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CHANGES IN ORGANIZATION OF THE INTERMEDIATE FILAMENT SYSTEM IN HUMAN FIBROBLASTS IN LYSOSOMAL STORAGE DISEASES AND THEIR EXPERIMENTAL MODELS

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UDC 616-055.5/.7-092:616-008.931:577.152.34]-07

KEY WORDS: lysosomal storage diseases; intermediate filaments

Hereditary insufficiency of one of the acid hydrolases in lysosomal storage diseases (LSD) leads to severe cell damage in various tissues of the body. As a result of progressive accumulation of unsplit compounds of varied nature, hypertrophy of the lysosomal compartment develops, and gradually fills the entire intracellular space. Extensive information has been obtained on the specific molecular biochemical changes lying at the basis of LSD, and is concerned chiefly with different forms of insufficiency of the lysosomal enzymes, the factors necessary for their function in the cell, and the receptor systems responsible for their transport into lysosomes, etc. [14].

However, information on nonspecific intracellular changes in LSD is extremely limited and consists mainly of a description of the morphological features of pathological cells and tissues [13]. Meanwhile, it can be claimed that blocking one stage of metabolism in LSD leads to a number of very serious disturbances in the target cells and, in particular, to changes in the intralysosomal pH [2], to activation of lysosomal enzymes unconnected with the primary defect [1], and others. The study of the cytoskeletal structures in pathological cells is particularly interesting from this standpoint. We know that under normal conditions various organelles, including lysosomes, are bound by some degree to different components of the cytoskeleton, and their dynamic interaction with microtubules (MT) has been studied in most detail [6, 8, 9,

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17]. In LSD, the continuous overfilling of the target cells with numerous lysosomes, heterogeneous as regards size, undoubtedly makes the organization of the intracellular space more complex. Accordingly, not only MT, but also the more stable system of cytoplasmic intermediate filaments (IF) attracts attention. These fibrils are less dynamic than actin microfilaments and microtubules. Evidence of the importance of the IF system is given by abundant data on regular changes in expression of IF proteins in the course of differentiation and during changes in intercellular contacts and contacts of cells with substrate [10].

The aim of this investigation was a comparative in vitro study of the organization of MT and IF in human skin fibroblasts under normal conditions, in two types of LSD (Fabry's disease, mannosidosis), and in an experimental model of LSD in vitro. Experiments were carried out on cells of stationary cultures in a compact monolayer and under conditions of reduced density, and during spreading of the pathological cells with different levels of accumulation over the substrate.

EXPERIMENTAL METHOD

Three strains of human cutaneous fibroblasts were used. Strain 1026 consisted of cells from a patient with Fabry's disease (deficiency of acid ceramide-trihexoside α-galactosidase, E.C. 3.2.1.22), which accumulate mannose-containing oligosaccharides [13]. Strain 1000 consisted of normal postnatal human cutaneous fibroblasts, and was used as the control and also in experiments with sucrose in the in vitro model of LSD [1]. All strains were obtained from the Cell Bank of the Institute of Medical Genetics, Academy of Medical Sciences of the USSR. The cells were cultured in Eagle's medium containing 10% bovine serum and human umbilical serum (1:1). The cells were dissociated with 0.25% trypsin solution ("Spofa," Czechoslovakia) in PBS buffer. For the experiments the cells were grown on coverslips in 35-mm Petri dishes. Intensive accumulation of undegraded compounds in the patient's tissues was known to begin during postnatal development, whereas in vitro it begins after proliferation of the target cells has stopped [13]. Cultures of pathological cells were therefore tested after a long stay in the stationary phase. In experiments to create a model of LSD normal fibroblasts were cultured for 7 days in medium with the addition of 0.04 M sucrose. Additionally, populations of defective cells (Fabry's disease) with a stationary phase 3, 10, and 17 days in duration and, correspondingly, with different levels of accumulation of undegraded compounds (stages I, II, and III) were obtained. These cells and the normal fibroblasts were subcultured on coverslips with lower density (1:4) and observations were made on the state of IF and MT after 1, 4, 5, and 24 h. The cells were fixed with ice-cold methanol (30 min at $-20°C$), and rinsed with PBS buffer. The preparations were then incubated with monoclonal mouse antibodies to cytoskeletal proteins: to vimentin, to IF protein in fibroblasts [3], and to tubulin, MT protein [18]. The preparations were then washed in PBS and incubated with the immunoglobulin fraction of goat antiserum to mouse immunoglobulins, conjugated with fluorescein isothiocyanate ("Sigma") in a dilution of 1:50. The preparations were studied and photographed by means of a photomicroscope ("Opton").

