The cytoskeleton of cultured skin fibroblasts from patients with Huntington's chorea

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Cultured skin fibroblasts from patients with Huntington's chorea were prepared for indirect immunofluorescence using monospecific antibodies to tubulin, actin, and fibronectin. The fibroblasts were also visualized by transmission electron microscopy. The fibroblasts were observed after 3 hours of plating and treatment with various concentrations of colcemid and cytochalasin B to test the reaction of the microfibrillar network to stressful conditions.

Disorders were not apparent in the cytoskeletal system (microtubules, microfilaments, and intermediate filaments) when compared with normal controls. Fibronectin was arranged in a fibrillar pattern similar to that seen with actin immunofluorescence. This colinear arrangement was not disturbed in Huntington's chorea cells. Microtubules, microfilaments, and 10-nm intermediate filaments became more parallel as the incubation period increased from 3 to 24 hours. This study showed that the cytoskeleton and the attachment of one surface protein (fibronectin) are not affected in Huntington's chorea.

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Huntington's chorea is an autosomal dominant disease that affects areas of the brain, resulting in severe dementia and loss of muscle control.¹ Observation of cultured skin fibroblasts from patients with Huntington's chorea offers a way to diagnose the disease. Several studies show changes in growth pattern, attachment problems, and abnormal morphology for such fibroblasts when compared with normal controls.²⁻⁶

Substrate attachment and cell morphology are affected by the status of both the cytoskeleton and cytoskeletal-associated proteins in various cultured cell lines.^{7,8} Furthermore, the intermediate filament network has received attention as being an information transfer system between the cell membrane and the nucleus.⁹

The present article details the methods and findings of the investigation of the cytoskeleton in cultured skin fibroblasts from patients with Huntington's chorea.

A transmembrane protein link appears to exist between actin stress fibers, a component of the cytoskeleton, and the extracellular protein fibronectin.¹⁰⁻¹² Other cytoskeletal-associated proteins are also involved in this linkage,¹³ and the microtubule-microfilament system could serve as a means to regulate many growth-controlling elements of the cell.¹⁴ To determine if fibronectin is lost from the Huntington's chorea fibroblast surface or if the fibrillar arrangement of fibronectin is affected, cells were also viewed for the presence of this high-molecular-weight surface glycoprotein.

Figure 1. Numerous parallel microtubules course through the cytoplasm of a normal and Huntington's chorea (HC) fibroblast after a 24-hour growth period in standard medium. Left, normal; right, HC. N indicates nucleus.

Figure 2. These fibroblasts, after a 24-hour growth period in standard medium, have long microfilament bundles in different depths of the cytoplasm. Geodesic dome structures are present near the cell edge. Left, normal; right, Huntington's chorea (HC). N indicates nucleus.

Figure 3. Microtubules intertwine near the periphery of both cell types fixed after 3 hours of incubation in standard medium. Both cells have a semi-flattened, rounded shape. The nucleus (N) is off-center. Left, normal; right, Huntington's chorea (HC).

Methods

Skin fibroblasts were obtained from the Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ. The six fibroblast strains (three normal and three Huntington's chorea) were matched as closely as possible according to sex, passage number, and age of donor. The fibroblasts were grown in standard conditions.¹⁵ The medium was F12FC20. For immunofluorescence, cells were seeded on sterile glass slides and processed for immunofluorescence at 3 or 24 hours after seeding (plating). These times were chosen because cells are still spreading out at 3 hours, while at 24 hours, most cells are flattened.¹⁶

Colcemid was applied in three concentrations of 1.0 $\mu\text{mol/L}$, and 8.0 $\mu\text{mol/L}$ for 4 hours. Cytochalasin B was applied in three concentrations of 2.0 $\mu\text{mol/L}$, 4.0 $\mu\text{mol/L}$, and 14.0 $\mu\text{mol/L}$ for 4 hours. These concentrations were found to give minimal, moderate, and maximal morphologic responses, respectively, in earlier studies.^{15,17}

A range of time exposures was also performed in early experiments; 4 hours was

found to be a good representation of the time of exposure for the dosages given. A cold methanol fixation (-20°C for 5 minutes) and standard procedure¹⁸ were used in the microtubule visualization. Method controls were performed in which normal rabbit serum was used in place of rabbit antitubulin serum.

