A possible involvement of endogenous polyamines in the TNF-α cellular sensitivity

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Abstract A critical step in the cytotoxic action mechanism of tumor necrosis factor-α (TNF-α) involves, among mitochondrial dysfunctions, an early change of the inner membrane permeability displaying the characteristics of permeability transition. Cytosolic polyamines, especially spermine, are known to inhibit it. Our results show that spermine is only detectable in the TNF-α resistant C6 cells while *N*1-acetylspermidine is present in the TNF-α sensitive WEHI-164 cells, and putrescine and spermidine are found in both. TNF-α treatment does not change this distribution but only induces a quantitative alteration in TNF-α sensitive cells. Omission of glutamine (energetic substrate) from the culture media alters neither the TNF-α responsiveness of both cell lines nor their polyamine distributions, only their quantitative polyamine contents.

Key words: Tumor necrosis factor-α; Polyamine; Cell

1. Introduction

Tumor necrosis factor-α (TNF-α) is a pleiotropic cytokine known to be capable of killing various tumor cells in vitro [1]. The participation of mitochondria in TNF-α induced cytotoxicity is inferred from the fact that these organelles present early metabolic dysfunctions such as impaired respiration [2,3], superoxide anion generation [4] and an activation of succinate dehydrogenase [5]. This cytokine generates an increase of PLA_2-released fatty acids in WEHI-164 cells which leads to a change in the inner membrane permeability linked to morphologic changes [6]. Both associated phenomena characterize the permeability transition which is a key step in the way leading to cell death. Cytosolic compounds such as polyamines are known to inhibit the permeability transition [7–10]. Most of the transformed cells display intracellular polyamine concentrations higher than those of normal cells. Polyamines prevent loss of respiratory control in aging mitochondria [11] and they have a protective effect against lipid peroxidation [12]. Their cytosolic contents, especially that of spermine, might be one of the critical factors in the cellular resistance to TNF-α cytotoxicity. Our interest in studying the effect of glutamine starvation on the polyamine content of tumor cells is mostly based on a substantial body of experimental evidence indicating that glutamine is one of the major substrates for generating energy in tumor cells [13,14]. In addition it has been reported that the omission of glutamine from the medium desensitizes the cells to TNF-α cytotoxicity while the lack of glucose does not alter the TNF-α response [15]. A possible linkage between glutamine catabolism and polyamine biosynthesis is mostly based on the associated ways of glutamine catabolism and citrulline synthesis through their common intermediate ornithine. It has been reported that in tumor cells glutamine oxidation is initiated by the conversion of glutamate to 2-oxoglutarate via the transamination pathway catalyzed by the glutamine-aspartic/glutamine-alanine transaminases rather than by its oxidative deamination catalyzed by glutamate dehydrogenase [13,14]. In order to show an expected relationship between cellular polyamine content and TNF-α sensitivity on the one hand and glutamine metabolism on the other hand, we report on the intracellular polyamine distribution in relation to TNF-α treatment and glutamine depletion in WEHI-164 cells, which are sensitive to TNF-α, and C6 cells, which are resistant.

2. Materials and methods

2.1. Reagents

More than 98% purified human r-TNF-α (specific activity: 2×10^7 U/mg) was purchased from Genzyme (Boston, MA, USA). Unless specified, the chemicals and the cell culture products used were respectively of HPLC grade and high purity grade.

2.2. Cell cultures and treatment

The TNF-α sensitive murine fibrosarcoma cell line WEHI-164 from ATCC (Rockville, MD, USA) and the TNF-α resistant C6 glioma cell line (Flow Laboratory, Les-Ulis, France) were respectively cultured in commercial RPMI 1640 and DMEM media (with L-glutamine) supplemented with 10% fetal calf serum and containing penicillin (100 U/ml), streptomycin (100 μg/ml) and non-essential amino acids (only used for WEHI-164 cells). The cell lines were routinely tested for mycoplasma. Trypan blue was used for manual cell counting. TNF-α was used in a concentration of 10 ng/ml and cells were incubated for 6 h. Adaptation of the cells to glutamine-free culture conditions was performed by a stepwise decrease of the glutamine (gln) concentration in the medium to zero over a 2 month period. The resulting C6/gln− and WEHI-164/gln− populations were routinely maintained in their respective DMEM and RPMI 1640 media free of glutamine and glutamate, supplemented with 10% fetal calf serum and antibiotics.

