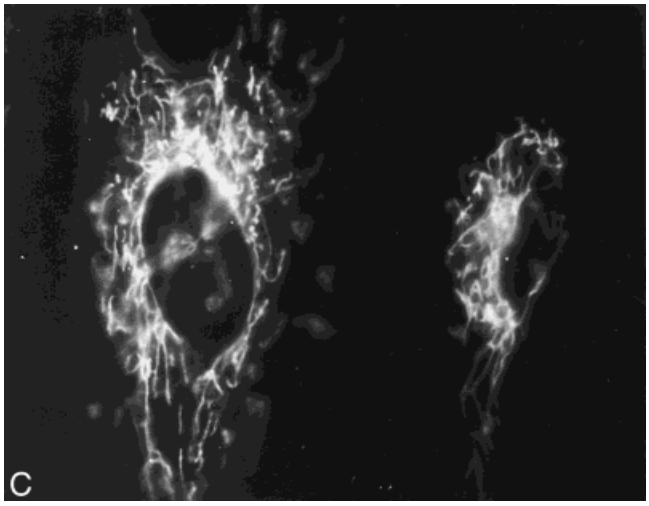
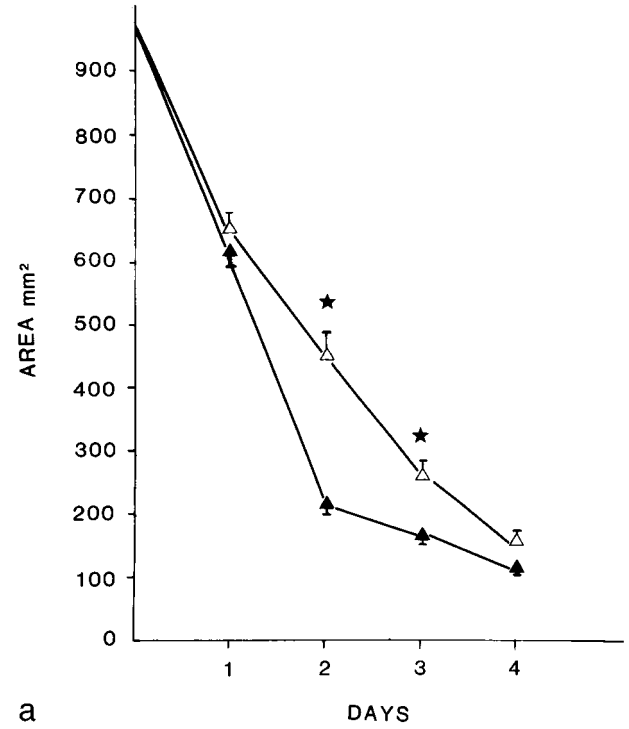


Fig. 4. Autofluorescence is shown within fibroblasts grown in monolayer which were inoculated with fluorescent tagged tubulin isolated from pig brain. **A:** Autofluorescence tubulin in representative untreated control cells. **B:** Autofluorescence tubulin in a representative fibroblast cultured with colchicine. **C:** Autofluorescence tubulin in a representative fibroblast cultured with cytochalasin B. The bar represents 12 μm.



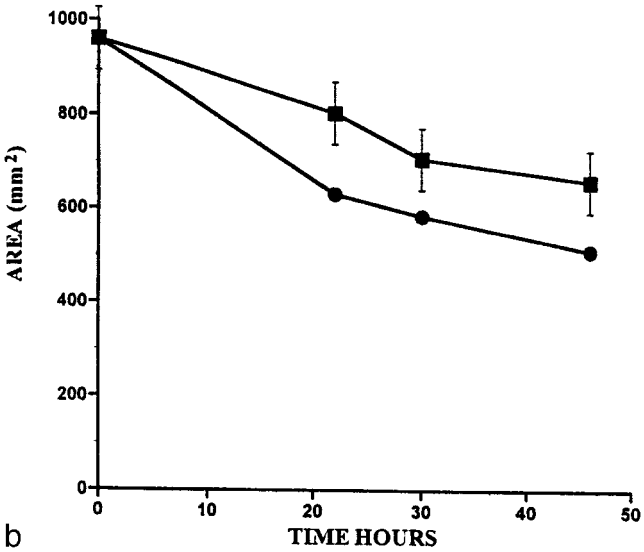


Fig. 5. A graphic presentation of the area change of FPCLs. **a:** FPCLs were measured from two treatment groups over a 4-day period. In one treatment group, fibroblasts were inoculated by electroporation with pig brain purified tubulin (solid triangles) prior to their incorporation into FPCLs. In the other treatment group, fibroblasts were inoculated by electroporation with sucrose alone (open triangles). The stars indicate statistical significance by the Student's t-test ($P \leq 0.05$). **b:** Fibroblasts were inoculated with purchased bovine brain tubulin (solid circles) or with bovine albumin (solid squares). There are no error bars with the tubulin inoculated fibroblasts because the areas of the FPCL were all the same at each time point measured.