Microfilament visualization involved 0.4% Triton X-100 treatment followed by formaldehyde fixation (1.0% for 30 seconds). Fibronectin visualization involved formaldehyde fixation (1.0% for 15 minutes) and cold acetone treatment (-20°C for 7 minutes).

Monospecific tubulin and actin antibody were prepared in rabbits or goats and vividly labeled the microtubular and microfilamentous system of mouse 3T3 cells, respectively. Method controls were done in which normal goat serum was used in place of goat antiactin serum. The initial gifts of antisera against tubulin and actin were furnished by Drs B. R. Brinkley and E. Lazarides, respectively. Later, antibodies to tubulin, actin, and fibronectin were prepared in our laboratory. Rabbit antihuman cold insoluble globulin was prepared

from human plasma in rabbits,¹⁹ and the specificity was tested by immunodiffusion on commercially prepared plates, immunoelectrophoresis plates, and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.²⁰

Slides were viewed with a microscope (Leitz Orthoplan UV [ultraviolet]) using epifluorescent illumination (magnification $\times 54$ using the oil-immersion objective [NA1.3]; overall magnification $\times 675$). From each slide, five areas of cells (approximately 50 cells per area) were viewed for staining morphologic characteristics. The figures presented in this article are representative of those cells. All immunofluorescent prints were exposed and developed under the same F-stop and time conditions.

For transmission electron microscopy, skin fibroblasts were seeded into four-chamber tissue culture slides. After fixation in 2.5% glutaraldehyde with 0.1 mol/L of cacodylate buffer (pH 7.0 at 37°C for 45 minutes), the fibroblasts were embedded in situ, preserving their relationship to the substrate and other cells.¹⁸ The layer of cells within the plastic was re-

moved by immersion in boiling water for only a few seconds. Areas of cells were selected, cut from the epoxy resin (Epon) sheet, and glued to capsules for thin sectioning. The sections were stained with uranyl acetate lead citrate, examined, and photographed with a transmission electron microscope (JEOL 100C) operated at 60 kV.

For a more complete description of the preparation of antibodies and experimental methods, see the appendix, beginning on page 249.

Results

Indirect immunofluorescence

Both normal human skin fibroblasts and skin fibroblasts from patients with Huntington's chorea (HC skin fibroblasts) displayed a full cytoplasmic microtubule complex after a 24-hour growth period in normal medium (Fig 1). Method controls for microtubules, as well as microfilaments and fibronectin, showed no staining or faint nonspecific fluorescence (not shown). Actin stress fibers were prominent in both normal and Huntington's chorea cells (HC cells) at 24 hours after plating, and occa-

Figure 4. Skin fibroblasts from a normal subject and a patient with Huntington's chorea after 3 hours of incubation in standard medium show a dense, juxtanuclear deposit of actin with some bundles radiating outward from these deposits. Peripheral fluorescence is apparent. Left, normal; right, Huntington's chorea (HC). N indicates nucleus.

Figure 5. The fibronectin of both cell types is arranged in long strands after 24 hours of growth in standard medium. Fibronectin bridges or strands link cells that are closely opposed. Punctate and juxtanuclear fluorescence is common. Left, normal; right, Huntington's chorea (HC). *N* indicates nucleus.

Figure 6. Peripheral microtubules are absent while some of the innermost microtubules are still intact after 4 hours of treatment with 4 $\mu\text{mol/L}$ of Colcemid. Left, normal; right, Huntington's chorea (HC). *N* indicates nucleus.

Figure 7. Skin fibroblasts treated with 4 $\mu\text{mol/L}$ of cytochalasin B for 4 hours are highly arborized and show foci of actin fluorescence. Left, normal; right, Huntington's chorea (HC). N indicates nucleus.

sionally the geodesic-dome pattern was seen along the periphery of cells (Fig 2). Most fibroblasts that were plated for only 3 hours were circular. These cells showed microtubules intertwining throughout the cytoplasm, especially near the cell periphery (Fig 3).

Microtubular patterns of HC cells were similar to those seen in normal cells and the spreading process seemed to be unaffected. Normal and HC skin fibroblasts that were prepared for actin visualization 3 hours after plating fluoresced brightly in the perinuclear region (Fig 4). No disturbances in the cytoskeletal pattern were detected in the actin arrangement of both normal and HC cells 3 hours after plating. Stress fibers were present after 3 hours in some cells. Stress fibers were present after 3 hours in some cells, while the periphery of cells did show the presence of actin, especially at regions of cell contact (Fig 4).