2.3. Lactate dehydrogenase activity

Adaptation of the cells to glutamine-free culture conditions was assayed by the spectrophotometric measurement of intracellular lactate dehydrogenase with a commercial diagnostic test (Boehringer-Mannheim, Meylan, France). For this purpose cells were harvested and washed with HBSS (5.3 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 4.2 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 5.5 mM glucose, 0.1% phenol red) then PBS (140 mM NaCl, 10 mM KCl, 10 mM sodium phosphate buffer pH 7.4). Cells were homogenized in 10 mM Tris pH 7.4 then solubilized with 0.5% Triton X-100. An aliquot of this preparation was used to measure the lactate dehydrogenase activity.

2.4. Polyamine determination

The intracellular polyamine content was determined by high performance liquid chromatography (HPLC) using a modification of the procedure described by Wickström and Betner [16]. Briefly, cells were cultured in a 75 cm^2 plate as described above. After treatment, the cells were scraped off the plate, collected by centrifugation and washed with HBSS then PBS. Cells were resuspended in distilled

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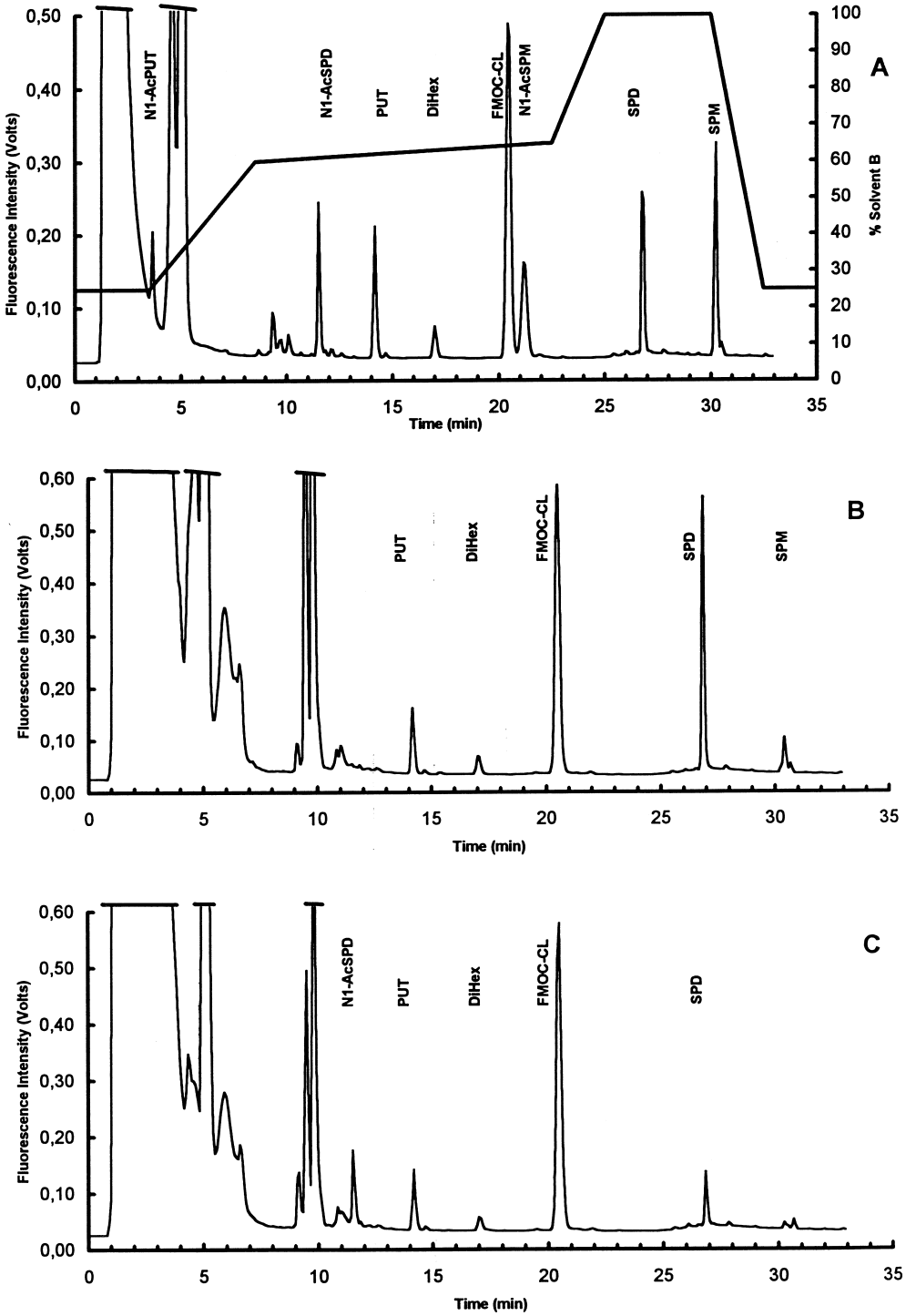

