The Cytoskeleton in Chediak-Higashi Syndrome Fibroblasts

By Richard E. Ostlund, Jr., Robert W. Tucker, Joyce T. Leung, Neil Okun, and Joseph R. Williamson

The Chediak-Higashi syndrome (CHS) trait is expressed in cultured human skin fibroblasts as an abnormal perinuclear concentration of moderately enlarged lysosomes. The cytoskeleton of CHS fibroblasts appears intact. Microtubules are normal in number and morphology, as assessed by colchicine binding studies, antitubulin immunofluorescence, and electron microscopy. Deformability by shear force is unaltered and microfilaments are abundant. However, CHS lysosomes appear to interact abnormally with the cytoskeleton, since the perinuclear aggregation partially disperses after depolymerization of cell microtubules with colchicine. These results suggest that CHS is associated with a defect of either the lysosomal membrane itself or of lysosomal membrane–microtubule interaction.

THE CHEDIAK-HIGASHI syndrome (CHS) is characterized by increased size of lysosomes and subcellular secretory and pigment granules without accumulation of any specific storage material. The etiology of this condition is unknown, but it has been suggested that subnormal microtubule subunit assembly is responsible. For example, granulocytes from CHS patients displayed spontaneous capping of cell surface concanavalin A, whereas normal granulocytes required pretreatment with the microtubule inhibitor colchicine in order to cap. In normal human granulocytes, concanavalin A produced an increase in microtubule number near the centriole, but the increase appeared blunted in human CHS granulocytes. Dibutyryl cyclic GMP and cholinergic agonists, agents that have been reported to increase the assembly of free tubulin subunits into microtubules, also corrected the spontaneous capping of concanavalin A in granulocytes from CHS patients and ameliorated the morphological appearance of fibroblasts from the CHS mouse. Finally, the ability of vinblastine to produce paracrystals of microtubule protein in intact human CHS fibroblasts was reduced, suggesting a decrease in the amount or precipitability of CHS tubulin.

Other evidence points away from a primary microtubule defect in CHS. Visualization of microtubules by immunofluorescence in CHS mouse skin fibroblasts and macrophages did not reveal any abnormalities. Application of vinblastine to soluble extracts of human CHS fibroblasts precipitated a normal amount of tubulin. Abnormalities other than those of microtubules have also been reported. A deficiency of neutral protease was seen granulocytes. Platelets concentrated serotonin poorly. Increased cellular cyclic AMP or decreased cyclic GMP have been suggested but not confirmed in all patients. Abnormally increased lysosome permeability to dyes was noted, raising the possibility of an abnormal lysosomal membrane. Recent evidence employing electron spin resonance spectroscopy has been obtained for a generalized increase in cell membrane fluidity.

To pursue the etiology of CHS we have studied microtubules, microfilaments and functional cytoskeletal integrity in skin fibroblasts from a CHS patient and normal subjects.

MATERIALS AND METHODS

Supplies

Colchicine, acidine orange grade II as the zinc chloride double salt, dibutyryl cyclic GMP, and concanavalin A were purchased from Sigma Chemical Co. (St. Louis, Mo.). 3H-colchicine (4 Ci/m mole) was obtained from Amersham Co. (Arlington Heights, Ill.). L-Aescorbic acid was purchased from Fisher Co. (Pittsburgh, Pa.). Lumicolchicine was prepared, as previously described, by ultraviolet irradiation.

Cells

Deltoid or scapular area skin fibroblasts were cultured in Eagle's minimum essential medium containing 15% fetal bovine serum and 50 U/ml penicillin with 50 µg/ml streptomycin. CHS fibroblasts were grown by Dr. W. Sly from a 1-yr-old female with suppurative cervical lymphadenitis and classic granulations of circulating leukocytes and have subsequently been submitted to the Human Genetic Mutant Cell Repository, Camden, N.J. (line GM 2075). Five control cells lines were obtained from subjects 2–51 yr of age. Cells were plated at 5 x 10⁵ cells/100-mm dish, fed twice weekly with 10 ml medium, and used after 7 days. Normal and CHS cells of similar passage were grown and examined or assayed simultaneously.

