Cadmium influences the 5-Fluorouracil cytotoxic effects on breast cancer cells

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Introduction

Cadmium (Cd) is one of the most toxic transition metals associated with air and water pollution, as well as cigarette smoking. The increase of industrialization and human activities have led to a greater use of metals, and, as a result; growing ecodispersion which involves considerable economic and social consequences. It is dangerous for human health. The absorption in the human organism occurs mainly through inhalation, ingestion and cutaneous contact. Cd has a long biological half-time; its values have been seen ranging from 15 to 40 years with an average around the 20-25 years. It is mainly accumulated in liver and kidneys.

Cd is considered to be one of the most dangerous metals, especially for human health, due to its teratogenic and carcinogenic effects. Indeed, there is increasing evidence demonstrating the, in vivo and in vitro, carcinogenicity of Cd, in both human and experimental animal models. The heavy metal Cd has been described to possess estrogen-like activity, as an endocrine disruptor. Moreover, in some countries it has been used in the past, and is still now, as a contaminant in pesticides and in fungicides. Unlike the inhalation of tobacco smoke representing the main source of non-occupationally exposed adult subjects, the metal is vaporized during combustion. Nevertheless, the main cause of exposure to Cd is through food ingestion. For example, the highest Cd concentrations, compared to other foods, are seen in sea food (molluscs), and kidney and liver of mammals. The International Agency for Research on Cancer (IARC) has classified Cd as a category 1 carcinogenic substance. Thus, the metal could induce liver cancer, hematopoietic cells neoplasm, bladder and stomach cancers. It is also believed that Cd may have a role in the origin of pancreatic carcinoma.

Breast cancer is the most prevalent cancer among women in many industrialized countries. The MCF-7 human breast cancer cell line has been used as an excellent experimental model to improve the efficiency of different therapies before they are used on patients. The MCF-7 cell line is a useful cell line that can be used in many cancer researches and it is also an estrogen receptor-negative. The case of glandular epithelial cells is important in the estrogen hormones study and in the parallel study of the cadmium-estrogens relationship due to the ability of Cd to mimic the effects of estrogen receptors tricking the cell membrane. 5-Fluorouracil (5-FU), are antimetabolite inhibitors of de novo purine and pyrimidines synthesis. 5-FU plays an important role in standard chemotherapy protocols for a range of solid tumors, including breast and colorectal cancers. Considering the carcinogenic and mutagenic effects of Cd on healthy living cells, it seems beneficial to use a cancer cellular line, such as MCF-7.3 It is particularly resistant to harmful solicitations of toxic metals, which provoke alterations to both cytoplasmic organelles and cytoskeleton proteins of different cellular lines. We investigated the potential antagonists effects of the antineoplastic drug 5-FU, using scanning electron microscopy (SEM), transmission electron microscopy (TEM) and immunofluorescence. The study of the modifications of cytoskeleton proteins is a parameter particularly indicative of the cellular and apoptotic cycle of the cellular lines. Cytoskeleton has been shown to be involved in the traffic of a membrane of membrane proteins. The effects of 5-FU were studied on MCF-7 cultured cells exposed to different concentrations of Cd, and, then, we evaluated the resulting morphological alterations, even at the ultrastructural level. Moreover, in order to clarify the mechanism of both Cd and 5-FU activities, we compared the effects of these elements on actin and tubulin, two cytoskeleton proteins, responsible not only for the intracellular transport of vesicles and cell organelles, but for their positioning and cellular integrity as well.

Materials and Methods

Cell culture

Human breast cancer MCF-7 cells were cultivated in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco) supplemented with 10% of fetal bovine serum (FBS) (Gibco), 2.0 mmol/L glutamine, 100 U/mL penicillin, and 100
mg/mL streptomycin. Cells were grown at 37°C in an atmosphere containing 5% CO2, then expanded for several days to become confluent in T75 flasks for cellular culture. They were then treated with Trypsin-EDTA (Invitrogen) and plated in a 24-well plate for immunohistochemistry and SEM preparation, and in T75 bottles for TEM preparation.

**Drugs and drug treatments**

Cells were treated with 5-Fluorouracil (5-FU) and Cadmium Chloride (CdCl2), purchased from Sigma-Aldrich. The drugs were dissolved in DMSO or water and stored at -20°C. For each experiment, the stock solutions were further diluted to obtain the desired concentrations. The final solvent concentration in cell culture was = 0.1% v/v of DMSO, a concentration without effect on cell replication. Parallel cultures of cells in medium with DMSO were used as controls.