EXPERIMENTAL RESULTS

Light-optical observations made on stationary cultures of pathological cells and normal fibroblasts, engaged in endocytosis of sucrose, confirmed the well-known fact of intensive vacuolation of the cytoplasm of the target cells in LSD and in its experimental models. Numerous vesicles with unmetabolized compounds filled the whole cytoplasm, or were distributed in it at different levels. The fact that the vesicles observed were lysosomes is shown by the accumulation of the specific vital dye neutral red [2]. The relatively small lysosomes, more homogeneous in size, were found in fibroblasts accumulating sucrose and in pathological cells of strain 936 and strain 1026 in stage I; the largest lysosomes (1-3 μ in diameter) were observed in Fabry's disease in the cells of strain 1026 at stages II-III of accumulation.

The study of the MT system in normal cells loaded with sucrose and in pathological cells by means of immunofluorescence staining revealed no marked differences between them: the shape and orientation of MT along the long axis were preserved (Figs. 1a and 2a). The arrangement of the network of vimentin-containing fibrils of IF in normal fibroblasts basically repeated the distribution of MT (Fig. 1b). However, in pathological cells with hyperaccumulation of lysosomes, the reticular organization of IF was partially disturbed, and dense ring-shaped structures with varied diameter were formed from the fibrils of IF (Fig. 2b). These closed bundles of IF were located at different levels in the cells and were most marked and massive in fibroblasts from the patient with Fabry's disease in stages II-III of accumulation. The arrangement of the altered structures of IF in the pathological cells under compact monolayer conditions made their organization difficult to study by immunofluorescence methods and photography. Cells of strain 1026 with different levels of accumula-

