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TNFα Alters Mitochondrial Membrane Potential in L929 but not in TNFα-Resistant L929.12 Cells: Relationship with the Expression of Stress Proteins, Annexin 1 and Superoxide Dismutase Activity

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Tumour necrosis factor α (TNFα) cytotoxicity is mediated, at least in part, by oxidative stress and phospholipase A2 activation. The first post-receptor events to be observed in TNFα-sensitive lines are the generation of superoxide anion (O_2^-) within the mitochondria and the activation of phospholipase A2. Using the lipophilic dye JC-1 to determine mitochondrial membrane potential, we showed that TNFα induces time-dependent alterations in mitochondrial membrane potential in L929 cells but not in the TNFα-resistant L929.12 subclone. Heat shock (HS) proteins (HSP) and superoxide dismutase (SOD) have been shown to protect cells from TNFα cytotoxicity, while glucose regulated proteins (GRP) and annexins might also be involved in cellular protection. We thus compared the expression of HSP, grp78 and annexin 1 as well as SOD activity in TNFα sensitive and resistant lines. We found no difference in the expression of HSP, grp78 or annexin 1, but an increase in the constitutive activity of SOD in the L929.12 cells as compared to L929. Furthermore, SOD was inducible by TNFα in L929 cells, but not in L929.12 cells. These data suggest that in TNFα-resistant lines, mitochondrial damage by TNFα is prevented by an increase in SOD rather than in overexpression of stress proteins or annexins.

Key words: tumour necrosis factor, oxidative stress, mitochondria, heat shock proteins, glucose regulated proteins, annexins, superoxide disumutase

INTRODUCTION

The cytotoxicity induced by TNFα is mediated, at least in part, by oxidative stress. Mitochondria are the main source of reactive oxygen species (ROS) in non-phagocytic cells and these organelles also are the first targets for the effects of the cytokine. Indeed, the earliest post-receptor event in TNFα sensitive cells is the mitochondrial generation of superoxide anion (O_2^-)[1].

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In the TNFα-resistant line L929.12, a subclone of the L929 murine fibroblasts usually used in biological assays for TNFα, mitochondrial production of O_2^- was abolished, although TNFα receptors were not different.[1,2] ROS may be toxic to cells and in particular to mitochondrial respiration.[3–5] Subsequent alterations in mitochondrial membrane potential (Δφ) can be measured by a cytofluorimetric assay using the lipophilic dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1).[6,7] This technique, which has been utilized in several recent studies,[8–13] has been now applied to analyse over time TNFα-induced mitochondrial damage in L929 and in L929.12 cells.

We also investigated potential protective factors involved in TNFα resistance. Both HSP and superoxide dismutase (SOD) have been shown to protect cells from TNFα cytotoxicity.[14–16] HSP are a set of conserved proteins, both constitutively expressed and inducible, which are classified into families according to their molecular weight and respective inducers, as well as their subcellular location. Various members of the HSP70 family are found in the cytoplasm, the ER, and the mitochondria.[17] While it has been proposed that TNFα induces hsp70, hsp70 has been shown to be involved in protection against TNFα-mediated cytotoxicity.[15,16,18] The inducible mitochondrial SOD is also induced by TNFα, and as for hsp70, has been shown to protect cells against TNFα-induced cytotoxicity.[14,19] Furthermore, stress leading to grp78 overexpression also confers resistance to TNFα-mediated lysis in murine tumor cell lines.[20]

On the other hand, the cytotoxicity induced by TNFα has been reported to be mediated by activation of phospholipase A2 (PLA2).[15,21,22] Annexins are potent inhibitors of PLA2[23] and a protective effect of extracellular annexin 1 has been reported.[24] We thus investigated a possible contribution of annexin 1 in the acquired TNFα-resistance of L929.12 cells and compared the expression of HSP, grp78 and annexin 1 and the activity of SOD in TNFα-sensitive and resistant cell lines.

MATERIAL AND METHODS

Cells

TNFα resistant L929.12 cells were selected by culture of murine L929 cell line in medium containing increasing concentrations of murine recombinant TNFα (Genzyme, Cambridge, UK) up to 1 ng/ml. After 10 passages, resistant clones were isolated as described elsewhere.[1] The cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 1% L-glutamine, 7% heat inactivated fetal calf serum and 1% penicillin-streptomycin.