Because the pig brain preparation contained other proteins besides tubulin, a more pure bovine tubulin protein preparation was purchased and tested. As shown in Figure 5b, the electroporation of bovine tu-

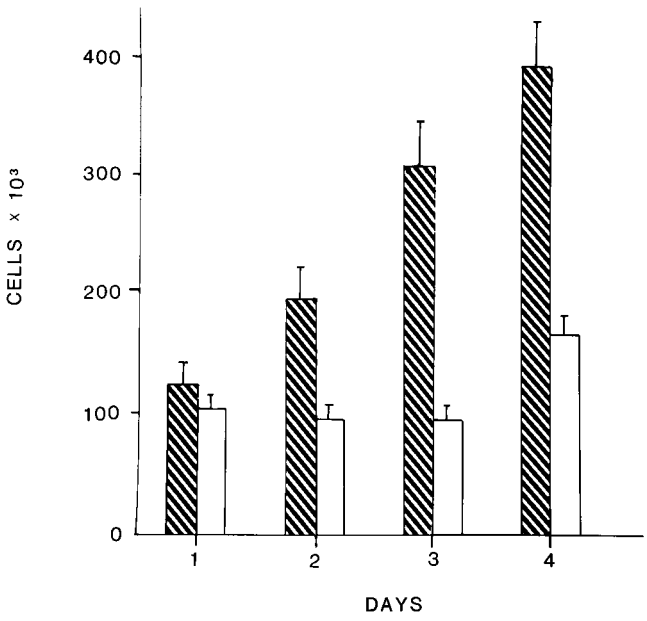


Fig. 6. A bar graph of the effect of tubulin introduction into fibroblasts by electroporation prior to their incorporation into FPCLs upon cell proliferation over a 4-day period. Fibroblasts either received 10 ng of isolated tubulin from pig brain or sucrose alone. At each time point, three FPCLs from each treatment group were collagenase digested to release cells which were then counted. The number of fibroblasts per FPCL is presented on the Y axis and the day of harvest on the X axis.

bulin into fibroblasts prior to their incorporation into collagen lattices increased FPCL contraction over a 2-day period. It was also effective at increasing the cell numbers in collagen lattices. The tubulin-FPCLs had 66,350 cells (128% of initial cell number) and the albumin control-FPCLs had 42,200 cells (73% of the initial number of cells). Lattice contraction was followed for only 2 days because FPCL had to be digested and cells counted at this early time point. These results supported that the findings from the pig brain tubulin preparation were related to tubulin and not some contaminates. Also demonstrated in this experiment was that albumin could not substitute for tubulin in affecting human fibroblasts in collagen matrices.

Tubulin-supplemented human fibroblasts maintained in FPCLs proliferated earlier (see Fig. 6). By day 2, cells in FPCL that received sucrose alone remained at the initial seeding, 102,000 ± 13,500 cells. The pig brain tubulin supplemented FPCLs had increased to 191,300 ± 21,000 cells. At day 4, the tubulin supplemented FPCLs had 384,000 ± 29,200 cells, while the sucrose-alone cells in FPCL had only 172,000 ± 16,000. The inoculation of pig brain tubulin into human fibroblasts in advance of their incorporation into FPCLs stimulated cell division.

The concentration of pig brain tubulin which maximized the initiation of fibroblast proliferation was explored. Aliquots of 400,000 cells were microinjected with either 2.5, 5.0, 10.0, or 15.0 ng of purified tubulin in sucrose or sucrose alone. Each treatment group contained three FPCLs seeded with 50,000 cells. The earliest stimulation of cell division was seen with 10 ng of tubulin. At day 1, there were 76,000 ± 14,000 cells counted and at day 4 the cell number had climbed to 269,000 ± 22,500 cells per FPCL. The 5.0 and 15.0 ng supplemented groups showed no change in cell numbers at day 1, but at day 2 there was 110,000 ± 23,000 cells with 5.0 ng and 98,000 ± 15,000 cells with 15 ng of tubulin. The 5.0 and 15.0 ng supplementation continued to stimulate cell division and on day 4 there were 314,000 ± 27,200 cells with 5.0 ng and 287,800 ± 27,300 cells with 15.0 ng. The 2.5 μg supplement fibroblasts showed a 2-day delay in the initiation of cell proliferation and there were 214,000 ± 44,700 cells counted on day 4. The controls, sucrose supplemented cells, did not divide until day 4, where there were only 99,000 ± 20,600 cells counted. All the tubulin supplemented groups had fibroblasts divide by day 2 compared to the sucrose controls which required 4 days to divide.

