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Kinetics of MTT-formazan exocytosis in phagocytic and non-phagocytic cells

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Abstract

MTT is taken up by cells by endocytosis and reduced to formazan in the endosomal/lysosomal compartment. Formazan is deposited intracellularly as blue granules and is later exocytosed as needle-like formazan crystals. The present study involves an analysis of the pattern of exocytosis of MTT in different cell types showing clearcut differences in the response that can be associated to their ability to phagocytose.

To further assess the characteristics of the exocytic mechanism of MTT/formazan, different experimental conditions were assayed. When culture medium with decreasing serum concentration was used as a metabolic modulator no variations were observed in the proportion of cells with formazan crystals. Conversely, the markedly sensitivity of phagocytic cells to increasing concentrations of genistein constituted a remarkable difference with non-phagocytic cells.

These results must be considered when the modulation of MTT exocytosis is used as a signal of the progress of human diseases.

Keywords: MTT; Exocytosis; Endocytosis; Tetrazolium; Formazan; Redox reaction; Formazan crystal

1. Introduction

The reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is an index of the redox status of the cell. The amount of formazan produced indicates the reductive potential of the cytoplasm and cell viability. It is one of the most frequently used methods to measure cell proliferation and cytotoxicity. At present it is also used to analyze metabolic alterations in normal or neoplastic cells (Liu and Schubert, 1997). MTT is taken up by cells by endocytosis, reduced in the endosomal/lysosomal compartment, and then transported to the extracellular space through exocytosis in the form of needle-like formazan (Mosmann, 1983; Nikkah et al., 1992; Shearman et al., 1995). Different types of cells have tremendous differences in their rate of MTT formazan exocytosis (Liu et al., 1997).

A detailed analysis of this phenomenon in different cell types and under different experimental conditions is of great interest particularly in the light of recent studies on the use of modulation of exocytosis of the MTT formazan in cells of neural and endothelial cells induced by amyloidogenic peptides. MTT reduction currently is considered a particularly sensitive indicator of β-amyloid protein (β-AP) mediated neurotoxicity.

Kerokoski et al. (2001), Soriano et al. (2003) and Shearman et al. (1994) have reported that the parameter of MTT reduction monitors both a specific mechanism and the site of action of the β-AP peptide and support the hypothesis that inhibition of MTT reduction represents an early indicator of β-AP toxicity. Moreover, Soriano et al. (2003)

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showed that the MTT signal was inhibited by β-AP and that the decrease in MTT formazan production appeared to be related to overproduction of dye crystals.

To gain deeper insight into this process and analyze the modifications in the exocytosis of tetrazolium reduction products, cells were cultured in the presence of decreasing concentrations of fetal calf serum FCS, a situation compatible with increasing cell growth inhibition.

Cells were also challenged with the flavonoid genistein which has been shown to have a wide range of inhibitory effects: among others, it restrains tyrosine-kinase, arrests cell cycle progression and reduces superoxide production (Record et al., 1995).

The aim of the present study is to perform a detailed analysis of the different patterns of exocytosis of MTT formazan in cells of different origins and functions and under diverse conditions of culture. Therefore, we analyzed the exocytosis of MTT formazan crystals in phagocytic and non-phagocytic cells. The pattern of response of cells depicting lineage-specific differences showed remarkable variations. The analysis of these variations will help to clarify the mechanisms of modulation of MTT exocytosis in different cell types.

2. Materials and methods

2.1. Drugs

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Thioglycollate broth, DMSO, RPMI-1640, eagle minimum essential medium with Dulbecco's modification (D-MEM), fetal calf serum, (FCS), 4,5,7-trihydroisoflavone phytoestrogen (genistein) and phosphate buffer saline (PBS) were purchased from Sigma Chem. Co.

2.2. Animals

Two-month-old Sencar mice were used for harvesting peritoneal and alveolar macrophages. They were bred at the National Atomic Energy Commission animal house facility, fed ad libitum and housed in a controlled environment.

2.3. Peritoneal and alveolar macrophages

Peritoneal macrophages (PMs) were obtained as previously described (Fernández et al., 1999). Briefly, mice were injected intraperitoneally with 2 ml of a solution of thioglycollate broth, 8% in PBS (pH 7.2). The animals were killed by cervical dislocation 4 days after injection. Macrophages were harvested by washing the cavity of each animal with 3 ml warm PBS.

Pulmonary alveolar macrophages (AMs) from Sencar mice were harvested by broncheoalveolar lavage (Tasat and Molinari, 1987). Briefly, lungs were cannulated and rinsed with 10 ml cold PBS (pH 7.2). Lavage fluid was collected and the viability of both kinds of macrophages was assayed by the trypan blue dye exclusion test (>95%).

Both PMs and AMs suspensions were centrifuged at 800*g* for 10 min at 4 °C and $4–6\times10^5$ cells were plated in 35 mm plastic dishes and cultured in RPMI-1640 supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. After 1 h incubation at 37 °C, in 5% CO_2, cells were washed to remove non-adherent cells. The remaining attached cells were 95% macrophages (as tested by 0.1% neutral red dye, 10 min). Except when otherwise stated, 48 h after seeding, cells were submitted to the MTT assays. The duration of the assay depends on each particular experimental condition.

2.4. V79 and MCF-7 cells and culture conditions

V79 embryo lung fibroblasts and MCF-7 breast carcinoma cells were grown in 35 mm dishes with D-MEM supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. In all cases, except when otherwise stated, 5×10^4 cells were seeded per dish and were incubated at 37 °C in an atmosphere of 5% CO_2. Twenty-four hours later, the MTT assay was performed. The duration of the assay depended on each set of experimental conditions.

2.5. MTT assay

After the time indicated for each experiment, cultures of macrophages, V79 and MCF-7 cells were carefully washed with PBS and 1 ml fresh complete growth medium supplemented with 50 μl MTT (4 mg/ml in PBS) was immediately added. Unless otherwise indicated, the assays were performed at different time-points after incubation in MTT-containing medium (0, 15, 30, 45, 60, 90, 120 and 240 min). Immediately, cells were thoroughly washed with PBS. Paraformaldehyde (4%) was then added to stop the MTT reaction and fix the cells. The cells were mounted in glycerine. Forty-five minutes of MTT incubation time were considered representative to evaluate the influence of cell density and culture age (Molinari et al., 2003).

To modulate the exocytosis of formazan macrophages and cell lines cell cultures were exposed to decreasing concentrations of FCS (10, 7.5, 5, 2.5 and 0%) during 24, 48, 72 and 96 h. Cells were also cultured in increasing concentrations of genistein (25, 50, 75 and 100 μM) for 24, 48 and 72 h.

2.6. Light microscopy

Reactive and non-reactive cells were observed by light microscopy. The reactive cells comprised one cell fraction with blue formazan/endosomes and another cell fraction depicting needle-like formazan/crystals. Cells without formazan/endosomes or formazan/crystals were considered as non-reactive cells. At least 200 cells per dish and three