Cytoskeletal changes in cultured human fibroblasts following exposure to 2,5-hexanedione

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In the present study, the effects of the 2,5-hexanedione (HD) on the various cytoskeletal components of human eukaryotic cells were investigated. Primary cultures of human fibroblasts from three healthy donors were exposed to 2.8 mM HD for 14 days; unexposed cultures were used as controls. At different time intervals, cells were counted and the growth curves compared. After 14 days of treatment, the cultures were processed for immunoblotting, immunocytochemistry and ultrastructural studies. Compared to controls, morphological abnormalities induced by HD consisted of modification of the cell shape, formation of tangles composed by 10 nm filaments and cytoplasmic segregation of microtubules and membrane-bound organelles. By immunocytochemistry, the tangles of cytoplasmic filaments were stained by an antibody specific for vimentin. By immunoblotting, the anti-vimentin antibody recognized only bands of 50-60 kDa. Comparison of growth curves between treated and control fibroblasts clearly revealed an interference of HD with the cell cycle. The present results demonstrate that the cytotoxicity of HD is not restricted to intermediate filaments, but affects other cytoskeletal components, such as microtubules, as suggested by the impairment of cell division cycle.

Keywords: cell cultures, 2,5-hexanedione, intermediate filaments, microtubules, cell cycle

Introduction

Previous in vivo and in vitro studies have provided evidence that intermediate filaments are the target of 2,5-hexanedione (HD) toxicity [4, 5, 14, 21, 22]. Following exposure to HD, formation of tangles of vimentin occurs in mesenchymal cells [5], glial fibrillary protein in astrocytes [6] and neurofilaments in neuronal cells in culture and distal axonal segments in vivo [19, 9, 3, 12, 10]. HD promotes pyrroilation of e-amino lysil residues of neurofilament proteins affecting their interaction with other cytoskeletal components [23, 17, 18, 13], thus producing an increased interpolymer spacing and a selective acceleration of neurofilaments transport along the axon. It has been suggested that this is the mechanism for the central-peripheral axonopathy observed in experimental models and, by extension, in human pathological conditions caused by HD exposure. However, evidence has been provided that other cytoskeletal components may be involved during HD intoxication [1, 2]: therefore, HD could affect a variety of cell functions other than translocation mechanisms.

In the present study we investigated the changes induced by HD in human cultured fibroblasts. The aim was to test the effects of HD on the spatial organization of IF and microtubules and, consequently, on cell viability, shape and cycle.

Materials and methods

Cell cultures

Primary cultures of human fibroblasts were obtained from skin biopsies of three normal individuals
undergoing surgery for inguinal hernia. Cells were grown from 0.5–1 mm explants in 60 mm plastic Petri dishes and after reaching confluence they were sub-cultured in flasks; cultures at the third passage were used for the present study. Cells were harvested by trypsinization and seeded into 35 mm plastic Petri dishes or on 9 mm glass coverslips. Feeding medium consisted of 60% DMEM (Gibco), 30% MEM 199 (Gibco), 5 mg/ml glucose and antibiotics. Twenty-four hours after plating (day 1), normal medium was replaced with medium containing HD at a final concentration of 2.8 μM. Medium containing HD was renewed every other day throughout the exposure time of 14 days.

Unexposed cultures were used as controls. Living cultures were daily checked under an inverted phase contrast microscope.

Ultrastructural study
After 14 days, cells growing in Petri dishes were fixed in 2.5% glutaraldehyde in PBS for 30 min at room temperature and post-fixed in 1% osmium tetroxide. After washing in PBS, cells were scraped with a rubber policeman, harvested and centrifuged; pellets were dehydrated in graded acetone and embedded in Spurr. Thin sections were stained with uranyl acetate and lead citrate and examined under a Zeiss 109 electron microscope.

