Effect of doxorubicin on actin reorganization in Chinese hamster ovary cells

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Exposure of Chinese hamster ovary (CHO AA8) cells to doxorubicin in doses of 75 μM resulted in reorganization of F-actin filaments and characteristic feature of apoptosis. Even increase in size of CHOAA8 cells was observed. Attached cells become more flattered and elongated than control ones. Intense labeling of F-actin around the nucleus and disrupted filaments in cytoplasm as well as stress fibres and bundles of F-actin were seen. Cells detached were rather rounded and there were not stress fibres present. In these cells the network of F-actin was weak and rather disrupted and cells with buds labelled for F-actin were observed. In section from confocal microscopy labeling of F-actin in nucleus was confirmed. Electron microscopy showed cells with multisegmented nuclei. Cells with intracellular area with small and large vacuoles and containing also electron-dense material were seen. Other cellular organelles were rather well preserve. There were margination and condensation of chromatin in nucleus. Immunogold labeling of actin was observed in cells whether or not treated with doxorubicin. Positivity for actin was localized in the nuclei and cytoplasm. In the nucleus gold particles were localized predominantly in the area of condense chromatin. Positive labeling for actin was not found after control incubation. These findings show that doxorubicin promotes changes in the distribution of actin in CHOAA8 cells and that reorganization of actin in these cells is involved in process of apoptosis.

Key words: CHOAA8 cells, actin, immunogold technique, fluorescence method, doxorubicin

Recently, studies of apoptosis in relation with actin cytoskeleton has been attracting researches attention [5, 7, 8, 15, 16, 20, 23]. The link between actin and apoptosis might offer in the future opportunities for therapeutic intervention, such as in the treatment of cancer or other disorders. Understanding not only the molecular control of apoptosis but also signalling to the actin cytoskeleton which is involved in apoptosis will undoubtedly yield in the future a new generation of drug targets. However, many key questions still remain to be answered. The problem of actin has been studied very extensively for decades and almost every well-defined role found so far has been localizes to the cytoplasm, but relation to nucleus still remains unknown [22]. Much of our knowledge of apoptosis in mammals comes from immune system models. It is clear that cells of the lymphoid type choose apoptosis in response to damage far more readily than do most other cells, for example fibroblasts [4]. Therefore, cells like HL-60 were often chosen as typical model for studying process of apoptosis on different level also in our studies [7–9]. Here, we used Chinese hamster ovary cell line (CHOAA8), which was described previously by THOMPSON et al [25]. BLANKENSHIP et al shown that apoptosis is the mode of cell death of nearly all Chinese hamster ovary cells also strain AA8 [2]. Apoptosis is the process, which is defined by morphological changes of cells and nuclei, as cell shrinkage, condensation and fragmentation of nuclei, and blebbing of the plasma membranes [11]. FANG et al in their work suggested that membrane and nucleus blebs probably are related to apoptotic chromatin condensation. They also suggest that membrane and nucleus blebs during apoptosis are related to microfilament reorganization and can accelerate apoptotic chromatin condensation [6]. In our earlier observation on leukemic K-562 and HL-60 cell lines we have shown that actin is involved in apoptotic chromatin condensation [7, 8, 9]. LUCHETTI et al also concluded in their work that is connection between actin and chromatin reorganization [16]. However, it is not yet clear whether actin is involved in regulation of nu-

clear process as chromatin reorganization during apoptosis. Studies on different cell types will be required to established this problem. We used the Chinese hamster ovary cells CHOAA8 to investigate whether changes seen by us in human leukemic cells especially in HL-60 cells which are model line for apoptosis are also present in mammalian normal fibroblast. We recorded here the effect of doxorubicin on fibroblast of CHOAA8 cell line in relationship with reorganization of actin which is especially involved in characteristic morphological features of apoptosis.