**Cytotoxicity assays in vitro**

The effect of anticancer drugs on cell viability was assessed using the sulforhodamine B colorimetric assay. Aliquots of MCF-7 cells suspension (1×10^4 cells/well) were seeded onto 24-well plates and incubated for 24 h. The cells were then treated with different concentrations of drugs in the culture medium. Three days later, the wells were aspirated, fresh medium and treatment was added, and the cells were maintained for an additional three days. Thereafter, the cells were processed as described previously, using a Titertek Multiscan apparatus (Flow, Irvine, CA, USA) at 492 nm. We evaluated linearity of the SRB assay with cell number for each cell line before each cell growth experiment. The IC50 values were calculated from semi logarithmic dose-response curves by linear interpolation. All of the experiments were plated in triplicate wells and were carried out at least twice. For each of the experiments we used different concentrations and at different time points (Table 1).

**Electron microscopy**

MCF-7 cells were treated with Cd (5 µM, 20 µM, 40 µM) and 5-FU (1.5 µM) at different time points (24 h, 48 h) (Table 1). The cells pellet, to be observed by TEM, were fixed in a 2% glutaraldehyde solution in a 0.1 M cacodylate buffer (1 h), and washed three times, 15 min each, in the same buffer. Cells post-fixation was done with 1% osmium tetroxide (OsO4) (1 h), washed again in buffer and distilled water, and then embedded in 1.5% low-melting-point agarose. Small blocks (1 mm x 1 mm) were cut out after the agarose had solidified. After dehydration by graded ethanol, the blocks were embedded in Spurr’s low-viscosity resin. After ultra thin sections were prepared by the LKB-8800 ultratome, with 4% uranyl acetate and lead citrate, the samples were stained. Photographic images were examined using a Zeiss 902 electron microscope at 80 kV. Culture cells, grown on circular coverslip, were fixed in 2.5% glutaraldehyde solution in phosphate buffer saline (PBS) for 90 min, then washed three times for 5 min each in PBS, and post-fixed in 1% OsO4 for 2 h. After three washes, 5 min each in PBS, the samples were dehydrated through graded alcohol solutions, air-dried with hexamethyldisilazane (10 min), examined under an ESEM (environmental scanning electron microscope), and photographed in low vacuum using both SEM FEIInspect S and Quanta 200.

**Immunohistochemistry**

Confluent cells were treated with trypsin-EDTA (Sigma-Aldrich) and plated on round coverslips (12 mm) in 24-well culture plates. We incubated them for 24 and 48 h, after which an additional concentration of Cd (5 µM, 20 µM, 40 µM) and 5-FU (1.5 µM) (Table 1) was added. After fixation with formaldehyde 4%, membrane permeabilization with Triton 0.2%, and saturation with BSA 1%, the cells were incubated for 1 h with a monoclonal anti-tubulin (Sigma-Aldrich), and then conjugated with Ab anti-mouse FITC (Sigma-Aldrich). In order to study actin, we incubated cells with TRITC-phalloidin conjugated (Sigma-Aldrich). The nuclei were observed by DAPI, and finally the coverslips, mounted by Mowiol on an object slide were studied using fluorescence microscopy Olympus BX61 (objective UplanApo 40x/1.35oil/iris, objective UplanApo 40x/1.35oil/iris), with digital B/W camera FViewII (Soft Imaging System), and analyzed using Cell software (CELL^P ver 1.20 Soft Imaging System, Olympus Soft Imaging Solution GmbH, Munster, Germany).

**Results**

**Cytotoxicity assay**

To evaluate the cytotoxicity of Cd and 5-FU human breast cancer cell line MCF-7 we used the SRB staining method. IC50 values, defined as the drug concentration resulting in 50% loss of cell viability relative to the untreated cells, were determined for the compound. The IC50 value for Cd was 5±0.25 µM and for 5-FU 1.5±0.3 µM.

**Electron microscopy**

TEM analysis showed that control MCF-7 breast cancer cells were polymorphous. The centrally placed nucleus showed indentation, usually contained one or two prominent nucleoli, and possessed an evident nuclear membrane. The cytoplasmic organelles appeared well defined and possessed a large number of mitochondria with globular aspect and lamellar cristae. Furthermore, the cell surface was characterized by a high microvillus density. SEM showed a variety of elongated and polygonal shapes with numerous slender and thick cytoplasmic projections and an increased number of filopodia (Figure 1 a,b,c).