Antibodies to plasma fibronectin decorated fibrillar strands on both normal and HC skin fibroblasts (Fig 5). In these cells, intense perinuclear fluorescence was present, in addition

to fibrillar strands connecting adjacent cells. All three concentrations of colcemid had the same general effect on the microtubule network in both normal and HC cells. Microtubules completely disappeared in the periphery, while in the central or perinuclear area of the cell, a few microtubules were observed, but usually only a diffuse fluorescence was seen near the nucleus (Fig 6).

A similar response by the different cell types was seen at each cytochalasin B concentration. The 2.0- $\mu\text{mol/L}$ concentration of cytochalasin B produced little effect in normal and HC cells but, at the 4.0- $\mu\text{mol/L}$ concentration, the cells became stellate with highly arborized processes (Fig 7). Actin aggregates were seen along some of the processes (Fig 7).

Transmission electron microscopy

Observation of thin sections from normal and HC skin fibroblasts revealed the additional cytoskeletal component, 10-nm intermediate filaments. Microfilaments, microtubules, and intermediate filaments ran parallel to one an-

Figure 8. A bundle of microfilaments (Mf), at least $0.25\ \mu\text{m}$ in thickness, runs parallel to the cell membrane in this micrograph of a normal skin fibroblast after 24 hours of growth in standard medium. Intermediate filaments (If) course through the cytoplasm in the same direction as the microfilaments and microtubules (arrowheads).

other and to the long dimension of the cell in cultures that were plated for 24 hours (Figs 8 and 9). The 6-nm microfilaments were closely applied to the cell membrane, while the intermediate filaments, arranged in loose bundles, were evident throughout the cytoplasm, sometimes coursing near the cell membrane or the nucleus. The intermediate filaments occasionally whorled about one another or were oriented perpendicular to the cell membrane, intermingling with the microfilaments.

Fibroblasts that were incubated for only 3 hours (3 hours after plating) showed an arrangement of microtubules and microfilaments that concurred with results obtained by immunofluorescence (Figs 10 and 11). Whorled intermediate filaments were observed in the cytoplasm and were occasionally grouped into parallel bundles. These filaments approached the cell mem-

Figure 9. Microfilaments (Mf) and intermediate filaments (If) parallel one another in this Huntington's chorea (HC) skin fibroblast after 24 hours of growth in standard medium. Densities, which may represent sites of α -actinin (arrowheads), are present along the microfilament bundle.

brane at right angles and seemed to intermesh with the microfilaments. Intermediate filaments were also closely related to the nucleus (Fig 11), some appearing to end on or near the nuclear membrane. The small-diameter microfilaments were again closely applied to the cell membrane, and some bundles were apparent within the cytoplasm. Microtubules radiated from the center of the cell to the periphery, occasionally crisscrossing one another, and were also found near the cell membrane.

Discussion

This study provides evidence that the cytoskeletal system of skin fibroblasts from patients with Huntington's chorea is not diminished or structurally altered. A full microtubular complex is apparent in cells that are flat and well spread. Visualization of the microtubule ar-

Figure 10. Two micrographs of normal skin fibroblasts incubated for only 3 hours in standard medium. Intermediate filaments generally run perpendicular to the microfilament bundle (Mf) and cell edge. Near the cell edge the microtubules (arrowheads) change in depth along their length. Intermediate filaments course around mitochondria and rough endoplasmic reticulum.

Figure 11. Views of Huntington's chorea (HC) fibroblasts incubated for only 3 hours in standard growth medium. Microfilaments (Mf) are present near the cell surface as well as deeper within the cytoplasm. Bundles of intermediate filaments (If) are seen near the center of the cell, with some closely related to the nuclear membrane (right). Intermediate filaments also weave among mitochondria and other organelles and approach the cell membrane. N indicates nucleus; arrowhead, microtubules.

range 3 hours after plating (3-hour cells) reveals a different disposition of microtubules with respect to a 24-hour incubation, but nothing that would suggest an abnormality in the functioning of the tubules. Problems with cells becoming spread was noted in cells that had cytoskeletal defects or disturbances.^{21,22} The large number of microtubules in spreading HC cells, extending into the peripheral and lamellar protein of the cell, indicates that lengthy, normal microtubules can be formed.