Immunocytochemistry
On day 14 of treatment, day 15 in vitro, cells growing on glass coverslips were fixed in 4% paraformaldehyde in PBS, pH 7.4, for 30 min at room temperature, washed in 0.1 M Tris buffered solution (TBS) and incubated overnight at 4 °C with the following antibodies: mouse IgG to vimentin (Labsystems) (diluted 1:50), rabbit Ig to tau (ICN) (1:100) and rabbit Ig to tubulin (Miles) (1:100). After washing in TBS, cells exposed to anti-vimentin were incubated with biotinylated goat anti-mouse IgG (Amersham) and Streptavidin-horseradish peroxidase (Amersham); swine anti-rabbit Ig (Dako) and rabbit PAP (Dako) were used for tau and tubulin. Reaction products were visualized by 3,3′-diaminobenzidine.

Immunoblotting
Fibroblasts growing on dishes were scraped in PBS, centrifuged at 2000 r.p.m. for 10 min, washed and resuspended in sample buffer. Five milligrams of proteins were separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose paper [26]. Blots were washed with TBS and exposed to anti-vimentin, as above. Molecular weights (MW) were obtained by comparison with standard MW (Boerhinger).

Quantitative analyses
At day 2, 3, 4, 6, 7, 8, 10, 13, and 15 exposed and control cells were harvested by trypsin treatment, centrifuged and the pellets were resuspended in PBS. Nutrient medium from each dish of control and exposed cultures was collected, centrifuged and floating cells were counted. Three samples of each cell suspension

were counted with a standard haemocytometer and the mean number of elements was obtained. This procedure was repeated three times: cell density at plating time was $8.2 \times 10^4$, $8.3 \times 10^4$, and $8.4 \times 10^4$, respectively. The growth curves of treated and control cells were estimated from triplicated experiments. Numbers obtained in treated and control cultures were statistically compared at each time point using a paired $t$-test (one tail). The null hypothesis was rejected when $P > 0.05$.

**Results**

**Morphology and immunocytochemistry**

Light microscopy examination showed that fibroblasts exposed to HD underwent changes in their morphology after the third day of treatment: by the end of the study, cells appeared as 'glial-like elements' provided with a large, swollen cytoplasm, an eccentric nucleus and thin

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Figure 3. HD exposed cultures: electron micrograph showing accumulation of tangles of intermediate filaments in central areas of the cytoplasm, the microtubules being segregated in the periphery. × 8000. Inset: higher magnification of a marginal area (*) containing exclusively microtubules. × 34800.

elongated processes. By immunocytochemistry, antibody to vimentin intensely stained the cytoplasm and processes (Figure 1a), whereas reactivity to tubulin and tau was less intense and not uniform. Control cultures exhibited a flat or elongated morphology and were uniformly stained by antibodies to vimentin, tubulin and tau (Figure 1b). At the ultrastructural level, the cytoplasm of exposed cells contained tangles of disorganized 10 nm filaments (Figure 2); packed mitochondria and other membrane-bound organelles were segregated with microtubules radiating from the centrosome. A typical example of cytoskeletal derangement induced by HD is

Figure 4. Control cultures: electron micrograph of two fibroblasts showing a normal arrangement of intermediate filaments and Mt. × 13,200.

illustrated in Figure 3 and inset, where intermediate filaments form disorganized cytoplasmic masses whereas microtubules are displaced around the perinuclear region. Exposed cultures showed no signs of cytotoxicity, such as vacuolization, swelling of Golgi apparatus and mitochondria abnormalities. Unexposed fibroblasts showed a normal distribution of cytoskeletal and cytoplasmic constituents (Figure 4).

Immunoblotting

Immunoblot analysis showed that both in treated and untreated cultures antibody to vimentin recognized several bands of apparent MW 50–60 kDa. No bands of higher MWs, corresponding to dimers or polymers of vimentin were seen in treated cultures (Figure 5).

Figure 5. Immunoblot showing that the antibody to vimentin recognizes bands of 50–60 kDa in control (lane 1) and HD (lane 2) cultures.

Quantitative analysis

The growth curves of control and treated cells were similar up to the fourth day of the study. From the sixth day on, HD affected the cell cycle (Figure 6), the difference being statistically significant (P=0.01 at day 8 and 13; P<0.01 at day 15. By the end of the study, the number of control cells increased threefold as compared to exposed cultures. No differences in the number of detached cells or cellular debris in the nutrient medium were observed between control and intoxicated cells, indicating that no cell death occurred in treated cultures.