Microscopy

Lysosomes were visualized in cells grown on glass coverslips by removing the medium, staining for 7 min at room temperature with 0.1 mg/ml acridine orange in 0.15 M NaCl buffered to pH 7.4 with 15 mM hepes, rinsing quickly with the buffer, mounting the inverted coverslip in a drop of culture medium, and observing the cells with...
fluorescein optics. Freshly prepared acridine orange in higher concentration than previously employed was used in order to visualize lysosomes diffusely throughout the cytoplasm of normal cells.

Visualization of low density lipoprotein (LDL) was performed by overnight preincubation of the cells with growth medium containing 5% lipoprotein-deficient human serum in order to induce LDL receptors, followed by 2 hr incubation at 37°C with 100 μg protein/ml of human LDL (density 1.019–1.063). The cells were placed at 4°C, washed with 0.15 M NaCl buffered to pH 7.4 with 50 mM Tris, and the surface-bound LDL was eluted with a 1-hr incubation at 4°C with 10 mg/ml heparin in saline G. 

That medium was removed and the cell coverslips were air dried, treated at –20°C with methanol for 4 min and acetone for 2 min, and then reacted with rabbit anti-human LDL serum followed by fluoresceinated goat anti-rabbit gamma globulin as previously described.

Antitubulin antibody was prepared against vinblastine-induced microtubule crystals from sea urchins, and the cells were processed for tubulin immunofluorescence as previously described. Cells for electron microscopy were removed from the dish with trypsin-stained with uranyl acetate and lead citrate for examination on a JEOL-100-c electron microscope.

**Determination of Microtubule Assembly by Colchicine Binding**

An assay for microtubules in tissue culture cells has been presented in detail elsewhere. Fibroblasts on 100-mm dishes were washed 6 times at room temperature with 0.15 M NaCl and once quickly with 1 ml microtubule stabilization buffer consisting of 10 mM sodium phosphate (pH 6.95), 0.5 mM MgCl₂, 0.5 mM guanosine 5′-triphosphate, 0.5 mM EDTA, 50% glycerol, and 5% dimethylsulfoxide. The cells were washed and postfixed in 1% osmium tetroxide for 1 hr, pretained with 0.5% uranyl acetate, dehydrated in alcohol, and embedded. Thin sections on grids were stained with uranyl acetate and lead citrate for examination on a JEOL-100-c electron microscope.

**Cell Deformability**

Fibroblasts were plated at 5 × 10⁵/100-mm dish and grown for 7 days before being removed from the monolayer by a 5-min incubation with calcium-magnesium-free saline G containing 0.05% trypsin and 0.02% EDTA. The cells were taken up in 10 ml growth medium to inactivate the trypsin, sedimented, suspended in saline, G, centrifuged at 875 g for 3.5 min, and resuspended in saline G containing 6% polyvinylpyrrolidone (Sigma Chemical Co., mol wt 360,000). Polyvinylpyrrolidone increased the viscosity of saline G to 30 centipoise at 37°C and prevented fibroblast fragmentation. The cells were exposed to shear stress in a siliconized stainless steel Couette-type concentric cylinder viscometer similar to one previously described except smaller such that the inner cylinder was of 2 cm diameter and the gap between inner and outer cylinders was 0.025 cm. Eight milliliters of cell suspension (5 × 10⁵ cells/ml) was used to charge the viscometer followed by injection of air to clear the viscometer bottom of cells that would otherwise not be sheared. Fibroblasts pushed from the viscometer top by this procedure were saved as unsheared controls. The 2.5 ml of cell suspension in the viscometer gap was sheared at 1000 dynes/sq cm (397 rpm) for 5 min at 37°C and then fixed while still under shear stress by injection of 1.5 ml 10% glutaraldehyde in 6% polyvinylpyrrolidone saline G. After 1 min, the viscometer was stopped, and the fixed cells were isolated by centrifugation, washed 3 times with distilled water, applied to glass slides, stained with methylene blue, and photographed. The ratio of length to width of 75 cells was quantitated in enlarged prints by means of a Hewlett Packard 9107 digitizer. Recovery of intact cells after shearing was over 75%.