Fig. 1. Chromatograms of polyamine standards and their counterparts in C6 and WEHI-164 cells. A: A mixture of polyamine standards of 7.5 pmol per polyamine. B: Deproteinized samples of C6 cells. C: Deproteinized samples of WEHI-164 cells. Polyamines were analyzed under conditions as follows: injection volume: 20 μl, flow rate: 1.5 ml/min. Isocratic conditions: 0–3.5 min 25% B. Gradient: 3.5–8.5 min 60% B; 8.5–22.5 min 65% B; 22.5–25 min 100% B; 25–30 min 100% B; 30–32.5 min 25% B; 32.5–35 min 25% B. Fluorescence detection. Polyamine compounds were identified by their retention times as compared to authentic standards. N1-AcPUT (*N*1-acetylputrescine), N1-AcSPD (*N*1-acetylspermidine), PUT (putrescine), diHex (dihexanolamine), N1-AcSPM (*N*1-acetylspermine), SPD (spermidine), SPM (spermine).

water and subjected to three repeated cycles of freezing and thawing, then centrifuged at 250 000×*g*, +4°C for 10 min. The supernatant was deproteinized with two volumes of acetonitrile, centrifuged and derivatized with 9-fluorenylmethyl chloroformate (Fmoc) according to Wickström and Betner [16]. The internal standard dihexanolamine was added prior to deproteinization. The chromatographic system was a gradient system consisting of the model 625 LC system (Millipore, Waters, Milford, MA, USA). Fluorescence was monitored with a Waters 474 scanning fluorescence detector where the excitation wavelength was set at 260 nm and the emission wavelength at 315 nm. A Waters Baseline 810 chromatography data system was used for peak integration. The column was a reverse-phase C-18 column (ultrasphere-ODS, Beckman, Palo Alto, CA, USA) 150×4.6 mm, 5 μm with a precolumn packed with the same material. Eluent A consisted

of 70% acetate (50 mM) buffered to pH 4.2 (with 50% NaOH solution) and 30% acetonitrile and eluent B was 100% acetonitrile.

2.5. Statistical analysis

All results are expressed as means ± S.D. unless stated otherwise. The non-parametric Mann-Whitney test was used to evaluate the significance of differences between groups accepting $P < 0.05$ as the level of significance.