Was the earlier proliferation of tubulin inoculated fibroblasts due to their enhanced progression through the cell cycles? Flow cytometry of nuclei from fibroblasts inoculated with either 10 ng of pig brain tubulin or sucrose was compared from 2-day-old FPCLs (Fig. 7). FPCL made with sucrose inoculated cells showed 42% of the cell population in the 4N state, while tubulin supplemented cells showed only 24% of the cells in the 4N state. This reduction of nuclei in the 4N state appeared due to the promotion of fibroblasts progressing through the cell cycle. Increasing the intracellular levels of tubulin in human fibroblasts prior to their incorporation into FPCLs promoted cell progression through the cell cycle.

DISCUSSION

Human dermal fibroblasts demonstrate a retardation in cell proliferation when suspended in a collagen matrix. It is reported: 1) When suspended in collagen, the progression of fibroblasts derived from human dermis through the cell cycle is retarded. 2) Added HA promotes cell proliferation by eliminating the block in the cell cycle and only modestly increases DNA synthesis. 3) Added HA increases the intracellular concentration of tubulin in human fibroblasts. 4) It is speculated that the introduction of isolated tubulin protein by electroporation into human fibroblasts prior to their incorporation into FPCLs promotes their progression through the cell cycle.

Microtubules are required for lattice contraction (Bell et al., 1979). They are also required for forming mitotic spindles during cell mitosis (Sorger et al., 1997). The speculation is that the levels of tubulin in human fibroblasts are not sufficient for both cell proliferation and lattice contraction. About 4 days are required for enough tubulin to accumulate for the completion of both mitosis and lattice contraction. The addition of HA and the direct addition of tubulin both increase intracellular pools of tubulin which is associated with the earlier progression of human fibroblasts through the cell cycle.

Schor (1980) reports that fibroblasts on a collagen-coated surface divide at a rate equivalent to fibroblasts plated on glass or tissue culture plastic. However, he reports that when suspended within a collagen lattice, there was a 4-day delay in cell division. We report that fibroblasts derived from human or gorilla dermis sus-

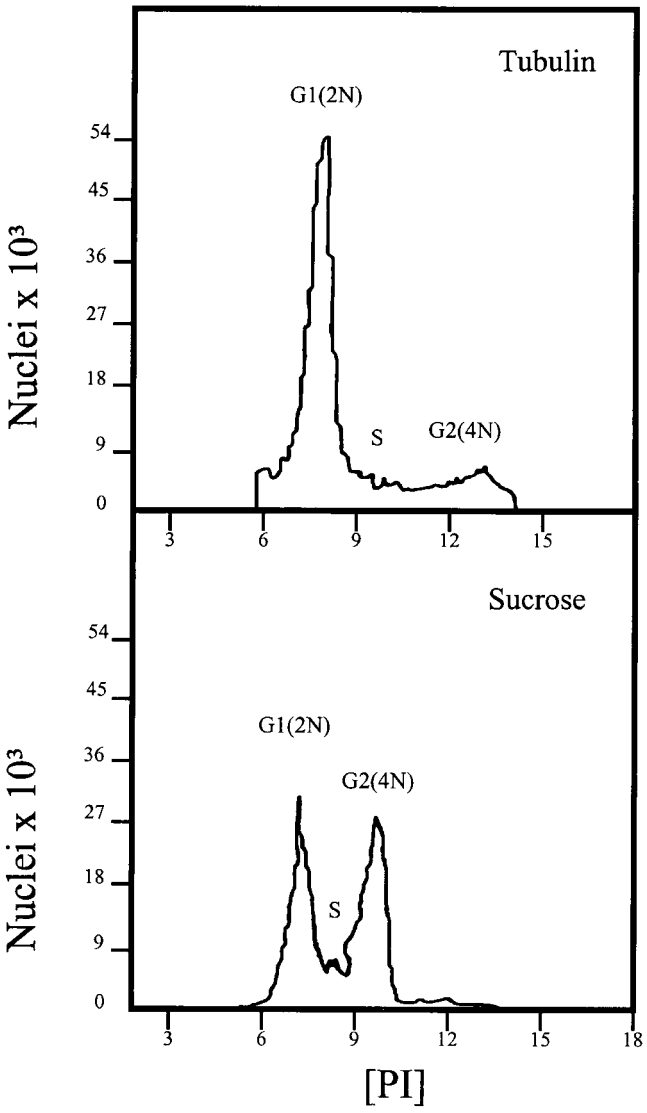


Fig. 7. Flow cytometry analysis of nuclei released from fibroblasts that were incorporated in an FPCL for 2 days. FPCLs were cast with cells that were inoculated by electroporation with either 10 ng of tubulin in sucrose or sucrose alone. At day 2, cells were freed from FPCLs by limited collagenase digestion and the cell nuclei collected then stained with propidium iodide. The stained nuclei were analyzed by a flow cytometer.