Actin antibody staining of HC cells 24-hours after plating shows numerous cytoplasmic stress fibers, a result that was also reported by Geary and associates,²³ in a study of two HC cell strains. Ruffles and an area near the nucleus are the regions with the greatest actin fluorescence in 3-hour HC cells and normal cells. Hynes and Destree¹⁰ reported that in most 3-hour, reseeded NIL8 hamster cells, the actin staining pattern showed up in ruffles and circumferential fibers.

Definite, straight microfilament bundles are apparent in the spreading normal or HC fibroblasts. When such bundles are present, they occasionally emanate from perinuclear fluorescent areas. The significance of these brightly staining regions is not known. Observation of 3-hour cells with transmission electron microscopy reveal 10-nm intermediate filaments in the perinuclear area; however, the limits of the intermediate filaments are not as circumscribed as that seen with antiactin fluorescence. Further studies are necessary to determine if this region (1) represents a pool of G-actin from which F-actin is organized; (2) is 10-nm filaments that react with actin antibody; or (3) is artifactual.

The position and arrangement of microfilaments, microtubules, and intermediate filaments in the fibroblasts are influenced by the amount of time that normal cells are allowed to incubate following seeding. In the spreading cells, the intermediate filaments are seen at right angles to the cell membrane and in a whorled arrangement. In 24-hour cells, the three elements of the microfibrillar system are, in most cases, parallel to one another and the long dimension of the cell. Differences of this pattern are not seen in the HC cells. The in-

termediate filaments and microtubules thus seem to have a more active role in spreading, whereas the actin cables become established after the cell has reached more of an elongated shape. These cables or stress fibers maintain the shape of the normal cell that quickly collapses when cytochalasin B is added to the medium. When the microtubules are abolished with colcemid treatment, the general cell shape does not change. Cells remain spread out and possibly become even more flattened, indicating that microtubules are not as important as microfilaments in the final shape configuration or tension of the cell. When these two organelles of HC fibroblasts are "disturbed" with colcemid and cytochalasin B, these cells respond like normal controls. Therefore, the HC cell seems to have the same threshold for drug response as the normal cell.

The fibrillar pattern of fibronectin fluorescence resembles that of the actin fluorescence in HC cells. This arrangement seems to be the norm for most cells observed in sparse cultures.^{10,11} A linear arrangement of fibronectin implies that the transmembrane linkage¹³ between fibronectin and actin in the HC cell is intact. Punctate fibronectin fluorescence is also present in 24-hour-plated cells. These fluorescent patches might be fibronectin-filled vesicles being transported to, or emptying onto, the outer surface of the cell because of the abundant number of membrane-related vesicles seen in sections of HC and normal cells.

Immunofluorescence procedures such as those described here offer a way to rapidly diagnose a change in cytoskeletal architecture. Cytoskeletal abnormalities may exist in cultured skin fibroblasts from patients with genetic diseases, but it is apparent that in order for these differences to be detected so that early diagnosis will become a possibility, a more defined approach and careful handling of the cells must be implemented.

Appendix

Materials and methods

Fibroblasts were cultured in F12 medium supplemented with 20% uninactivated fetal calf serum (FCS), penicillin (100 U/mL), and streptomycin (100 U/mL), and incubated at 37°C in an atmosphere

of high humidity and 5% carbon dioxide in air. Confluent cultures, growing in 60-mm plastic tissue culture dishes (Lux Scientific Corp, Newbury Park, Calif) were treated for 5 to 10 minutes with trypsin (0.05% in saline D with 0.02% edetic acid).

The fibroblasts were removed from the culture dish by slowly pipetting the trypsin-edetic acid solution back and forth over the cells. The cell suspension was then added to 3 mL of F12FC20, thus stopping the tryptic action, and centrifuged at $28 \times g$ for 5 minutes. The medium was aspirated and the cells were resuspended in 1 mL of F12FC20.