3. Results and discussion

3.1. Optimum conditions for separation of polyamines and their N1-acetylated derivatives

In order to adjust the HPLC analysis procedure described by Wickström and Betner [16] to the column and HPLC apparatus used, we made some modifications. Optimization of the analysis conditions was achieved by varying the flow rate, the gradient steps and the length of the run. In initial experiments we had difficulty in recovering *N*1-acetylspermine and spermine. *N*1-acetylspermine and the excess of Fmoc which was not neutralized by glycine were not separated. The other major problem was the shortness of the run which did not allow elution of the spermine. As shown in Fig. 1A a run length of 35 min and a flow rate of 1.5 ml/min made it possible to elute spermine in a sharp peak 5 min before the end of the run and to improve the separation of the *N*1-acetylated spermine and the excess of unneutralized Fmoc (Fmoc-Cl). Under the same conditions, the polyamines from the cellular extracts are eluted and separated with similar efficiency as the standard counterparts (Fig. 1B,C), whose detection limit is 0.5 pmol, the analytical coefficients of variation for retention time and peak area vary from 0.3% to 1.1% and 6% to 18%, respectively.

3.2. Comparative studies of the polyamine species in TNF-α resistant C6 cells and in TNF-α sensitive WEHI-164 cells

In order to check whether the degree of TNF-α sensitivity of each tumor cell line might be related to its polyamine distribution, we analyzed the polyamine contents of C6 and WEHI-164 cells. Numerous studies have reported that polyamine levels fluctuated throughout the cell cycle [17]. Conse-

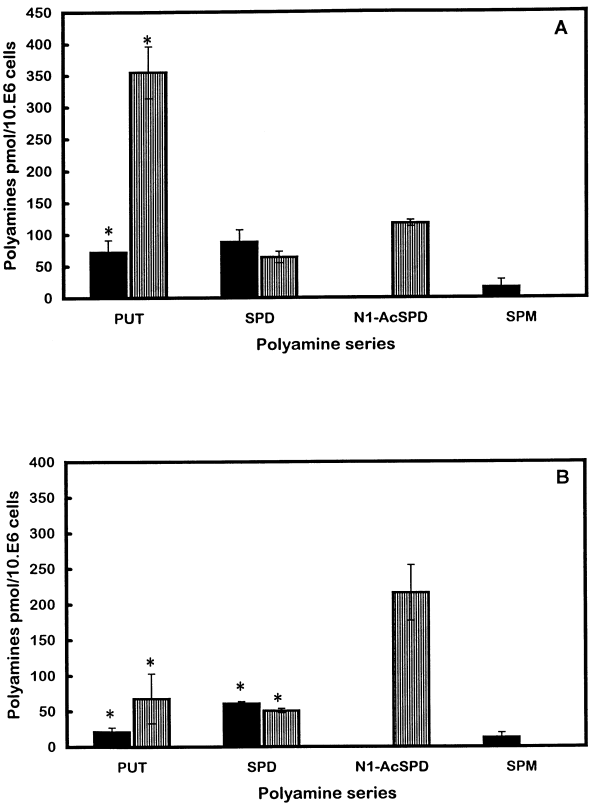

Fig. 2. Comparative study of the polyamine series in C6 and WEHI-164 cells. A: 48 h culture growth: C6 black bars; WEHI-164 hatched bars. B: 72 h culture growth: C6 black bars; WEHI-164 hatched bars. The values are the mean ± S.D. ($n = 4$), $^*P < 0.050$. PUT (putrescine), SPD (spermidine), N1-AcSPD (*N*1-acetylspermidine), SPM (spermine).