Exposure to HS, TNFα or herbimycin A

L929 and L929.12 cells were preincubated with different concentrations of recombinant murine TNFα for 3 or 24 h. Herbimycin A (HA) (Calbiochem, San Diego, USA) was dissolved in dimethyl sulfoxide and used at the final concentration of 1μg/ml for 4 h before labeling. For HS, cells in 25 mM Hepes-buffered RPMI without methionine (Gibco) were incubated in a waterbath at 45°C for 20 min. After HS cells were allowed to recover at 37°C for 2 to 4 h before labeling.

Determination of Δφ

Cells were prepared and mitochondrial membrane potential measured as described using the lipophilic cation JC-1.[6,7] JC-1 selectively enters mitochondria,[25] where it resides in a monomeric form emitting at 527 nm after excitation at 490 nm. Depending on the membrane potential, JC-1 forms the so-called J-aggregates that are associated with a large shift in emission (590 nm). The changes in the colors of the dye can be detected using the filters commonly mounted in flow cytometers.

Cytofluorimetric analysis

Flow cytometry (FCM) was performed using a FACScan flow cytometer (Becton Dickinson, San

José, CA, USA) equipped with a single 488 nm argon laser. A minimum of 10 000 cells per sample were acquired in list mode and analyzed by Lysys II software, as previously reported.[6,7]

Protein synthesis and Western blot analysis

DMEM was replaced by RPMI without methionine. After HS and recovery, cells were labeled with 6 μCi/ml ^{35}S-methionine (specific activity >1000 Ci/mmol; Amersham Laboratories, Buckinghamshire, England) for 90 min, then washed and lyzed in SDS. Proteins from samples corresponding to equal cell numbers were resolved by SDS-PAGE as described[26] and revealed by autoradiography. HSP70s were characterized by mouse monoclonal antibody against the human constitutive and inducible protein (SPA820 and SPA810 respectively, StressGen Biotechnologies, Victoria, Canada), and grp78 with SPA827 (StressGen). Annexin 1 was identified using a polyclonal antibody raised against recombinant annexin 1 produced as a GST fusion protein in *E. Coli*; this antibody is highly specific for annexin 1 and does not cross-react with other annexins (Russo-Marie, unpublished).

Superoxide dismutase measurement

Total enzymatic activity was measured in a cytochrome c-reducing xanthine-xanthine oxidase system according to the method described by Crapo *et al.*[27]

Statistical analysis

The two tail Student's *t* test was used to analyze the difference in SOD activity between L929 and L929.12 cells.

RESULTS AND DISCUSSION

Mitochondria have been proposed to be the first targets of TNFα damage.[1,28] By using a cytofluorimetric approach which allows the study of mitochondrial function within intact cells, we found a time-dependent depolarization of the mitochondrial membrane and thus provide direct evidence of the ability of TNFα to interfere with mitochondrial activity. Figure 1 shows that incubation with TNFα induced profound changes of Δφ in L929 but not in L929.12 cells and Figure 2 illustrates the differences between the two cell types, both under basal conditions and after addition of TNFα. It can be seen from Figure 2 that some degree of depolarization existed in untreated L929 cells (more than in L929.12), which is in agreement with the concept of functional heterogeneity of mitochondria.[13] As shown in Figures 1 and 2, the alterations in Δφ induced by TNFα were time-dependent. During the period we have considered, however, no significative alterations of mitochondrial mass took place in the two cell types (data not shown).

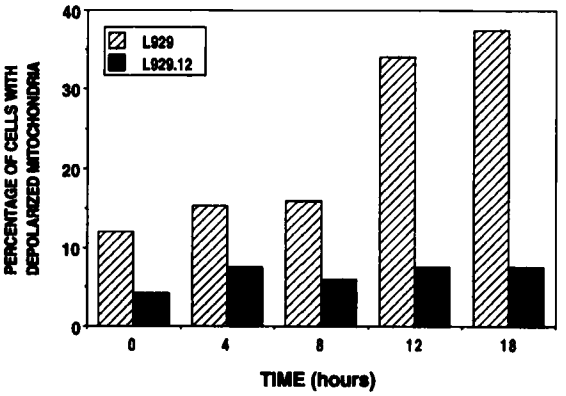

FIGURE 1 Effect of TNFα on Δφ in L929 and L929.12 cells. The histograms refer to the cytofluorimetric analysis of cells stained with JC-1, and represent % cells with depolarized mitochondria. TNFα induced profound changes of mitochondrial membrane potential in L929 but not in L929.12 cells. Data presented refer to one experiment representative of 3, in all of which 10 000 cells were analyzed. Statistical analysis comparing L929 to L929-12 cells by Student's T test indicated that $p<0.01$ in all cases.