Discussion

Morphological abnormalities and impairment of the cell cycle were the main changes observed in primary cultures of human fibroblasts following exposure to 2,5-hexanediol (HD). The effects of HD have been extensively studied in experimental and human pathological conditions [7, 8, 14]; yet the precise molecular mechanisms of HD toxicity have not been completely elucidated. However, consistent evidence has been provided that intermediate filaments, which together with microtubules and actin filaments form the cytoskeleton of higher eukaryotic cells, represent the main target of HD [4, 6, 10]. In the nerve fibre, the interaction between
HD and neurofilaments induces modifications of the ε- amino groups of the lysine sidechains with formation of pyrroles; this is followed by disruption of the microtubules-neurofilaments network with increased interpolymer spacing and accelerated transport of neurofilaments [13, 16]. As a consequence, neurofilaments accumulate at sites of axonal constriction such as the node of Ranvier [24] but especially in the distal regions of the axons, leading to the formation of giant axonal swellings [17, 18, 19]. Proximal segments of the fibres show a reduction in neurofilament number and decrease in axonal calibre, thus indicating that neurofilaments are the main determinant of axonal size and that neurofilaments are the main target of HD toxicity.

The present results strongly suggest that cytoskeletal changes are likely to occur also for vimentin, the intermediate filament of fibroblasts. Although in fibroblasts the microtubules-neurofilaments network has not the dynamic and architectonic features observed in axons, evidence has been obtained that vimentin filaments are spatially organized and pulled towards the cell periphery throughout interactions with microtubules [25]. Therefore, following exposure to HD, impairment of polymer sliding mechanisms between microtubules and vimentin filaments may occur and intermediate filaments accumulate in disorganized cytoplasmic masses [22]. Vimentin filaments, which unlike neurofilaments, are not provided with side-arms, maintained their ultrastructural and immunoreactive properties after HD treatment, thus confirming that HD affects only their interaction with microtubules. Therefore, the effects of HD could be explained by an interference of this compound with microtubules side-arms or, alternatively, by changes in tubulin polymerization rates. Support for both these mechanisms has been obtained in previous ultrastructural and pulse-labelling studies [17, 18].

At variance with other reports [6, 21], evidence for formation of cross-linked vimentin filaments was not obtained in the present study. Instead, we found that the spatial organization of microtubules is affected by HD. Under physiological conditions, microtubules emerge from the centrosome as single, dynamically unstable filaments and radiate outward through the cytoplasm, aligning themselves along the major axis of elongated cells. Such a spatial organization regulates cell shape and locomotion and, in addition, provides a scaffold along which vesicles and other membrane-bound organelles are transported [11]. In fibroblasts exposed to HD, microtubules appeared segregated in given areas of cell cytoplasm: this abnormal organization may account for changes in cell polarity and shape and for the impairment of the intracellular trafficking of organelles, which were co-segregated with microtubules. Such a spatial redistribution of microtubules may be the consequence of a mechanical derangement induced by the tangles of vimentin filaments, but changes of microtubules proteins cannot be ruled out [1, 2].

Severe impairment of the cell cycle was another main effect of HD intoxication. In fact, at the end of the experiment, the number of treated cells was threefold lower than controls; this was not due to cell death but rather to a prolongation of the cell cycle. It is well known that the cell cycle requires a number of complex mechanical processes and that microtubules play a major role in each of these processes, particularly during early phases when centrosome division and mitotic spindle formation occur. Thus, the microtubules derangement could be a possible explanation for the anti-mitotic activity exhibited by HD in human fibroblasts. Other proposed mechanisms include alterations of the integrity and function of intermediate filaments [15] or the inactivation of some enzymes of the glycolytic pathway [20].

In conclusion, this study provides evidence that cytotoxicity of HD is not restricted to intermediate filaments but it also involves other cytoskeletal components such as microtubules, producing morphological abnormalities as well as functional changes in human fibroblasts in vitro.

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