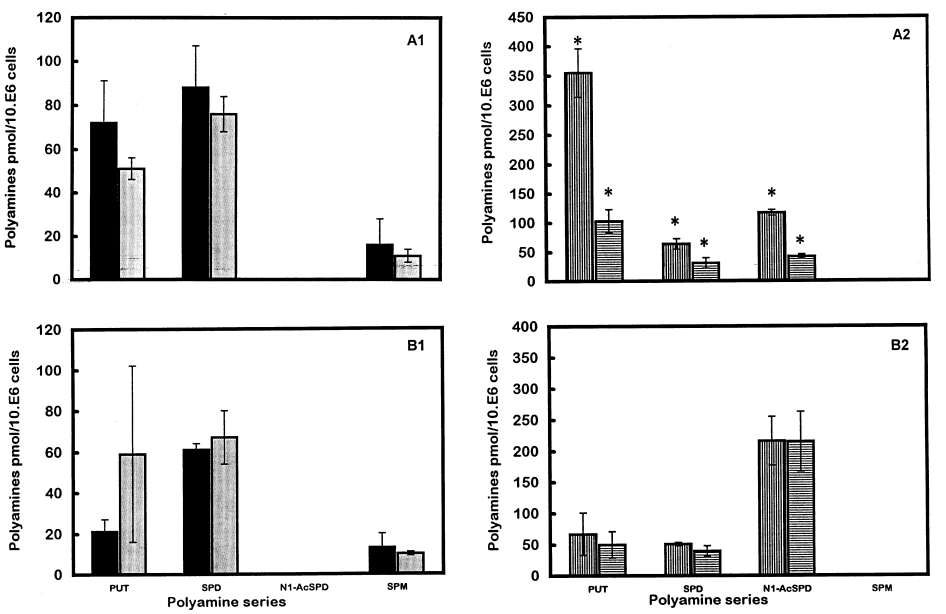

Fig. 3. Effect of TNF-α treatment on the polyamine content of C6 cells and WEHI-164 cells. After 48 h (A1, A2) or 72 h (B1, B2) of culture growth, cells were treated with TNF-α 10 ng/ml for 6 h. C6 cells: control black bars, assay gray bars. WEHI-164 cells: control vertically hatched bars, assay horizontally hatched bars. The values are the mean ± S.D. ($n = 4$), $^*P < 0.050$. PUT (putrescine), SPD (spermidine), N1-AcSPD (*N*1-acetylspermidine), SPM (spermine).