Cells were counted with a Coulter counter (Coulter Electronics, Inc, Hialeah, Fla) and seeded onto two types of glass slides, depending on the method of observation to be done, immunofluorescence or transmission electron microscopy. For immunofluorescence, 3×10^3 cells in 0.1 mL of F12FC20 were seeded within an area of approximately 11×22 mm on a sterile glass slide placed in a 100-mm Lux Petri dish. Some cells were processed for immunofluorescence after 3 hours of incubation. For 24-hour incubations, slides with 3-hour-attached cells were flooded, and hence, covered with culture medium.

After allowing cells to incubate for 24 hours, colcemid (Sigma Chemical Co, St Louis, Mo) was applied in three concentrations of 1.0 $\mu\text{mol/L}$ (0.37 $\mu\text{g/mL}$), 4.0 $\mu\text{mol/L}$ (1.48 $\mu\text{g/mL}$), and 8.0 $\mu\text{mol/L}$ (2.96 $\mu\text{g/mL}$) for 4 hours. Likewise, cytochalasin B was applied in three concentrations of 2.0 $\mu\text{mol/L}$ (1 $\mu\text{g/mL}$), 4.0 $\mu\text{mol/L}$ (4 $\mu\text{g/mL}$), and 14.0 $\mu\text{mol/L}$ (7 $\mu\text{g/mL}$) for 4 hours.

For visualization of microtubules, the slides were washed briefly in phosphate-buffered saline solution (pH 7.4, at room temperature, without Ca^{+2}), treated with cold methanol (-20°C) for 5 minutes, rinsed three times in phosphate-buffered saline solution at room temperature, incubated for 60 minutes at a humid 37°C with rabbit antitubulin antibody, rinsed six times in phosphate-buffered saline solution, incubated for 60 minutes at a humid 37°C with goat anti-rabbit fluorescein-tagged IgG (Mely Laboratories, Inc, Springfield, Va), rinsed six times in phosphate-buffered saline, and mounted with glycerol plus phosphate-buffered saline solution (9:1).

Method controls were done in which normal rabbit serum was used in place of rabbit antitubulin serum. An alternate procedure for the early steps in microtubule staining was as follows: (1) after the initial wash, cells were fixed for 20 minutes at room temperature in 1.0% formaldehyde dissolved in phosphate-buffered saline solution (2)

rinsed three times with phosphate-buffered saline solution at room temperature, (3) treated with cold acetone for 7 minutes at -20°C , (4) rinsed three times with phosphate-buffered saline solution at room temperature, and (5) antibody was applied. All photographs shown in the Results section are from the cold methanol fixation.

For visualization of microfilaments, the slides were washed briefly in phosphate-buffered saline solution (pH 7.4, at room temperature, without Ca^{+2} or Mg^{+2} , with 0.1 mol/L of citrate), dipped for 4 seconds in 0.4% Triton X-100 dissolved in the aforementioned phosphate-buffered saline solution fixed for 30 seconds in 1.0% formaldehyde solution (in the aforementioned phosphate-buffered saline solution). The slides were then transferred to 0.4% Triton X-100, and incubated for 13 minutes at room temperature, rinsed three times in phosphate-buffered saline solution, treated with cold methanol (-20°C) for 3 minutes, and rinsed three times in phosphate-buffered saline solution. They were next incubated for 60 minutes at a humid 37°C with goat antiactin antibody, rinsed six times in phosphate-buffered saline solution, and mounted with glycerol plus phosphate-buffered saline solution (9:1). Method controls were done in which normal goat serum was used in place of goat antiactin serum.

For visualization of fibronectin, the slides were washed briefly in phosphate-buffered saline solution without Ca^{+2} at room temperature, fixed in 1.0% formaldehyde solution for 15 minutes at room temperature, rinsed three times in phosphate-buffered saline solution at room temperature, treated with cold acetone (-20°C) for 7 minutes, rinsed three times in phosphate-buffered saline solution at room temperature, incubated for 60 minutes at 37°C in a humid atmosphere with 30 μL of rabbit anti-cold-insoluble globulin. Each slide was rinsed six times in phosphate-buffered saline solution, and 30 μL of a 1/20 dilution (in phosphate-buffered saline solution) of goat anti-rabbit IgG-FITC (Mely) was added. Slides were incubated an additional 60 minutes, after which they were rinsed six times in phosphate-buffered saline solution and mounted with a glass coverslip using glycerol-phosphate-buffered saline solution (9:1) as the mountant.