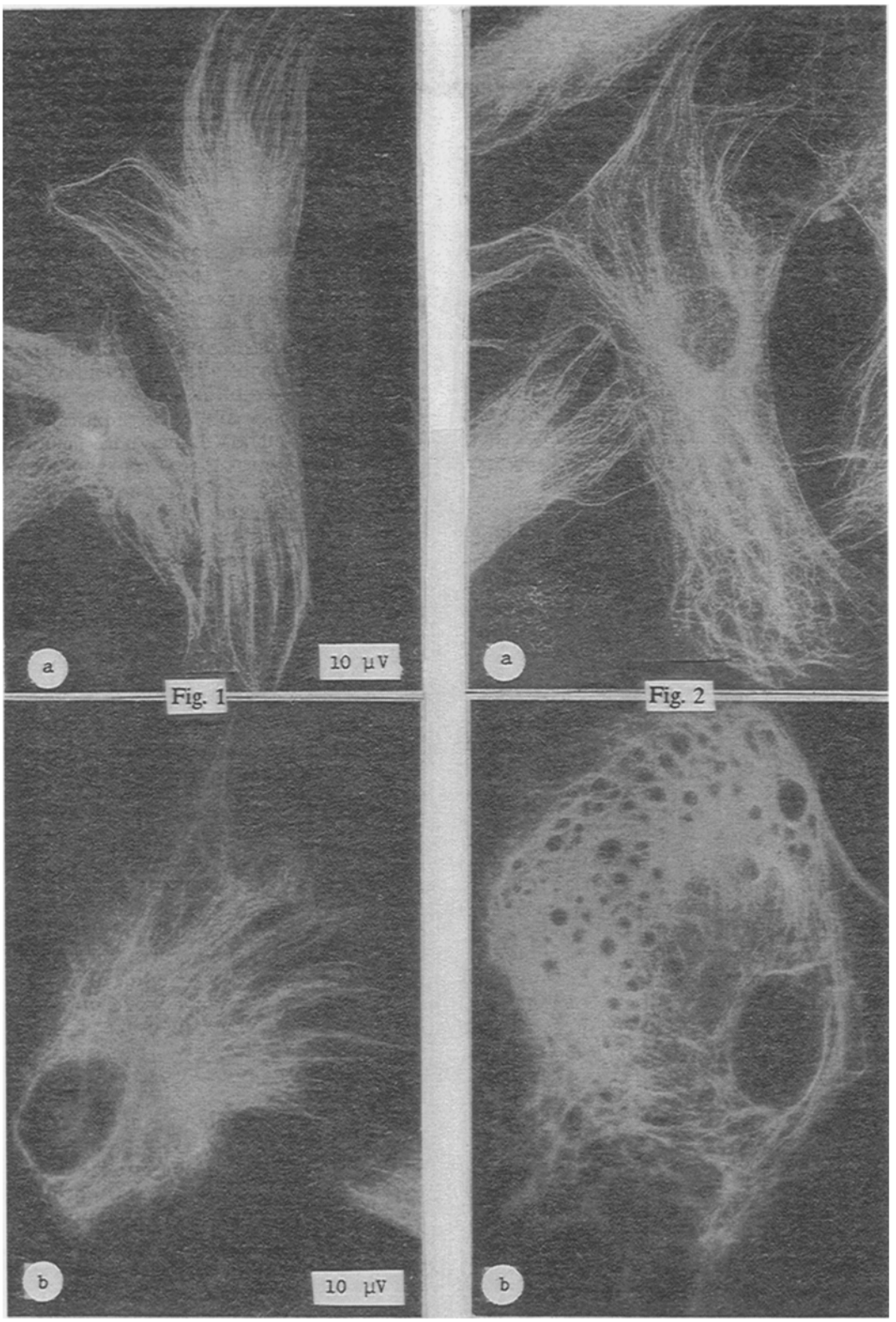

Fig. 1. Immunofluorescence microscopy of cytoskeleton of normal human cutaneous fibroblasts in vitro: a) microtubules, b) vimentin-containing intermediate filaments (radial fibrillar organization). Scale: 10 mm (objective ×40/1.6, ocular ×15).

Fig. 2. Immunofluorescence microscopy of cytoskeleton of pathological human fibroblasts (Fabry's disease) in vitro: a) microtubules, b) intermediate filaments (forming ring-shaped structures). Magnification and scale the same.

