ACID PHOSPHATASE AS A MARKER OF CELLULAR DAMAGE. CHARACTERIZATION OF TWO CELL LINES FOR TOXICITY STUDIES

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Summary. - Acid Phosphatase (AP) has been widely used as an indicator for xenobiotics toxicity in mammalian cells. In this study, two cell lines (HEp-2, IEC-17) have been characterized for their AP content during the growth cycle, measuring total and free activities. HEp-2 cell line has been exposed to a peptic-tryptic-cotazym® (PTC) digest from bread and durum wheat gliadins: PTC digest of gliadin derived from bread wheat induced a significant increase of AP activity in exposed cells. The use of AP as index of toxicity is discussed in relation to the compartmentalization and the latency of the enzyme. A more standardized approach to estimate AP activity in cells exposed to xenobiotics is suggested, due to the very different methods used for its determination.

Introduction

The lysosomes are the sites where materials coming from both the outside and the interior of the cells are broken down. They are characterized by the presence of several hydrolytic enzymes such as acid phosphatase, ribonuclease, aryl sulphotase, cathepsin, whose specific activity is very high in lysosomes purified by differential centrifugation. The enzymes are present mostly in their latent form (70-90%), because in part are probably bound to the inner membrane of lysosomes. Free forms of the enzyme are usually present in the cytoplasm. The lysolemma acts as permeability barrier. Labilization of lysosomes is found to precede cell division (1), but usually the lability of lysolemma is taken as a signal of pathological damage (2).

Acid phosphatase (AP) has been the most studied of these hydrolytic enzymes and many investigations have been performed on its compartmentaliza-
tion and latency (3-6). Moreover AP has been measured in several toxicity studies on cell culture with different chemicals, although the methods used and the sites of measurements were different. Some essays, in fact, have been performed in the culture medium (7-9), others in the total cell homogenate or sonicate (8,10-13); moreover, the total and free activities have been measured (6) and either histochemical reactions or biochemical essays have been used; an increase in AP has been recovered in the total homogenate or inside the cells, following exposure to crysotile (11), lead (14), DDT (12) and in the medium following exposure to asbestos (8), acetaminophen (7), tetracycline (9); a decrease has been observed in the homogenate or inside the cells after exposure to vanadium, cadmium, nichel, manganese, chromium (13), mercury chloride (15), paraquat (16).

In this work, we have characterized two cell lines (HEp-2 and IEC-17) for their total and free AP content with the purpose of standardizing a methodology which could be useful in toxicological investigations aiming at an early detection of xenobiotics' damaging effects. Moreover, one of this cell line (HEp-2) has been exposed to a PTC digest of gliadins derived from durum and bread wheats and variations in AP content have been measured.

Materials and Methods

Cell culture

HEp-2 cells were purchased from the Virology Laboratory of the Istituto Superiore di Sanità, Rome, Italy. These cells, which derive from human carcinoma of the larynx, have an epithelial-like morphology. They were cultured as previously described (17).

IEC-17 cells are intestinal epithelial rat cells. They were kindly supplied by Dr. A. Quaroni and were cultured according to his technique (18).

PTC exposure

HEp-2 cells were seeded at 4x10^5 in 60 mm Falcon dishes in a medium containing 1 mg/ml digest prepared from bread (var. Mentana) and durum (var. Azizia) wheat gliadins. The cultures were incubated at 37°C in CO₂ air for 5 days. PTC preparations were kindly provided by Dr. M. De Vincenzi (17).

Enzyme essay

Monolayers of cells were washed three times with cold Phosphate Buffered Saline (PBS) pH 7.2 and removed with a rubber policeman. The cell suspensions were homogenized in 0.1% Triton-X100 using a Dounce homogenizer with 10 strokes. The homogenates were centrifuged at 3.000 rpm for 15'. To measure the free activity the procedure was the same as for the total activity, except the cells were detached and homogenized in 2 ml of sucrose 0.25 M. After centrifugation, 0.2 ml of the supernatant were used for the essay by a colorimetric method (Sigma Chemical Co., St. Louis, MO.) using p-nitrophenylphosphate as a substrate (1). The reaction is expressed in Sigma Units, that is defined as the amount of the enzyme activity that will liberate 1 μmole of p-nitrophenol per hour under the test conditions. Each measure was performed in duplicate and the results are the mean of three experiments ± SE.

Results

Differences in total AP content and variations in AP activity during the cell cycle have been recorded in our cell lines. Concerning the HEp-2 cells (Fig. 1) the total activity/10^6 cells was 0.42 SU (± 0.0082) and remained stable during the cell cycle.

As to IEC-17 cells (Fig. 2), the total activity/10^6 cells was 0.82 SU (± 0.026) in early cell cycle and 0.97 SU (± 0.018) in late cell cycle, showing a
Fig. 1. - Correlation between cell growth and acid phosphatase content on HEp-2 cell line.