Material and methods

The hamster fibroblasts CHO AA8 kindly provided by Prof. M. Zdzienicka from Leiden University Medical Center were studied. Cells were diluted to $5x10^5$ cells/ml and were grown in RPMI 1640 medium supplemented with 15% fetal calf serum and 125 μl gentamycin in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were incubated with 75 μM of doxorubicin (Sigma, St. Louis MO, USA) for 72 hours. Control cells incubated in absence of doxorubicin were treated identically. Cell viability was assessed by the trypan blue dye exclusion method. F-actin filaments in cells were labeled with phalloidin conjugated to rhodamine (TRITC; Sigma) as previously described [1]. Cells for fluorescent microscopical analysis were collected directly onto microscopical slides using a cytocentrifuge and were fixed with 4% paraformaldehyde. Then, they were incubated in 2 μM rhodamine-phalloidin calcium and magnesium free in phosphate-buffered saline (PBS-A) containing 20% methanol for 20 min at room temperature in the dark, rinsed twice in PBS-A and mounted in gelvatol (Monsanto, St. Louis MO, USA). DAPI (Sigma) was used to show nuclei. The organization of F-actin filaments and DNA staining was examined with an Eclipse E600 microscope equipped with Y-FL epifluorescence equipment (Nikon, Tokyo, Japan) and especially in the nucleus by using confocal microscope (inverted microscope, Nikon, Eclipse TE 300). For conventional electron microscopy cells were fixed with 3.6% glutaraldehyde in phosphate buffer, postfixed in OsO_4 in the same buffer and after dehydratation with alcohol embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate. To demonstrate actin at the ultrastructural level, a postembedding streptavidin-gold method was used. Cells were fixed in 4% paraformaldehyde in PBS for 1 h at 4 °C, and washed overnight in PBS at 4 °C. Dehydration was performed in an ascending series of ethanol and embedding of the cells was performed in LR White. Sections (60 nm thick) were cut and placed on nickel grids (Sigma). The grids were floated on a drop of nonimmune rabbit serum (Dako, Glostrup, Denmark) for 20 min and then transferred onto drops of a 1:100 dilution of a monoclonal anti-actin antibody (AC-40; Sigma). After incubation with primary antibodies for 30 min, the grids were rinsed in PBS. The grids were then exposed to biotinylated rabbit anti-mouse immunoglobulins (Dako) diluted 1:100 and then again washed in PBS. Afterwards, the grids were transferred onto drops of a 1:20 dilution of 10 nm gold particles conjugated to streptavidin (Sigma) and incubated for 30 min. The grids were then washed in PBS and dried. All incubation steps were performed at room temp. Control specimens were incubated with nonimmune antiserum (normal mouse serum, Dako). The preparations were examined using a transmission electron microscope JEM 100 CX (JEOL, Tokyo, Japan) at 80 kV.

Results

Doxorubicin treatment of CHOAA8 cells resulted in size increase and cells become more flattered compared to elongated form of control cells (Fig. 1). The bright F-actin labelling around the nucleus and disrupted filaments in cytoplasm treated cells were observed. Attached cells showed also stress fibres and bundles of F-actin. Detached cells were rounded without stress fibres and network of F-actin was weak and rather disrupted (Fig. 2a–d). The cells with characteristic feature of apoptosis with buds labeled for F-actin were also observed (Fig. 3a, c). Cells labeled with DAPI in Figures 3 b, and d confirmed the presence of chromatin in buds. The nucleus in apoptotic cells undergo disruption either by budding or cleavage what can be seen in Figure 4a–d. In sections from confocal microscopy labeling of F-actin in nucleus was also confirmed (Fig. 5). After treatment with 75 μM of doxorubicin multisegmented nuclei which appeared often as isolated ones were seen at the ultrastructural level (Fig. 6). The cells containing intracel-

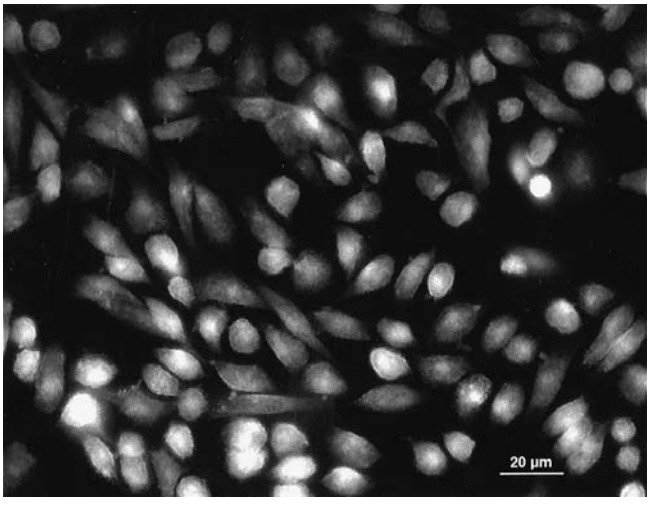

Figure 1. Control CHOAA8 cells cultured in medium free from doxorubicin. Spindle-shaped cells are present.