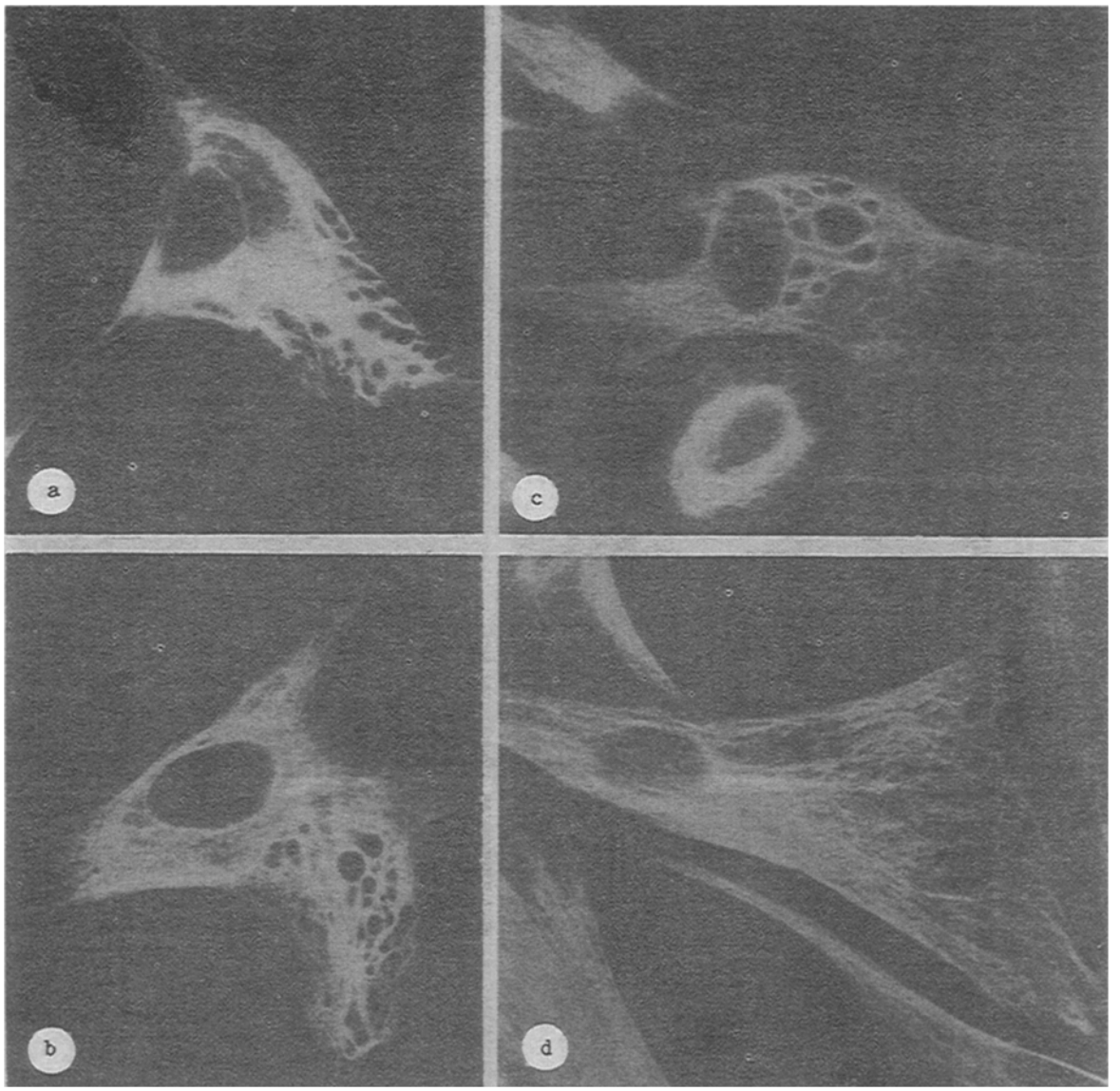

Fig. 3. Immunofluorescence microscopy of vimentin intermediate filaments of pathological cells (Fabry's disease) at different stages of spreading: a, b) centrifugal displacement of ring-shaped structures of intermediate filaments during polarization of cells, c) ring-shaped structures, heterogeneous in size, in cells in stage III of accumulation, d) restoration of radial organization of intermediate filaments (24 h after subculture of cells). Scale: 10 μ (objective $\times$40/1.6; ocular $\times$15).

tion of granules (stages I-III) were therefore subjected to repeated passage in lower density. The effect of hypertrophy of the lysosomal compartment on the velocity of spread and morphology of IF and MT was investigated in these cells.

During spreading of the layer of normal fibroblasts changes took place in the shape of the cells [5]. In the control, 1 h after transplantation the spherical cells were converted into disk-shaped. Further spreading of the monolayer led to polarization of the cytoplasm. After 4.5 h and later the fibroblasts acquired the typical elongated, polarized form. At this stage they had a radially organized system of MT and IF (Fig. 1a, b).

By analysis of the process of spreading of the pathological cells we found negative correlation between the degree of hypertrophy of the lysosomal apparatus and the rate of spreading over the substrate. Cells with minimal accumulation of glycolipids spread fastest, and after 4.5 h most of the cell population was diskshaped, and their lamellopodia were spread out evenly virtually around the entire perimeter, while in remaining cells the polarization process had begun (Fig. 3a). Spreading of cells with stage II of accumulation, many of them irregularly diskshaped, spread more slowly, and in some

areas the arrangement of the lamellopodia was disturbed (Fig. 2b). Meanwhile, the elongated, polarized shape of these cells, typical of fibroblasts, was restored after 24 h. Pathological fibroblasts with hyperaccumulation (stage III), exhibited delayed spreading, and after 4.5 h their appearance corresponded to morphology of the cells in state I 1 h after transplantation. After 24 h half of this cell population was still in the process of spreading, whereas the remaining cells, after completing their spreading, had become completely polarized (Fig. 3c, d).