Slides were viewed with a Leitz Orthoplan UV microscope. (E. Leitz, Inc, Rockleigh, NJ) using epifluorescent illumination (through a $54 \times$ oil immersion objective, [NA1.3]). Images were recorded on Tri-X film and developed in Acufine (Acufine, Inc, Chicago, Ill). All immunofluorescent prints were exposed and developed under the same F-stop and time conditions.

Preparation of antibodies

Bovine brain tubulin was prepared according to the method of Shelanski and associates.²⁴ Briefly, brain tissue obtained from freshly slaughtered animals was homogenized with a blender in reassembly buffer 0.1 mol/L of 2-(N-morpholino) ethane sulfonic acid, 1 mmol/L of ethylene glycol-bis-(β -amino ethyl ester) N, N'-tetraacetic acid (EGTA), 0.1 mmol/L of guanosine triphosphate, and 0.5 mmol/L of magnesium chloride (pH 6.9) at 4°C. The homogenate was centrifuged at 10,000 $\times$ g, and the supernatant was subjected to two polymerization cycles using reassembly buffer containing 4 mol/L of glycerol. The final step consisted of fractionating the twice-polymerized tubulin on a Sepharose 4B column (2.5 $\times$ 60 cm) to separate out high-molecular-weight proteins. Samples of tubulin obtained from the column were found to be electrophoretically pure when assayed on polyacrylamide gels using a 5% to 20% gradient and staining with Coomassie blue.

Antitubulin antibody was prepared by immunizing rabbits using the procedure of Fuller and associates²⁵ and later the method of Jockusch and associates.²⁶ Purified bovine tubulin was cross-linked by adding 15 mg to 20 mg to 2% glutaraldehyde solution and heating for 2 hours at 37°C. A small amount of precipitate formed that was pelletized by centrifugation and washed twice with 0.9% saline solution. The material was resuspended in saline solution, emulsified in Freund's complete adjuvant, and injected intramuscularly into New Zealand white rabbits on days 0, 14, and 28. Each rabbit received 3.5- to 5.0-mg cross-linked tubulin. Rabbits were bled 10 days after the last injection and the serum was processed for monospecific antibody. However, the antiserum failed to show significant antibody activity when tested on the microtubular system of mouse 3T3 cells. To increase the concentration of humoral antibody, each rabbit received a series of intravenous injections. The purified tubulin was precipitated with 10% alum, pH 7.3, at room temperature. The precipitate was collected by centrifugation and resuspended in 0.9% saline solution. The aforementioned rabbits received the following amounts of alum-precipitated tubulin on the indicated days: 0.1 mg on days 60, 63, and 66; 0.2 mg on days 71, 74, and 77; and 0.3 mg on day 80. The rabbits were bled on day 94. The antiserum that now contained high activity was sterilized by filtration and stored at -20°C until processing for monospecificity.

Crude γ -globulin fractions of the rabbit immune serum were prepared by precipitation with 18% so-

dium sulfate followed by extensive dialysis against borate saline buffer, pH 8.4. The crude anti- γ -globulin antibody was purified to monospecificity on a Sepharose 4B-cyanogen bromide (CNBr) tubulin affinity column.

Sepharose 4B (Pharmacia, Piscataway, NJ) was activated with CNBr using the procedure of Cuatrecasas and associates.²⁷ Briefly, 25 mL of Sepharose 4B was mixed in 3.5 of CNBr at 0°C for 15 minutes while keeping the reaction mixture at pH 11 with 4 mol/L of sodium hydroxide. The activated Sepharose 4B was washed thoroughly with cold, distilled water and with cold borate saline buffer at pH 8.4. Purified bovine brain tubulin (50 mg) was added to the activated gel and allowed to react for 20 hours at 4°C. The slurry was washed with 0.1 mol/L of hydrochloric acid to elute the unbound protein. Approximately 94% of the tubulin remained bound to the activated jet as determined by OD₂₈₀ readings on the washings. The material was then washed with borate saline buffer and poured into a 20-mL glass syringe. The column was washed with several column volumes of borate saline buffer and poured into a 20-mL glass syringe. For the preparation of monospecific antibody, 4 mL of the crude γ -globulin fraction (28 mg/mL of protein) was added to the affinity column and allowed to slowly filter through in a 2-hour period. The column was washed with borate saline buffer until the eluent OD₂₈₀ dropped to zero. The attached antibody was eluted with 4 mol/L of magnesium chloride while collecting 1-mL fractions. The protein-containing fractions were collected and dialyzed against 8 L of phosphate-buffered saline solution at a pH of 7.4. The monospecific antibody was stored in small aliquots at -20°C. A solution containing 50 μ g/mL of the monospecific antibody vividly labeled the microtubular system of mouse 3T3 cells using immunofluorescent techniques.