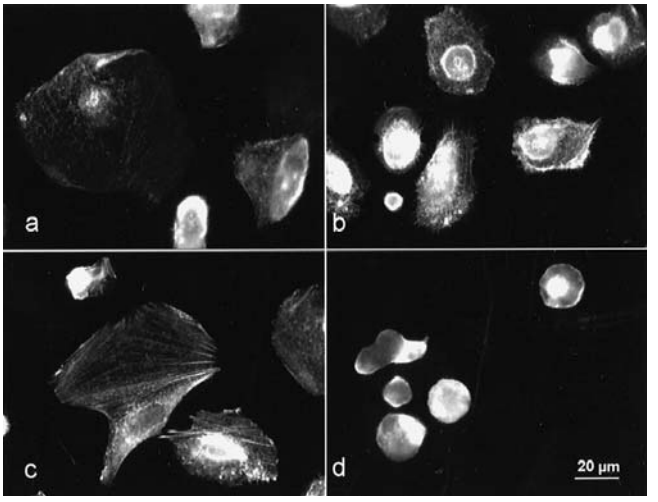

Figure 2. CHOAA8 cells treated with doxorubicin labeled for F-actin. F-actin around the nucleus and disrupted filaments are seen (a, b). Cells with stress fibres and network of F-actin (c) and detached rounded cells with weak network of F-actin (d).

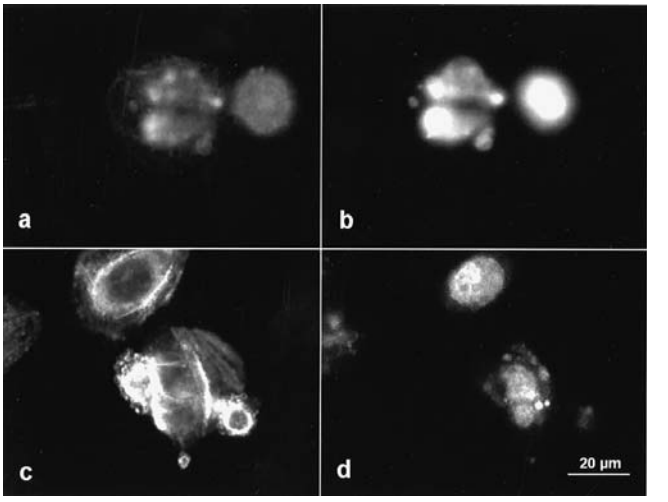

Figure 4. CHOAA8 cells treated with 75 μM of doxorubicin. Cells labeled for F-actin with phalloidin conjugated to rhodamine (a, c). Figures b and d are showing labeling with DAPI. Cleaved nucleus (a) and nucleus with buds (a, c).

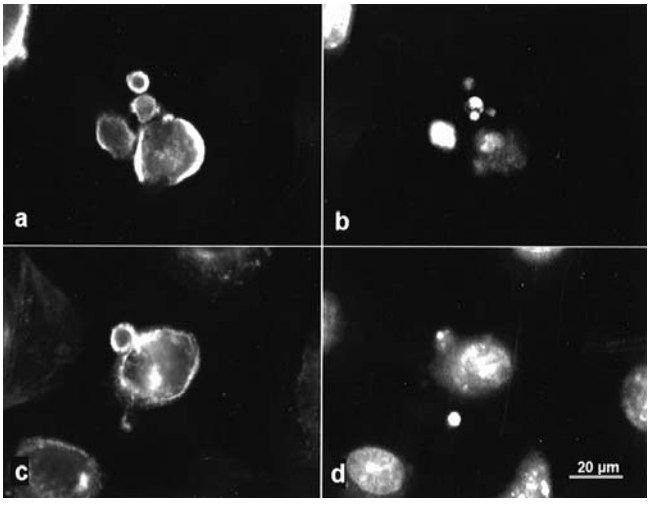

Figure 3. Apoptotic CHOAA8 cells. Labeling for F-actin (a, c) and DAPI staining confirms in buds (b, d).