**Table 1. Time and concentrations of experimental conditions.**

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>Time point</th>
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<tbody>
<tr>
<td>Ctrl</td>
<td>-</td>
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<tr>
<td>Cd</td>
<td>5 µM/20 µM/40 µM</td>
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<tr>
<td>5-FU</td>
<td>1.5 µM</td>
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<tr>
<td>Cd+5-FU</td>
<td>Cd 5 µM/Cd 20 µM + 5-FU 1.5 µM</td>
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Figure 1. a) TEM 3000X, MCF-7 control; the cells, with polymorphous aspect, present numerous globular mitochondria with lamellar cristae (big arrow) and microvilli (thin arrow); b) SEM 3000X, MCF-7 control; the cells with polymorphous cytoplasm, are rich in microvilli (big arrow) and long filopodia (thin arrow); c) detail of b), MCF-7 control, the filopodia (thin arrow) are evident. Scale bar: 5 µm.
Cadmium

Cd-treated cells at low concentration (5 µM) and for short times of incubation (24 h) showed an intense metabolic activity with larger, clearer and globular mitochondria characterized by thin and numerous dilated cristae. Additionally, a large number of glycogen deposits in clusters were found. The nuclei of all the cells contained completely dispersed chromatin and one or two large nucleoli, which were peripherally located (Figure 2a). The surface of Cd-treated cells at low concentrations observed under SEM looked similar to the surface of the control cells, displaying short and abundant microvilli and infrequent blebs (Figure 2b). At high concentrations of Cd (20 µM) and 24 h of treatment, the nuclei became very evident, and moved sideways in the cellular membrane. Numerous cytoplasmic vesicles were seen, including abundant mitochondria with lamellar cristae sometimes altered. The vesicles were close to the plasmalemma and showed signs of exocytosis. We observed a reduction of the superficial filopodia, although, under extreme conditions (Cd 40 µM, 48 h), they increased significantly in number and showed an increase of cellular death. Furthermore, numerous blebs of different sizes were noticed and cells became more rounded and less attached to the dish.

5-FU

5-FU induced cytotoxicity in MCF-7 cells showed cytoplasmic alterations, an increased number of distended vesicles appearing, and swollen mitochondria with partial crista loss. Components of cell nuclei displayed evident signs of degeneration. There was an absence of nucleoli, and the chromatin condensed into dense granules located near the nuclear membrane (Figure 2c). SEM analyses showed that 5-FU-treated cells had lengthened, became more irregular, and there were numerous intercellular spaces. However, the most striking feature was the pore-like alterations in the cell membrane, which were absent in both parental MCF-7 cells and Cd-treated cells. Also, we observed cells more rounded and less attached to the dish (Figure 2d).

Cadmium plus 5-FU

The addition of 5-FU to cells treated with Cd (5 µM - 20 µM) did not induce significant ultrastructural changes compared with cells treated only with Cd. In cells treated with Cd 40 µM, the cellular death increased. In Cd+5FU-treated cells, numerous mitochondria with globular aspect and regular cristae were present. The nucleus was regular in shape with dispersed chromatin and one or two nucleoli. These observations indicate a cell system in an active metabolic state. SEM analyses showed flat cells with short filopodia (Figure 2 e,f), that after 48 h of treatment were made shorter and tended to disappear.

Immunohistochemistry

In control cells both tubulin and actin appeared distributed in the cytoplasm showing...
a well-organized filamentous structure (Figure 3 a,b,c).

**Cadmium**

In cells treated with Cd (5 µM and 20 µM) for 24 h, tubulin did not undergo structural changes; it remained quite distributed in the nuclear and cytoplasmatic zone (Figure 3 d,e,f,g,h,i). After treatment with Cd the fine architecture of actin filaments appeared changed, consisting of large bundles of filaments which, in some points, thickened to form smeared patches. This finding was quite similar to that observed after incubation with Cd 5 µM (Figure 3 d,e,f) and became more pronounced with Cd 20 µM. High concentrations of Cd induced a strong, evident, actin expression in the plasmatic membrane. Tubulin, especially in MCF-7 cells treated with Cd 20 µM, appeared with an intense perinuclear and compact fluorescence from which separate arrays of filaments departed, branching out throughout the cytoplasm (Figure 3 g,h,i). Cells treated with Cd 40 µM, as already said, showed a cellular death increase.