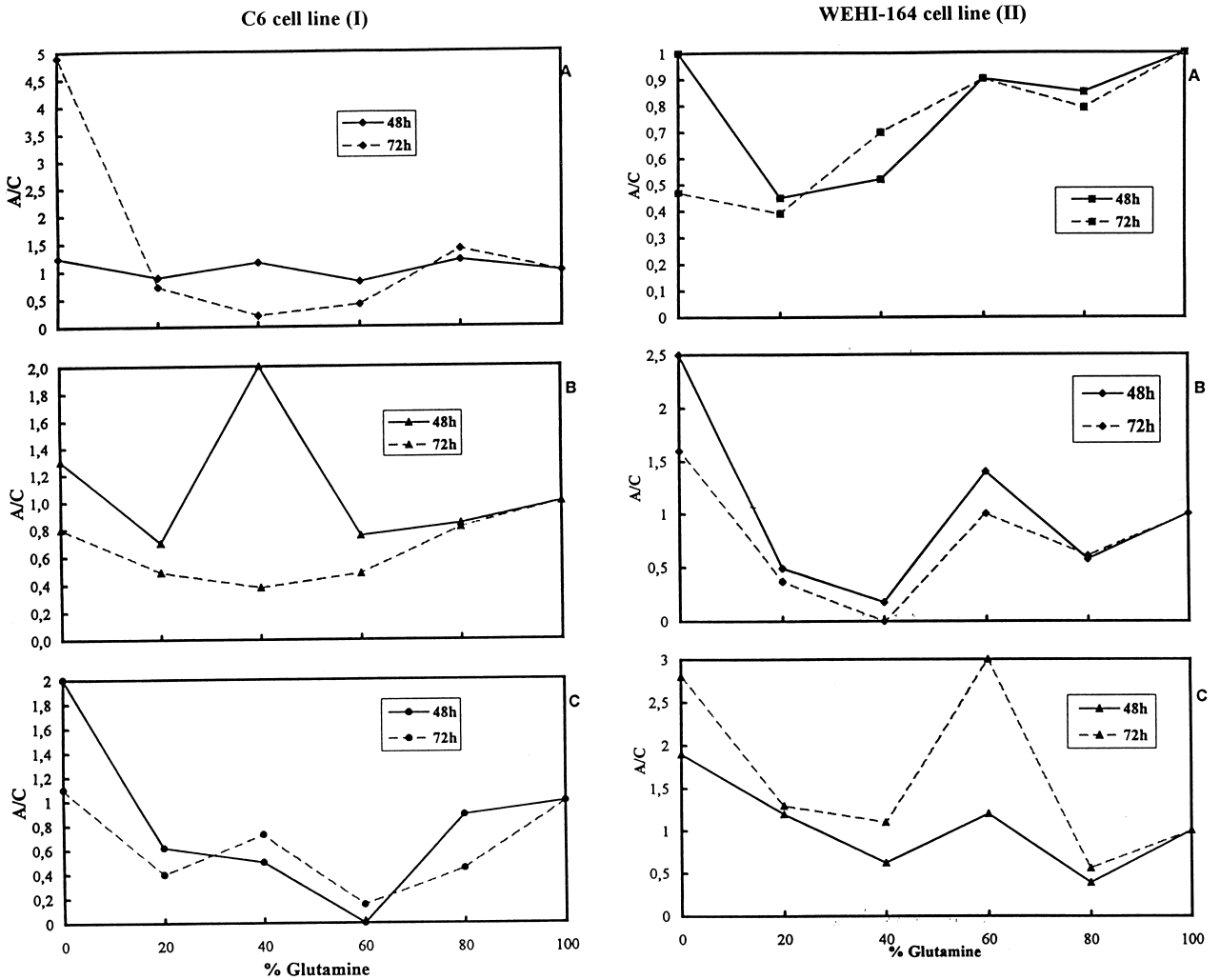

Fig. 4. Effect of glutamine depletion on the polyamine content of the C6 and WEHI-164 cell lines. The content of each polyamine species was determined at intermediate stages of glutamine depletion and at 48 h and 72 h of cellular growth. A: Putrescine. B: Spermidine. C: Spermine. The ratio A/C is calculated as follows: [Assay (gln−) polyamine pmol$\times 10^{-6}$ cells]/[Control polyamine pmol$\times 10^{-6}$ cells].

quently the polyamine measurements were done at 48 h and 72 h of cellular growth. As can be seen in Fig. 2, the qualitative analysis shows that spermine is only detectable in C6 cells while putrescine and spermidine are found in both cell lines. Of the *N*1-acetylated derivatives only *N*1-acetylspermidine is present in WEHI-164 cells, its amount is particularly large at 72 h of cellular growth (Fig. 2B). At 48 h of cellular growth (Fig. 2A) the putrescine content is significantly higher in WEHI-164 cells than in C6 cells while at 72 h it falls in both cell lines. These results show that TNFα sensitive and resistant cells do not display the same polyamine distribution. This striking difference in composition of their intracellular polyamine contents could be due to a disturbance of the regulation of polyamine biosynthesis. The cellular polyamine content depends on a highly regulated complex metabolism which results from the activities of several key enzymes and two major amino acids, ornithine and methionine. Spermine synthase, which acts by adding a propylamine moiety to spermine, is deeply inhibited by 5′-methylthioadenosine [18] which results from the decarboxylation of *S*-adenosylmethionine, the source of the propylamine groups. The inhibition of this enzyme by the accumulation of 5′-methylthioadenosine could be due to a deficit of 5′-methylthioadenosine phosphorylase [19,20], though being widely distributed in all normal tissues [21], certain tumor cell lines including some derived from humans have lost this enzyme. Consequently, the expected inhibition of spermine synthase could lead to an absence of spermine and an accumulation of spermidine [22]. In order to prevent an accumulation of intracellular polyamines, the biosynthetic pathway is coupled to the interconversion pathway which takes place by the actions of the *N*1-acetyltransferases. By this way spermidine is converted into *N*1-acetylspermidine which is rapidly degraded by polyamine oxidase under physiological conditions. However, it must be noted that in mouse tissue, larger amounts of *N*1-acetylspermidine and *N*1-acetylspermine can be found because they have 20 times lower levels of polyamine oxidase than in rat tissues where this enzyme has a much greater activity. Our results corroborate these statements because WEHI-164 cells are from murine lungs and C6 from rat brain.