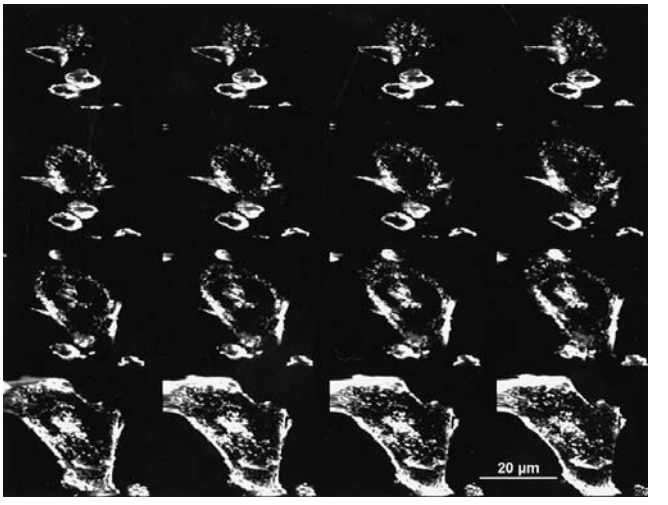

Figure 5. Z-scan series through doxorubicin treated CHOAA8 cell with TRITC-phalloidin labeling for F-actin. There are rich F-actin in cell cortex and buds. In some sections nucleus labeling can be clearly observed.

lular areas with small and large vacuoles (Fig. 7) exhibited nucleus with margination and condensation of chromatin (Fig. 8). The control cells showed normal morphology with oval or kidney-shaped single nucleus (Fig. 9). We estimated also the distribution of actin at the ultrastructural level using a streptavidin-gold immunoelectron microscopical technique. Immunogold labeling of actin was observed in cells whether or not treated with doxorubicin. Positivity for actin was localized in the nuclei and cytoplasm. In the nucleus gold particles were localized especially in the area of condense chromatin (Fig. 10). Positive labeling for actin was not found after control incubation with non-immune antiserum (normal mouse serum, Dako) (Fig. 11).

Discussion

Apoptosis is believed to be one important mode of action of chemotherapeutic drugs used in cancer treatment as well as in therapy for fibro-proliferative disorders. Involvement of actin cytoskeleton in apoptosis can provide a new insight in this process and may be therefor of significant clinical interest. There are still no effective therapeutical approaches to reduce the proliferation of fibroblast cell population within existing fibrotic lesions [17, 24, 27]. The mechanisms by which fibroblasts undergo apoptosis are still not well known. Presented results demonstrate the effect of

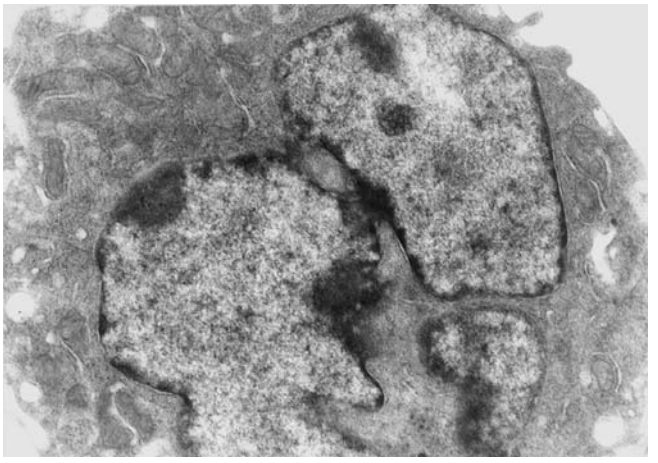

Figure 6. A part of CHOAA8 cell treatment with 75 μM of doxorubicin. The segmentation of nucleus is seen. x 17,000.