**RESULTS**

**Morphology**

The Chediak-Higashi syndrome was expressed in cultured human skin fibroblasts stained with the lysosome-seeking supravital dye acridine orange. In normal fibroblasts, lysosomes appeared evenly dispersed throughout the cytoplasm (Fig. 1A). Average lysosome size in CHS fibroblasts was moderately increased, but the most striking abnormality was the marked perinuclear location of the lysosomes (Fig. 1B). Large portions of the cytoplasm were nearly devoid of lysosomes. This pattern was not seen in five control cell lines, including one matched for age and sex. Rare giant lysosomes were found in both normal and CHS cells. The spatial distribution of lysosomes also can be studied by immunolocalization of low density lipoprotein (LDL), a substance known to be taken up into fibroblast lysosomes. Figure 1 D–F shows that LDL was concentrated around the nucleus in CHS fibroblasts compared to a more diffuse pattern in normal cells.

The abnormal lysosomal distribution was not altered by preincubation for 2 or 3 days with 1 mM carbachol, 10 mM dibutyryl cyclic GMP, 1 mM ascorbate, 1–100 μg/ml vitamin E, 0.2–1.0 μg/ml biotin, 0.1–1.0 μg/ml vitamin K, or 10⁻⁸ M leupeptin. Because of a previous report that chronic carbachol treatment restored normal morphology to the CHS mouse fibroblast, control and CHS cells were plated at 2000 cells/35-mm dish and grown for 14 days in 10⁻⁸ M carbachol with daily medium changes. Staining with either 1 μg/ml or 100 μg/ml acridine orange revealed no change in the abnormal lysosome morphology.

When the CHS cells were pretreated with colchicine and then stained with acridine orange, there was a distinct peripheral dispersion of the lysosomes (Fig.
Fig. 1. Fibroblasts on glass coverslips were stained for 7 min at room temperature with 0.1 mg/ml acridine orange as described in Materials and Methods. Washed, and mounted for fluorescence microscopy. (A) Normal fibroblasts. Bright punctate areas represent acridine orange-filled lysosomes. (B) CHS fibroblasts. Lysosomes are seen in perinuclear clumps. (C) CHS fibroblasts after treatment for 3 hr with 10⁻⁴ M colchicine. Some dispersion of lysosomes is seen compared to B. (D) Normal fibroblasts immunostained for LDL after incubation for 2 hr at 37°C with LDL. (E) CHS fibroblasts immunostained for LDL after similar incubation. (F) CHS fibroblasts prepared as in E except that normal rabbit serum was substituted for anti-LDL serum.

The effect was observed with as little as 5 × 10⁻⁷ M colchicine for 3 hr but not with up to 10⁻³ M lumicolchicine. However, lysosomes remained large, and complete dispersion of CHS lysosomes did not occur. Normal fibroblasts under these conditions had a slight clumping of acridine orange granules, but the general cellular distribution of the granules was not changed. These data suggest that microtubules may maintain the perinuclear lysosome pattern of CHS fibroblasts.
CHEDIAK-HIGASHI SYNDROME FIBROBLASTS

Table 1. Microtubule Assembly in Human Skin Fibroblasts

<table>
<thead>
<tr>
<th>Microtubule-derived tubulin (pmole/colchicine bound/mg protein)</th>
<th>Normal</th>
<th>CHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>23.0 ± 2.0 (24)</td>
<td>24.2 ± 0.9 (15)</td>
<td></td>
</tr>
<tr>
<td>Percent of total cell tubulin assembled into microtubules</td>
<td>34.3 ± 3.9 (8)</td>
<td>37.7 ± 0.7 (4)</td>
</tr>
</tbody>
</table>

Cells were plated at 5 x 10^6/100-mm dish, grown as described for 7 days, and then analyzed for microtubule assembly by the colchicine binding method.