Fig. 2. - Correlation between cell growth and acid phosphatase content on IEC-17 cell line.
small accumulation of the enzyme in the stationary phase. No AP release in the medium could be observed in either case. The free activity has been shown to be about 65% of the total for both cell lines.

For which concerns the AP activities of the HEp-2 cells treated with PTC digest from durum and bread wheat, toxic peptides caused an increase of the enzymatic activity, that was greater for the PTC digest prepared from bread wheat gliadins (+64% ± 18) than for that prepared from durum wheat gliadins (+29% ± 12).

Discussion

HEp-2 and IEC-17 cell lines have been studied for change in enzyme activity during the growth cycle. Studies on the variation of the lysosomal enzyme activity at different cell densities have shown, indeed, that it may differ in various continuous cell lines and in primary cultures, according with what we observed with HEp-2, a continuous cell line and IEC-17, a cell line with a limited life span.

The free activity resulted to be about 65% of the total for both cell types, higher than expected according to other studies (19,20), probably due to our technical conditions.

Investigating toxic effects due to gliadins (responsible in vivo for coeliac disease) on HEp-2 cell cultures, we found a significant increase in AP content for bread wheat treated cells and a lower and not significant increase for durum wheat. These results are in agreement with our previous findings showing greater toxic effects due to bread wheat than to durum wheat on morphological and growth characteristics of HEp-2 cells, (17, 21). Since we have measured the total activity, it seems that gliadins induce a new synthesis of the enzyme. No activity has been found in the medium. As consequence of our results we believe that the measure of intracellular AP can give an earlier and sensitive indication of functional conditions of the cells after exposure to toxic agents, therefore we suggest the following strategy:

a) the AP levels can change during the cell cycle (HeLa), and after addition of serum. AP increase is sensitive to cycloheximide, showing that the increase induced by serum is due to the induction of protein synthesis (20). In 3T3, 3T6 and Hela cells the specific activity is greater in confluent cultures. That is not the case for BHK cell cultures and in normal diploid chick embryo fibroblasts (22). For this reason the cell system to be used must be characterized for AP content in relation to the growth phase;

b) most of the studies on AP have been conducted on rat liver, but from the results on other tissues or cells, or liver from other species, differences between the enzymes from various species and tissues can be expected (23-26). So that the conditions of the essays should be optimized for the system in use;

c) AP seems to exist in more than one form (at least for rat liver lysosomes): one easily dissociable from the structure and the other more tightly bound. Cristofalo (3) suggests that one is typically lysosomal and the other is confined to the supernatant; also for WI 38 cells more than one form may exist, as shown by different pH optima and by different sensitivities to inhibitors (3,20). In fact two substrates are generally used to measure the AP activity: the β-glycerophosphate and p-nitrophenylphosphate. The first one gives a good localization of the enzyme in the lysosome fraction, the second one is also a good substrate for microsomal phosphatase (27,19).

For toxicity studies the p-nitrophenylphosphate can be used as a substrate, although it is less specific than β-glycerophosphate, which has low affinity with the enzyme and often contains impurities from α-glycerophosphate (27). According to Kaplan (22) the data obtained with β-glycerophosphate are less reproducible than with p-nitrophenylphosphate and anyhow, the pattern of enzyme distribution is substantially similar. 4-Methylumbelliferyl phosphate is a good substrate too, but is the less hydrolyzable of all;
d) in rat liver 70-80% AP is recovered in lysosomes, the other activity being recovered in other cellular fractions and in the supernatant (3). The measure of the "bound" and soluble forms is usually very sensitive to the preparation conditions such as ionic strength and cation species (4). According to Baccino and coworkers (4), the latency of the enzyme cannot be ascribed completely to the compartmentalization of the enzyme: non latent activity is present in the sedimented fraction, whereas, an increase in active enzyme does not correspond to significative increase in the soluble fraction. After ethionine treatment, for instance, AP is no longer latent but still bound (6); the same observation has been performed by Osborne after freezing (5). Viceversa the damage of lysosomes, according to Ruth (25) does not correspond always to a loss of latency. The exposure to Amanita phalloides toxin results in a complete shift to soluble fraction (28). As a consequence, Baccino (19) suggests that the measure of free/total is more significant than the measure of soluble/bound activities, and also allows to avoid the very delicate and time consuming isolation of purified lysosomes.

In conclusion, the total AP activity in the cells should be the first measure to be performed to detect any variation in the synthesis of the enzyme, using the homogenization in presence of Triton-X100 0.1%. If no differences are seen between control and treated cells it is worth while measuring total and free activities to see if there is any activation of the latent form. The free activity is measured in the presence of 0.25 M sucrose which protects lysosomes structure and latent sites. The measure of the leakage in the medium seems to be the least meaningful parameter, as leakage preceeds immediately the cell death and thus cannot be exploited for an early detection of toxic damage.

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REFERENCES

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