As was pointed out above, intensive intracellular accumulation of undegraded compounds was accompanied by the formation of well-marked ring-shaped bundles of IF, whose internal diameter corresponded to the size of the lysosomes. The relatively small ring-shaped structures of IF, uniform in size, were found in fibroblasts in stage I of accumulation (Fig. 3a). The largest ring-shaped bundles of IF, heterogeneous in size and shape, accumulated in cells with hyperaccumulation (stage III) (Fig. 3c). Thus an altered organization of the IF system was characteristic of polarized pathological cells in a compact monolayer, and was preserved in them at the spreading out stage, with a decrease in cell density.

However, this change in the structure of IF was found to be reversible: on further polarization of the fibroblasts a centrifugal shift of the ring-shaped bundles of IF took place into an active border, whereas in the endoplasm the radial organization of the IF fibrils was restored. After restoration of the typical structure of IF toward 24 h after transplantation was observed in cells in stage I and in most of the population in stage II of accumulation; single closed bundles of IF still remained in the cytoplasm of individual polarized cells. The whole spectrum of transition from one organization of IF to the other could be seen in the population of fibroblasts with the highest level of accumulation after 24 h (Fig. 3). An example of the displacement of bundles of IF, interweaving around granules, into the active border is shown in Fig. 3a, b.

The results of these investigations showed that in LSD the formation of a hypertrophied lysosomal compartment is accompanied by considerable reorganization of the IF system. Under these conditions the radial structure of the vimentin-containing fibrils of IF is transformed into ring-shaped bundles, interweaving around enlarged lysosomes. The changes observed in the cytoskeleton had definite similarity with those described in the literature during accumulation of lipid granules in the course of differentiation of adipocytes [7], endocytosis of asbestos crystals by mesothelial cells [16], and virus infection of cells [11]. This can be taken as evidence of the nonspecific functional reorganization of the structure of IF. It may be due to the necessity for the optimal distribution of a large number of lysosomes or other vesicles, with contents of endogenous or exogenous origin, in the cytoplasm and their stabilization. Slow spreading of pathological cells over the substrate may perhaps also be connected with a disturbance of the structure of IF. They have definite similarity with the slowly spreading cells, incubated in medium with colcemid, which causes depolymerization of MT and collapse of IF. In this case, IF interweave around the cell nucleus, twisting around it in the form of thick flagella [5]. Thus fibroblasts with hypertrophied lysosomes and with a ring-shaped structure of IF may prove to be a successful model with which to study the role of IF, since the MT in this case (unlike in the situation with colcemid) are intact.

Meanwhile, our findings showed that the change in the organization of IF is a reversible process. When cells with a hypertrophied lysosomal compartment were subcultured, reducing the cell density, at the polarization stage following the stage of maximal spreading, a centrifugal shift of the disk-shaped bundles of IF and gradual restoration of the typical radial organization of IF were observed. When studying differentiation of adipocytes, the authors also observed disappearance in some cases of massive tangles of IF, but it was impossible to determine precisely at what stage, before or after passage, this effect was observed [7]. In LSD or other states connected with intensive accumulation of a variety of compounds, intracellular accumulation of membranes also takes place. It can be tentatively suggested that on removal of contact inhibition, under free spreading conditions, membranes accumulated in the form of vesicles may be transported into the zone of the active border of the cells, maintaining the membrane flow [5]. This centrifugal shift of a significant number of lysosomes into the active border we observed previously during spreading of normal fibroblasts, but on complete polarization of these cells the lysosomes were concentrated around the nucleus. There are reports in the literature that target cells in LSD can be purified under rapid proliferation conditions [12]. It is also known that during logarithmic growth of cells the intensity of lysosomal proteolysis declines sharply [4], and cell proliferation can be stimulated by inhibition of lysosomal enzyme activity [15]. It can therefore be postulated that on removal of contact inhibition a large part of the hypertrophied lysosomal compartment is "lost" by cells at the polarization stage during formation of an active border of cytoplasm. This is accompanied by a centrifugal shift and subsequent disappearance of massive tangles of IF, with gradual restoration of the radioorganization of IF fibrils. Preliminary clearance of the excess of lysosomes may be a necessary step in the transition of the target cells to proliferation under low density conditions.

Thus a change in the organization of IF in pathological fibroblasts in LSD is one additional important characteristic of these cells. It is becomming increasingly evident that deficiency of one lysosomal enzyme leads not only to the accumulation of undegraded compounds in the cell, but also to serious changes in other cytobiochemical parameters.

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