Figures in parentheses represent the number of dishes of cells analyzed. Data from two control cell lines were combined.

**Microtubule Assembly**

The polymerization of cell tubulin was studied by biochemical and morphological techniques in CHS and normal fibroblasts. As seen in Table 1, neither the mass of microtubules nor the percent of total tubulin assembled into microtubules was reduced in CHS cells when assessed by colchicine binding assays. Furthermore, both colchicine and concanavalin A, substances that depolymerize normal fibroblast microtubules, had the expected effect in CHS cells (Table 2). Dibutyryl cyclic GMP, sodium butyrate, and ascorbate had no effect on fibroblast microtubules in either the mutant or normal cells. Microtubules in CHS fibroblasts decreased to 2.6% ± 0.8% of the original value after incubation for 60 min at 4°C (normal cells, 3.2% ± 0.5%) and reassembled to 34.0% ± 6.5% of original after heating at 37°C for 15 min (normal cells, 40.4% ± 3.4%). Thus, microtubule assembly kinetics and mass of polymerized microtubules were similar in CHS and normal cells.

The biochemical studies were complemented by immunofluorescent and electron microscopic techniques. Figure 2 shows immunofluorescent pictures of selected cells stained with antitubulin antibody. A qualitatively normal distribution of cytoplasmic microtubules was present. Figure 3 demonstrates many morphologically normal microtubules in a CHS fibroblast.

**Cell Deformability**

Fibroblasts were removed from cell monolayer by trypsin, and the rounded cells were exposed to shear stress in a concentric cylinder viscometer as described in Materials and Methods. Table 3 shows that shear force elongated both CHS and normal cells similarly, increasing the length/width ratio by 16%–19%. Cytochalasin B, which reduces cytoplasmic viscosity in vitro, caused an additional 28% increase in the sheared length/width ratio in normal cells.
The cytoskeleton of CHS fibroblasts was studied because of evidence that it may regulate the intracellular distribution of organelles. For example, the phasic movement of pigment granules in fish melanophores and erythrophores between the cell periphery and the nucleus is dependent on microtubules.27,28 Perinuclear groups of lysosomes in transformed fibroblasts can be dispersed by microtubule destruction,29 while in normal fibroblasts under other culture conditions microtubule destruction appears to cause perinuclear aggregation of lysosomes.17 Microtubules30 and actin filaments31 are known to bind in vitro to secretory granule membranes and might thus influence granule distribution in the intact cell.

Cell deformability was studied as a functional assay for cytoskeletal integrity. Deformability under 1000 dynes/sq cm shear stress was normal in CHS fibroblasts (Table 3). Hence, nonmicrotubular components of the cytoskeleton also appeared grossly intact by this measure. In addition, actin-like microfilaments were abundant in electron micrographs (Fig. 3). It was previously suggested that CHS cells might be excessively rigid because of poor granulocyte migration through small pore filters,1 but no such rigidity was noted in cultured fibroblasts.

Our data show an abnormal interaction between microtubules and CHS lysosomes (Fig. 1). Microtubules appeared to hold the lysosomes near the nucleus; destruction of microtubules with colchicine caused significant (but not complete) dispersion of the lysosomes. Lumicolchicine, a mixture of colchicine isomers unable to disrupt microtubules, was without effect. Such an abnormal microtubule–lysosome interaction might be due to a defect of either the lysosomal membrane or microtubules. A gross alteration of microtubules is not apparent from these studies. Whether the defect lies in the lysosome membrane or in a subtle qualitative abnormality of the microtubule is the subject of ongoing investigation.

**ACKNOWLEDGMENT**

Gratitude is expressed to Dr. Shirley Hajek, Alice Williams, and Kay Zorn for assistance in these studies.
REFERENCES


The cytoskeleton in Chediak-Higashi syndrome fibroblasts

RE Jr Oslund, RW Tucker, JT Leung, N Okun and JR Williamson