Because of the well described protective effects of HSP against TNFα-mediated cytotoxicity, we hypothesized that TNFα-resistance may be associated with an increased expression of HSP, either constitutive or inducible. We thus compared the induction of HSP in L929 and L929.12 cells after exposure to HS, to TNFα, as well as to the tyrosine

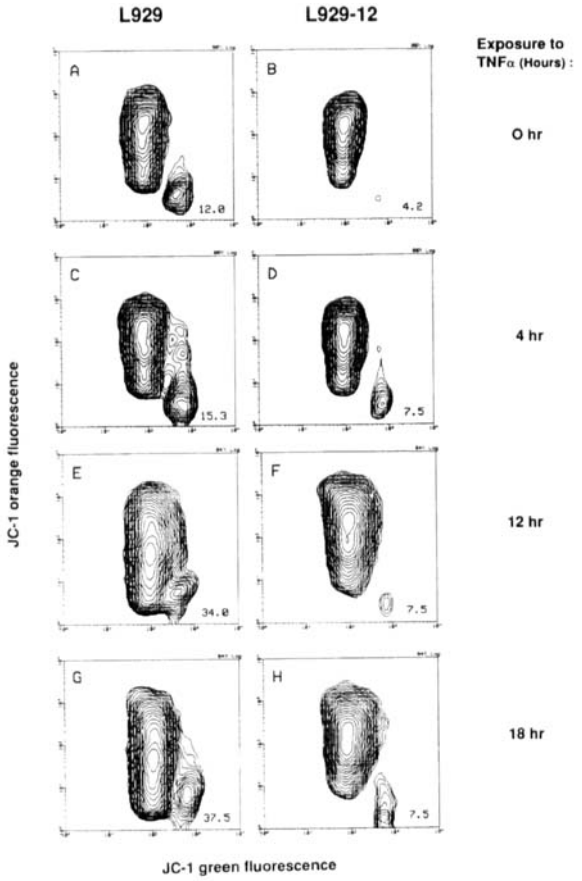

FIGURE 2 Cytofluorimetric analysis of $\Delta\varphi$ in L929 and L929-12 cells. Cells were untreated, or treated with TNFα for the indicated times. Numbers indicate the percentage cells with depolarized mitochondria. In this representative experiment, it can be seen that even under basal conditions, there are more cells with depolarized mitochondria in the L929 than the L929.12 cells.

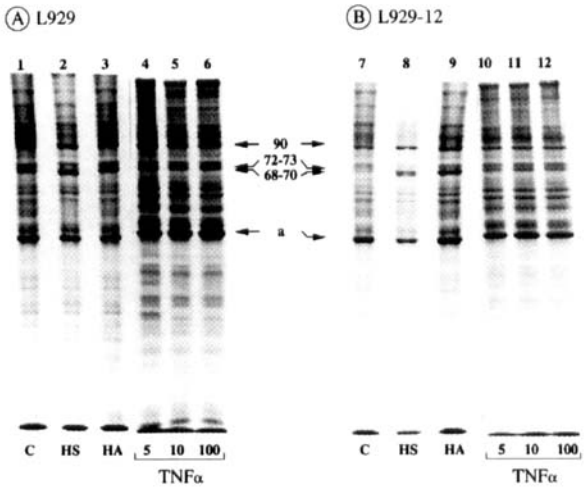

FIGURE 3 Effects of HS, HA and TNFα on protein synthesis in L929 and L929-12 cells. L929 (panel A) and L929-12 (panel B) were incubated for 20 min at 45°C (lanes 2 and 8), for 4 h with 1 μg/ml HA (lanes 3 and 9) or for 24 h with TNFα (lanes 4–6 and 10–12) before labelling with ^{35}S-methionine as described. Proteins were resolved by SDS-PAGE (10% polyacrylamide) and revealed by autoradiography.

kinase inhibitor and thiol reactive compound HA, another inducer of HSP[29] (Figure 3). The synthesis of hsp70 and hsp90 was upregulated after exposure to both HS and HA, while TNFα had no effect on HSP synthesis. There was no difference between L929 and L929.12 cells.

We then examined the expression of the constitutively expressed hsc70, of hsp70, grp78 and annexin 1 in L929 and in L929.12 cells, under control conditions and after exposure to HS or HA. Neither hsc70 (a constituitively expressed member of the hsp70 family)[30] nor hsp70 were increased in L929.12 cells as compared to L929 cells (Figure 4, panels a and b). Although we observed, using biometabolic labeling, an increased rate of synthesis of hsp70 after exposure to HA (Figure 3), Western blotting did not reveal increased expression of these proteins. The expression of grp78 and of annexin 1 were also similar under all conditions tested (Figure 4, panels c and d).