pended in collagen showed no delay in DNA synthesis, but a 4-day delay in cell division (Greco and Ehrlich, 1992). In contrast, fibroblasts derived from nonprimates, such as the rat, neither exhibit a delay in DNA synthesis nor in cell division when suspended in a collagen matrix.

HA added to human fibroblasts suspended in collagen matrices induces cell division by day 1. In the absence of added HA, 4 days are required for cell division. One possibility is that 4 days are needed for sufficient HA to accumulate for human fibroblasts to divide. That possibility is not supported by the study with added hyaluronidase, where the inclusion of hyaluronidase in the casting of FPCL did not further delay cell division. The breakdown of HA would be expected to further delay cell division.

Added HA promotion of human fibroblast proliferation is accomplished by the advancement of cells through the cell cycle. When human fibroblasts are grown in monolayer cultures, they show only a 6% increase in [^{3}H]-thymidine incorporation and a modest 15% increase in cell number. These same cells in FPCL with included HA show an increase in DNA synthesis of 31% and a 120% increase in cell number. Compared to cells in monolayer culture, HA stimulated DNA synthesis fivefold within fibroblasts contained in collagen lattices and cell division by eightfold. Flow cytometry confirms that the mechanism of HA promotion of cell division is through the advancement of fibroblasts through G2/M, rather than by the enhancement of the S-phase of the cell cycle.

HA has two well-studied cell surface receptors, CD44 and the receptor for HA-mediated motility (RHAMM). The binding of HA to its cell surface receptor RHAMM is critical for the progression of fibroblasts through the cell cycle (Mohapatra et al., 1996). The mechanism for the HA promotion of cells through the cell cycle is that HA-RHAMM signaling sustains the level of Cdc2/cyclin B1 complex kinase activity. The Cdc2/cyclin B1 complex is required to push cells through G2/M of the cell cycle. The shutdown or loss of RHAMM signaling leads to reduced levels of both Cdc2 and cyclin B1 and the loss of kinase activity. In regard to the CD44 cell surface receptor, there is a local accumulation of HA in vivo just beneath the epidermis in transgenic mice bearing the CD44 antisense gene in their keratinocytes. The affected keratinocytes do not express CD44. The absence of this cell surface HA binding protein leads to the lack of HA degradation and the accumulation of HA in the superficial dermis (Kaya et al., 1997). The fibroblast population in that region of dermis where HA accumulates demonstrates local increased proliferation. The fibroblast density can be reduced to normal in the superficial dermis of these affected mice by the local repeated administration of hyaluronidase.

Doillon et al. (1984) showed that HA enhanced chick tendon fibroblast growth in seeded collagen sponges. The inclusion of HA enhanced DNA synthesis as demonstrated by the fourfold increased incorporation of [^{3}H]-thymidine by fibroblasts grown on collagen sponges for 3 days. In the data presented here, human fibroblasts suspended in an uncrosslinked collagen matrix mimic the findings with chicken fibroblasts on chemically defined, crosslinked collagen sponges.

Synchronized human embryonic fibroblasts in monolayer culture demonstrate a rise in HA synthesis, associated with mitosis (Brecht et al., 1986). The addition of colchicine reduces both the levels of HA and cell mitosis, while the removal of colchicine increases HA synthesis and restores cell division. In our hands at 150 μg/ml, HA does not affect FPCL contraction or cell morphology. However, at concentrations greater than 1,000 μg/ml (sixfold higher), Huang-Lee and Nimni (1993) report that HA inhibited FPCL contraction.

As wound healing progresses in vivo, there are changes in the composition of the extracellular matrix. Initially, a wound contains a fibrin-rich matrix, followed by an HA-rich matrix (Bertolami and Donoff, 1978). As granulation tissue matures, the HA is re-

placed with chondroitin-6-sulfate. In early granulation tissue, an HA-rich environment may promote fibroblast migration and proliferation. As granulation tissue matures and HA is replaced with chondroitin-6-sulfate, fibroblast proliferation ceases. The speculation is that removal of HA from the extracellular matrix plays a role in the control of fibroblast proliferation in maturing granulation tissue.

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