Actin was prepared from bovine cardiac muscle by use of the methods of Spudich and Watt²⁸ and was a gift from Dr Ana-Maria Waltenbaugh. The actin was cross-linked with glutaraldehyde as described for tubulin.

Antiactin was prepared by emulsifying the cross-linked actin with Freund's complete adjuvant and injecting a goat intramuscularly according to the following schedule: 4 mg on day 0 and 4 mg on day 10. Bleeding was 10 days after the last injection. The goat antiserum was also fractionated with 18% sodium sulfate and applied to an actin affinity column.

Sepharose 4B was activated with CNBr according to procedures already described. The activated

gel was washed with 0.1 mol/L of cold sodium bicarbonate (pH 9.0) and then with 1 mmol/L of hydrochloric acid at room temperature. G-actin (50 mg) was added to the gel in the presence of coupling buffer (5 mmol/L of triethanolamine hydrochloride, pH 8.5) and allowed to react for 2 hours at room temperature. The gel was then washed with 2 L of coupling buffer followed by a 2-hour incubation with 1 mol/L of ethanolamine. Finally, the gel was washed alternately with several changes of 0.1 mol/L of borate with 0.3 mol/L of potassium chloride, pH 8.5, and 0.1 mol/L of acetate with 0.3 mol/L of potassium chloride, pH 4.0. The material was resuspended in phosphate-buffered saline solution, pH 7.4, and packed in a glass syringe.

Monospecific actin antibody was prepared as described for tubulin antibody.

Cold-insoluble globulin was prepared from human plasma on a gelatin affinity column as described by Engrall and Ruoslahti.¹⁹ After elution with 8 mol/L of urea, the protein was dialyzed against two changes of phosphate-buffered saline solution, pH 7.4, and concentrated via vacuum dialysis. Purification analysis using sodium dodecyl sulfate (SDS) polyacrylamide slab gel electrophoresis with a 5% to 20% gel gradient with 3.5% stacking gel revealed a nearly homogeneous preparation.

Antibody was prepared by immunizing New Zealand white rabbits with the purified cold-insoluble globulin according to the following protocol: days 0, 3, and 7, 0.1 mg (in 1.0 mL of phosphate-buffered saline solution); days 10, 14, and 17, 0.2 mg; day 23, 0.3 mg; and day 30, 0.5 mg. All injections were given intravenously. Rabbits were bled by cardiac puncture on day 36. After collection, the serum was filtered, heated at 56°C for 30 minutes, and stored at -20°C.

Rabbit anti-cold-insoluble globulin was precipitated with 18% sodium sulfate at room temperature followed by extensive dialysis against phosphate-buffered saline solution. This crude γ -globulin preparation was then subjected to affinity chromatography by coupling purified cold-insoluble globulin to cyanogen bromide-activated Sepharose 4B (Sigma Chemical Co, St Louis, Mo) and packing in a glass column. Ten milliliters of the crude γ -globulin preparation (30 mg/mL) was allowed to slowly filter through the column over a 2-hour period at room temperature. The column was thoroughly washed with phosphate-buffered saline solution and the bound protein was eluted with 4 mol/L of magnesium chloride, dialyzed against phosphate-buffered saline solution, concentrated with vacuum di-

alysis, and stored at -20°C. This antibody solution was used in all experiments described at a concentration of 50 μ g/mL.

The specificity of the purified antibody was determined by immunodiffusion, immunoelectrophoresis, and SDS-polyacrylamide gel electrophoresis.

Immunodiffusion was accomplished on commercially prepared plates (ICN Irvine, Ca) of 1.5% agarose in phosphate buffer at a pH of 8.0. Reactants were added to each well and diffused for 60 hours at 23°C. After washing for 40 hours with 0.9% saline solution, the plates were stained with 0.1% amido black (Sigma Chemical Co).