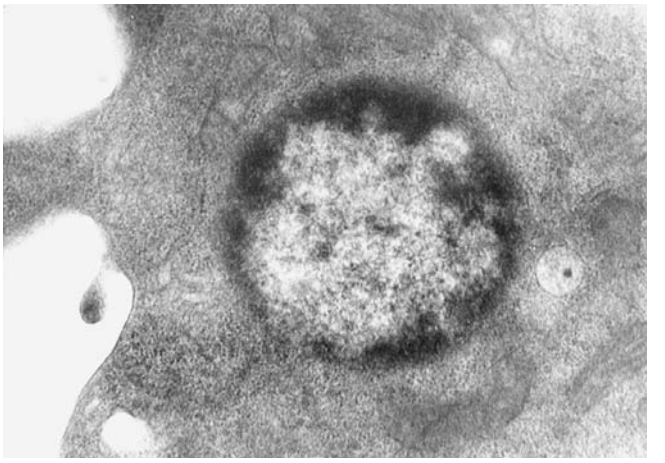

Figure 8. Electron micrograph a part of CHOAA8 cell treated with 75 μM of doxorubicin. The nucleus with margination of chromatin is seen. x 23,000.

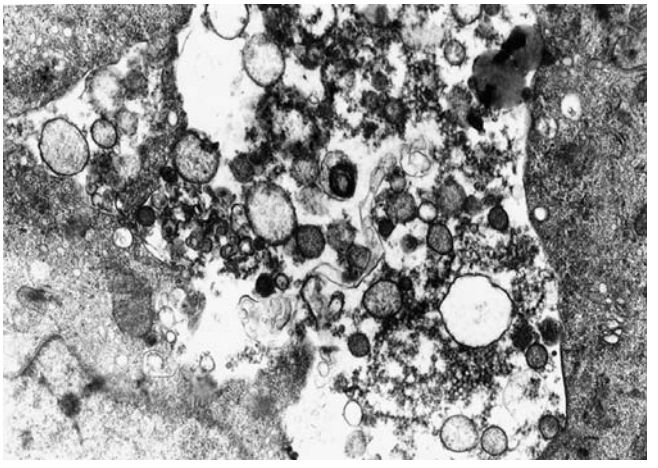

Figure 7. A part of CHOAA8 cell with intracellular area where small and large vacuoles and also electron-dense material can be observed. x 17,000.

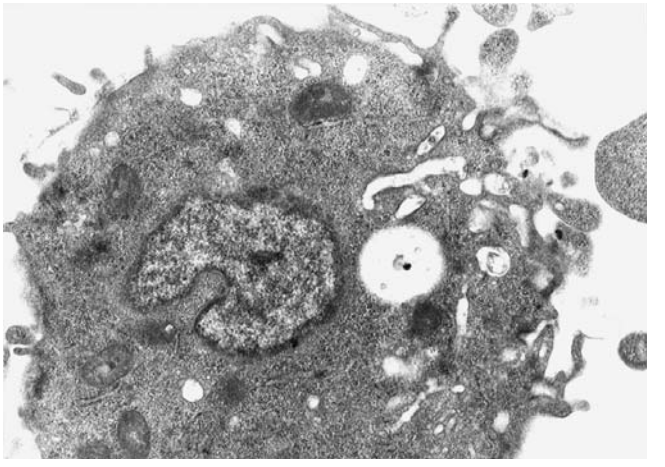

Figure 9. Electron micrograph of CHOAA8 cell without doxorubicin treatment. The kidney-shaped single nucleus is seen (a). x 8000.

doxorubicin on fibroblast of CHOAA8 cell line in relationship with reorganization of actin which is especially involved in characteristic morphological features of apoptosis. Doxorubicin is topoisomerase II inhibitor which above all intercalate into DNA and is one of the most active anti-cancer agents showing also its cytotoxic effect on malignant cells by generating free radicals with subsequent induction of apoptosis [18, 26]. HASINOFF et al in their studies showed that topoisomerase II inhibitor dexrazoxane inhibits the division of CHOAA8 cells but allows them to increase in size. They suggest that dexrazoxane may be useful in treating tumors having defects in their apoptosis-transduction mechanism. Dexrazoxane growth inhibitory effect seems to be done through the induction of endoruplication rather than through apoptosis [10]. Presented data showed that doxorubicin treatment not only allowed cells to increase in size but also induced apoptosis in CHO AA8 cell line which is functionally defective in p53 [13, 14]. There are also other reports on induction of apoptotic cell death in CHOAA8 cell line [2, 3]. Our previous results have provided insight into reorganization of actin and other main proteins of cytoskeleton in relationship with death of K-562 and HL-60 leukemia cell lines after treatment with doxorubicin and etoposide [7–9]. We had previously found not only apoptotic cells but also cells which stopped to divide and continued to increase in size what is in agreement with observations HASINOFF et al. We suggest that changes in the reorganization of actin seen in our previous and these studies are independent on type of cells used. The results with CHOAA8 cells showed also disruption of F-actin filaments integrity by doxorubicin which was often promptly associated with characteristic features of apoptosis. The clone AA8 contain defects in its apoptosis-transduction mechanism in spite of that doxorubicin toxicity induced death of