**5-FU**

In 5-FU-treated cells actin appeared less organized and filamentous. Tubulin filaments showed a regular halo of labeling around the nuclear membrane that did not spread into the cytoplasm. The times of incubation of 24 and 48 h did not show substantial differences (Figure 4 a,b).

**Cadmium plus 5-FU**

In Cd (5 µM-20 µM) +5-FU-treated cells for 24 h actin expression was less filamentous compared to parental non-treated cells; nevertheless, the distribution was clearly better than in the Cd-only-treated cells. Tubulin showed expression and distribution patterns similar to those seen in control and Cd-treated cells (Figure 4 d,e,f). In cells treated for 48 h with Cd (5 µM - 20 µM) +5FU, actin was progressively breaking, whereas tubulin maintained a stable and ordered structure (Figure 4 g,h,i). Therefore, the association Cd+5-FU induced less alterations in both actin and tubulin proteins. Under extreme conditions (Cd 40 µM, 48 h), the addition of 5-FU did not significantly modify the cellular disorder observed with only Cd.

**Discussion**

Cadmium is a heavy metal present in the environment with proven carcinogenic effects on several organs including lung, liver, kidneys and prostate, among others. The damaging effects in the cytoskeleton bring about a complete disappearance of microtubules and the disruption of the filamentous actin protein. The action of Cd on the proliferation of MCF-7 breast cancer cell line has been proved. Yet, 5-FU is an antitumor drug, with an important role in standard chemotherapy protocols, for a range of solid tumors including breast cancer and colorectal cancers. Our results demonstrated that in the cells treated only with Cd, there are ultrastructural changes in MCF-7 cells, including the appearance of secretory vacuoles in the cytoplasm, probably lysosomes. Cells can produce these vesicles as a response to the exposure to Cd. The increase of lysosomes after exposure of Cd is known as a mechanism of sequestration and excretion of substances taken up by cells from the environment. Moreover, Cd induced mitochondrial swelling with a dense matrix in comparison to control non-treated MCF-7 cells, which presented globular mitochondrial with regular lamellar cristae. In breast cancer cells it can be supposed that, besides the implication of mitochondria in phenomena of oxidative stress, the morphological alterations of membranes being a possible consequence of variations in transcription and, consequently, transduction to the block of metallothionein.

Moreover, in Cd-treated cells, nuclei were usually moved toward the cellular membrane and a large number of vesicles and organelles were present from the opposite part of the cell. Actin filaments were arranged as big bundles in the cytoplasmic membrane, while the tubulin branched out throughout the cytoplasm. All of this would explain the perimembranous position of the nucleus. It has been demonstrated that Cd increases proliferation in MCF-7 breast cancer cells and disrupt the normal cell homeostasis triggering signals otherwise switched off.

5-FU-treated cells displayed characteristic signs of degeneration in both cytoplasm and nucleus such as the lack of membrane integrity and the appearance of exocytotic bodies. Moreover, an increased rate of spherical and non-adherent cells was present after treatment with a high dose of 5-FU. These effects indicate the anticancer properties of the antimiabolite 5-FU, which acts hindering the cellular growth and increasing the secretion of vesicles of lysosomal nature. Combined treatment of Cd and 5-FU did not alter ultrastructural components showing similar characteristics to MCF-7 cells, which were only treated with Cd, independently of 5-FU dose. We observed that the tubulin aspect is comparable to control non-treated cells and that the actin presents a non-filamentous organization like in Cd-treated cells. These results suggest that Cd pre-
vents the anticancer effects of 5-FU. This blockage acts preventing the formation of lysosomes in the cytoplasm, that is in relationship with oxidized stress at mitochondrial level. With the combination Cd+5FU there are few and small number of lysosomal vesicles.

In conclusion, in the present study we demonstrated the importance of the interaction between the heavy environmental metal Cadmium, and the antitumor drug 5-FU from a morphological point of view. Although the mechanism remains unknown at present, our findings suggest that Cd prevents the cytotoxic effect of 5-FU on breast cancer cells. Further research is required to fully analyze correlations between our morphological results and the bimolecular aspects involved in this phenomenon.

Our results suggest the study of patients exposed to environmental pollution, including cigarette smoke, and treated with chemotherapies with the determination of cadmium levels in breast cancer patients with negative estrogen receptor before 5-FU treatment, in order to highlight a possible interaction in vivo between Cd and anticancer drugs.

References