Since it has already been established that TNFα-resistance of L929 cells does not relate to differences in receptor number or binding affinity[1] and that the first distinct and measurable post-receptor event which is distinct in L929 as compared to the L929.12 is mitochondrial production of O_2^-, we then examined the activity, in the two cell lines, of SOD. Indeed, it has been reported that TNFα induces MnSOD in a variety of cell types and this induction acts as an autoprotective mechanism against TNFα-mediated cytotoxicity.[14,19] The analysis of the SOD activity is reported in Figure 5. The treatment for 2 h with this cytokine increased SOD activity in the TNFα-sensitive but not in the TNFα-resistant cell line, in which the basal activity of SOD was constitutively higher; the difference in SOD activity between L929 and

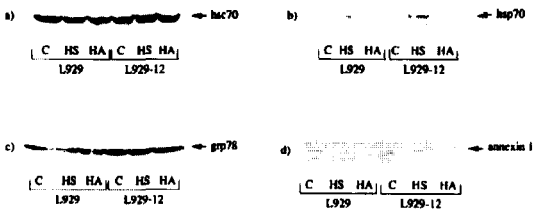

FIGURE 4 Effects of HS and HA on HSP, grp78 and annexin 1 expression in L929 and L929-12 cells. Western blots for hsc70 (panel a), hsp70 (panel b), grp78 (panel c) and annexin 1 (panel d) in L929 and L929-12 exposed to HS or HA. There were no significant differences between the two cell types in the expression of these proteins in any condition tested.

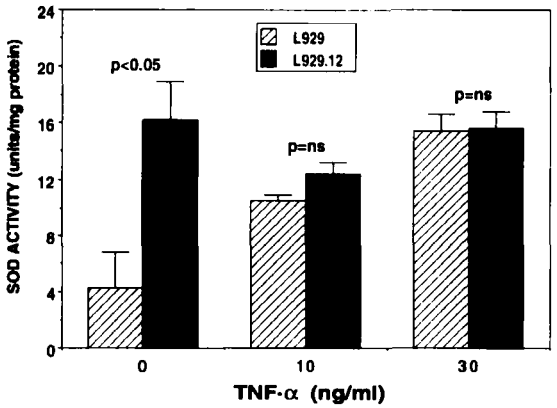

FIGURE 5 Constitutive and TNFα-induced SOD activity in L929 and L929-12 cells. Basal SOD activity was significantly higher in L929.12 cells than in L929 cells while upon exposure to TNFα, SOD activity only increased in L929 cells.

L929.12 cells in the absence of TNFα was statistically significant (Figure 5). It is generally accepted that the SOD isoform induced by TNFα is the mitochondrial, inducible isoform, Mn-SOD, which is in good agreement with our results, indicating mitochondrial protection in TNFα-resistant cells.[14,31,32] Experiments we performed (not shown), including KCN as a selective inhibitor of Cu, Zn-SOD, are in agreement with these previous observations.[4,32] The possibility that TNFα resistance results in L929 cells from a mutation in the SOD promotor, from inducible to constitutive, deserves consideration.

TNFα resistance of L929-12 cells appears thus to parallel an increased activity of SOD, while it did not result from a modulation of the expression of hsp70, grp78 nor annexin, which however have all been described, along with SOD, to be involved in protection against TNFα. One explanation could be that these different stress proteins exert their protective effects within specific intra-cellular targets distinct from mitochondria, such as the cytosol (hsc70), the ER or the nucleus (grp78), or the plasma membrane (annexin 1). For example, while induction of grp78 mRNA has been reported to correlate with the development of resistance against TNFα-mediated cell lysis, DNA rather than mitochondria appeared to be the target organelle for the protective effect of grp78.[20,33]

Activation of PLA2 is among the post-receptor events induced after TNFα interaction with cells, implicating both the cytosolic PLA2 (cPLA2) and a secreted PLA2 (sPLA2).[34–36] Annexin 1 could participate in the protection against TNFα-induced cytotoxicity by inhibiting the action of either the cPLA2 or the sPLA2. Although our study indicates that there is no increased expression of annexin I in TNFα-resistant cells, we cannot preclude an increased secretion of annexin I, which could, along with other mechanisms including overexpression of mitochondria-specific HSP,[37] of hsp27[16] or of the mitochondrial membrane associated protein product of the *bcl*-2 oncogene,[38,39] contribute to the likely SOD-mediated TNFα-resistance of L929.12 cells.

Acknowledgments

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