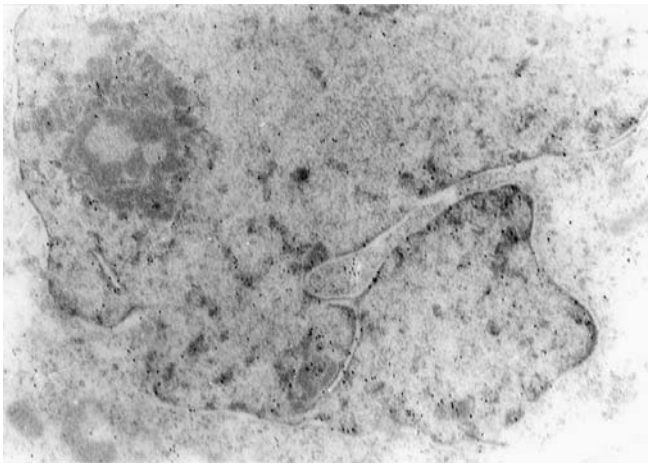

Figure 10. Immunogold labeling of CHOAA8 cell treated with 75 μM of doxorubicin. Gold particles are seen in areas of condensed chromatin. x 25,000.

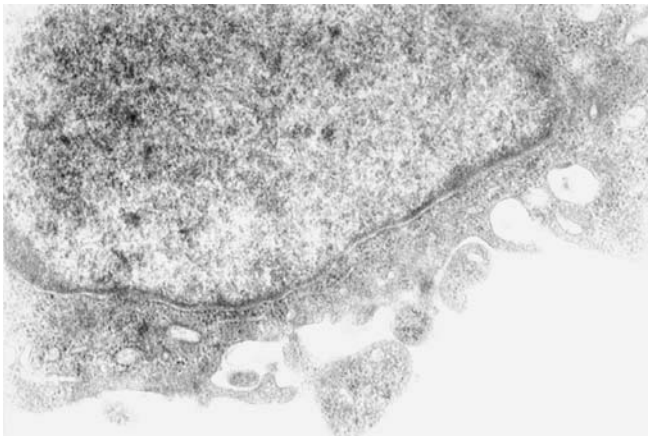

Figure 11. Electron micrograph of control cell incubated with nonimmune antiserum. Gold particles are not seen. x 20,000.

studied cells, however changes in reorganization of actin in association with characteristic features of apoptosis were observed. Morphologically, multisegmented nuclei were often seen in doxorubicin treated CHOAA8 cells. In numerous cells the segmentation appeared as isolated nuclei. Other reports also showed that treatment of cells with anticancer drugs and other agents as retinoic acid can produce multisegmentation of nuclei [10, 19]. These observations showed that doxorubicin as topoisomerase II inhibitor induced characteristic apoptotic features in CHOAA8 cells which are not sensitive to apoptosis as it was seen in HL-60 cells used in our previous studies [7, 8]. We agree with conclusions of other authors that induced toxicity is an extremely complex process, involving multiple pathways leading to apoptosis [3]. The main death pathway initiated by activation of CD95 involves a series of death associated molecules including the Fas-associated death domain containing protein (FADD) [12]. Recently PARLATO et al showed a linkage of CD95 to actin system in human T lymphocytes [21]. KULMUS at al speculated that rearrangement of the cytoskeletal actin filaments forms the basis for cell death receptor clustering. They showed in their studies that CD95 clusters colocalize with distrupted actin filaments [12]. The actin system may be notice as an additional pathway involved in the process of apoptosis and can be promising target for development of new cytostatic agents. Further investigations would be necessary to evaluate the potential clinical efficacy involvement of actin in apoptosis process for cancer therapy.

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