Immunoelectrophoresis plates (ICN) consisted of 1% agarose in 0.05 mol/L of barbital buffer, pH 8.6 and were run at a constant current of 35 mA for 75 minutes at 23°C. These plates were also washed in saline solution and stained with amido black.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Ames, using a 5% to 20% gel gradient and a 3.5% stacking gel. Separation was achieved using a constant voltage of 50 V for 3.5 hours. For visualization of individual bands, the gels were stained with 0.025% Coomassie blue. For elucidation of antibody specificity, the procedure of Olden and Yamada²⁹ was used.

Transmission electron microscopy

Skin fibroblasts were seeded at a density of 3×10^3 cells per chamber into four-chamber tissue culture slides (Lab-Tek Products, Westmont, Ill). The cells were incubated for either 3 or 24 hours. For 24-hour incubation periods, the initial 0.1-mL cell suspension was supplemented with 0.4 mL of medium to prevent drying of the cultures. After either 3 or 24 hours, the cells were fixed with 2.5% glutaraldehyde in 0.1 mol/L of cacodylate buffer, pH 7.0, for 45 minutes at 37°C. The cells were then washed twice at 10 minutes each, with the same 0.1 mol/L of cacodylate buffer and postfixed with 1% osmium tetroxide in 0.1 mol/L of cacodylate buffer (pH 7.0) for 20 minutes at room temperature.

Next, cells were briefly washed twice with three-times-distilled deionized water ($3 \times$ DI), then stained for 30 minutes at room temperature with saturated aqueous uranyl acetate followed by a $3 \times$ DI wash. Dehydration was performed by a graded acetone series (10-minute changes in 30%, 50%, 70%, and 95% and two 10-minute changes in 100%) followed by a 10-minute change in 1:1 acetone-epoxy resin (Epon 812) mixture, and a 10-minute change in epoxy resin (Epon 812). The slides were then flat-embedded in epoxy resin

(Epon 812). Slides were later removed from the plastic by immersion in boiling water for 10 to 20 seconds. Areas of cells, visible with the light microscope, were cut from the plastic and mounted on epoxy resin (Epon) bullets for this sectioning. Sections were cut with a Porter-Blum MT-2 ultramicrotome and stained with uranyl acetate-lead citrate. The resulting specimens were examined and photographed with a transmission electron microscope (Jeol 100C) operated at 60 kV.

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Your education is important to us!

*Left Photo: Don Middleton, D.O.,
Resident, Tracy Overton, D.O.,
Resident and "small" patient at FOH.*

Right Photo: Sucheta Kulkarni, D.O., Intern

"Flint Osteopathic Hospital has provided acute and general health care for the Flint community for 50 years. The hospital is affiliated with Michigan State University and provides a full range of medical, surgical, emergency, and intensive care services. All medical and surgical subspecialties are covered. At FOH the intern and resident receives training in both outpatient ambulatory care as well as inpatient practices.

FOH, with 359 beds, is the largest osteopathic hospital in Michigan offering intern and residency training programs for osteopathic physicians. The medical education program is designed to provide a structured curriculum and experience in diagnosis and treatment. Morning reports and guest physician lectures occur daily. Reading lists and objectives have been developed for each service. A monthly journal club is conducted by each clinical department. EKG conferences are scheduled twice monthly. The FOH Congdon Lecture Series brings both M.D. and D.O. physicians to the hospital each month. Prominent practitioners, representative of both medical communities, share expertise in research findings during these monthly, day-long seminar presentations.

Ambulatory clinics have been established and provide longitudinal continuity training for interns and residents. Both traditional and alternative track internships are available at FOH. The hospital is a charter member of the Consortium of Osteopathic Graduate Medical Education and Training (COGMET) in association with Michigan State University."

Residencies

- Anesthesia
- Family Practice
- Gastroenterology
- Internal Medicine
- Obstetrics/Gynecology
- Ophthalmology
- Orthopedics
- Otorhinolaryngology
- Pathology
- Pulmonary
- Radiology
- Surgery
- Urology

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- Medical Diseases of the Chest
- Gastroenterology

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Christopher T. Meyer, D.O.
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Education

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