3.3. *Influence of TNF-α treatment on endogenous polyamine levels in C6 and WEHI-164 cell lines*

In order to determine whether the action of TNF-α can induce a change in the intracellular polyamine content, cultures were set up to qualitatively and quantitatively analyze

polyamines in both cell lines under TNF-α treatment. As can be seen in Fig. 3 (A1,B1) TNF-α does not significantly change the distribution and the amount of polyamines in C6 cells. With regard to WEHI-164 cells (Fig. 3, A2,B2) it only induces a significant quantitative change in 48 h aged cells (Fig. 3, A2), the amounts of putrescine and *N*1-acetylspermidine decrease more than those of spermidine. These results show that TNF-α only affects the polyamine metabolism of the TNF-α sensitive cell line (WEHI-164) and especially at 48 h of cellular growth where the polyamine content is characterized by a high level of putrescine. The absence of spermine and the decrease of the polyamine content induced by TNF-α are two factors which could contribute to the sensitivity of the cells to TNF-α cytotoxicity. Besides that polyamines are essential for cellular growth, they stabilize various membrane systems including mitochondria [23]. The binding of spermine to mitochondrial external sites prevents the CA^{2+}-dependent permeability transition [10,24]. Some of our recent results have shown that in TNF-α treated C6 cells, mitochondria do not display the orthodox state characteristic of permeability transition. Our works [6] and those of others [25] have shown that the mitochondrial permeability transition is causally linked to the genesis of irreversible cell injury with TNF-α. Our present results provide evidence for the expected role of the polyamines, especially spermine, in the protection against TNF-α damage. This protective role is emphasized by the fact that the polycationic nature of spermine can prevent the saturation of anionic binding sites such as cardiolipin in mitochondria [26]. It can affect the formation and the metabolism of diacylglycerol and inositol 1,4,5-triphosphate which consist of intracellular second messengers located in plasma membranes and which belong to the major pathway of signal transduction during the response of the cells to external receptor ligands [27]. Consequently the intracellular polyamine content would be a critical factor for the TNF-α cell signaling pathway.

3.4. Influence of glutamine starvation on the endogenous polyamine content of TNF-α resistant and sensitive cell lines

The effect of glutamine depletion in the culture medium on the cellular polyamine content was investigated in the TNF-α sensitive and resistant cell lines. In order to follow the adaptation of each cell line to the glutamine depleted medium, we checked the use of glucose as the major energy source by measuring the lactate production through the activity of lactate dehydrogenase. In C6 cells the absence of glutamine does not change the activity of this enzyme and the cellular growth while both parameters decrease in WEHI-164 cells. These results indicate that glutamine is the 'energy fuel' for WEHI-164 cells but not for C6 cells. Though the glutamine omission does not change the TNF-α sensitivity of both cell lines (data not shown), their polyamine contents are profoundly altered. In C6 cells (Fig. 4-I) as in WEHI-164 cells (Fig. 4-II) they vary in accordance with the percentage of glutamine in the culture medium. Whatever the age of the cells complete glutamine starvation causes an increase of the polyamine content, especially for putrescine (Fig. 4-IA) and spermine (Fig. 4-IC) respectively in 72 h and 48 h aged C6 cells. In WEHI-164 cells putrescine (Fig. 4-IIB) and spermidine contents (Fig. 4-IIC) are also enhanced whatever the age of the cells while the level of *N*1-acetylspermidine (Fig. 4-IIA) is unchanged at 48 h of cellular growth but deeply decreased at 72 h. A greater availability of the ornithine pool could explain the high putrescine content of C6 and WEHI-164 cells cultured in gln− media, but in tumor cells, the complexity of the amino acid metabolism does not rule out a possible relationship between glutamine and the key amino acids methionine-ornithine. Tumor cells have a high turnover of divisions which needs an adapted amino acid metabolism whose mechanism is still unknown. Although there is no known direct way between the metabolism of glutamine and that of polyamines, these results suggest an expected relationship between them and show that according to our conditions glutamine is not the unique factor involved in cellular TNF-α sensitivity.

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