Inhibition by glucocorticoids of tumor necrosis factor-mediated cytotoxicity

Evidence against lipocortin involvement

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The role of the phospholipase inhibitor proteins, lipocortin-I and -II, in tumor necrosis factor (TNF)-mediated cytotoxicity against L929 fibrosarcoma cells was investigated. We previously reported that TNF-mediated cytotoxicity was inhibited by dexamethasone (DEX), suggesting an involvement of lipocortins [1]. Now we show that, despite inhibition by DEX of TNF-induced arachidonic acid release, DEX has no effect on the synthesis of these lipocortins. Moreover, TNF itself has no effect on the synthesis and phosphorylation of lipocortin-I and -II. Also there was no difference in expression levels of lipocortin-I and -II between TNF-sensitive and -resistant cells. These data strongly suggest that the protective effect of DEX and other glucocorticoids is not mediated by lipocortins.

Tumor necrosis factor; Lipocortin; Phospholipase; Dexamethasone

1. INTRODUCTION

TNF is a protein that exerts cytotoxic and cytostatic effects against certain tumor cell lines, both in vitro and in vivo, while sparing normal cells [2]. New information is beginning to shed light on the molecular mechanisms involved in TNF-mediated destruction of transformed cells in vitro.

Our in vitro studies with murine L929 cells indicate that one of the intracellular steps occurring after TNF treatment is activation of a PLA2 [1,3]. Inhibitors of PLA2 activation, such as DEX, quinacrine and high concentrations of indomethacin, blocked TNF-mediated cell destruction. In addition, we and others found an increased release of AA from phospholipids into the cell supernatant upon TNF-treatment ([3] and our unpublished results).

The mechanism of DEX action is thought to involve synthesis of lipocortins, which are phospholipase inhibitors [4]. Several authors postulated an involvement of lipocortins in TNF action [1,5,6]. Hence we were interested to determine whether DEX could inhibit the TNF-induced AA release and whether lipocortins played any role in the observed effects. We also wondered whether TNF could activate PLA2 by decreasing lipocortin synthesis or increasing lipocortin phosphorylation, the latter leading to its inactivation [7].

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Abbreviations: TNF, tumor necrosis factor; PLA2, phospholipase A2; DEX, dexamethasone; AA, arachidonic acid; EGF, epidermal growth factor

2. MATERIALS AND METHODS

2.1. *Source and cultivation of cells*

L929, HeLaD98/AH2, MCF7 and FS4 cells were obtained and cultured as described previously [8].

2.2. *Source of antibodies, lipocortin-II and TNF*

Anti-lipocortin-I was a gift from Dr B. Pepinsky (Biogen, Cambridge, MD, USA). Anti-murine lipocortin-II and purified lipocortin-II protein were kindly provided by Dr V. Gerke (Max-Planck-Institut, Göttingen, FRG). Anti-human lipocortin-II was a gift from Dr C.M. Isacke (Salk Institute, San Diego, CA, USA). Human TNF was prepared as described [9]. The preparation had a specific activity of $2\text{-}3 \times 10^7$ U/mg protein and contained less than 13 ng endotoxin/mg protein.

2.3. *TNF-mediated cytotoxicity and AA release*

Measurement of TNF-mediated cytotoxicity and TNF-induced AA release were performed as described previously [8].

2.4. *In vitro phosphorylation*

In vitro phosphorylation reactions were carried out as described by Pepinsky and Sinclair [10]. Cells were pretreated with 100 U TNF/ml or 200 ng EGF/ml (Boehringer Mannheim, Mannheim, FRG) before preparation of the membranes. The phosphorylation reaction was carried out in the absence or presence of 1000 U TNF/ml or 2 μg EGF/ml. The reactions were stopped by adding a 300-fold excess of cold ATP in lysis buffer and freezing at $-20°C$.

2.5. *Metabolic labeling experiments*

Cells in a 6-well plate were pretreated for different time periods with 1000 U TNF/ml, labeled with 100 μCi [^{35}S]methionine or 1 mCi ^{32}Pi/well and lysed as described previously [10].

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2.6. *Immunoprecipitation*

The appropriate antisera were added to equal amounts (10^7 cpm) of labeled lysate and the samples were incubated for 2 h at 4°C. The immune complexes were collected by adsorption to protein A-Sepharose for 1 h at 4°C with continuous mixing. The adsorbed immune precipitates were washed 4 times with 1 ml buffer (10 mM Na_2HPO_4, pH 7.2; 155 mM NaCl; 1% NP-40; 0.1% SDS; 1% sodium deoxycholate; 30 mM NaN_3; 1 mM NaF; 1 mM EDTA), suspended in 50 μl of 1.5 × Laemmli sample buffer [11], heated at 60°C for 10 min and subjected to electrophoresis in SDS-containing, 10% polyacrylamide gels. The gels were fixed with 50% trichloroacetic acid and fluorographed with a Fuji Rx Medical film (Tokyo, Japan). Molecular weight protein standards were from Amersham International (Amersham, England).

3. RESULTS

3.1. *DEX inhibits TNF-induced AA release*

L929 cells, prelabeled with [^{3}H]AA, were treated with 50 μM DEX for 1 h before control medium or TNF-containing medium was added. 5 h later, the amount of radioactivity in the supernatant was determined. The effect of DEX on TNF-mediated cytotoxicity was measured in a 36-h assay as described in section 2. Both TNF-induced AA release and TNF-mediated cytotoxicity were inhibited by DEX (table 1).

3.2. *DEX does not induce the synthesis or secretion of lipocortin-I or -II in L929 cells*

DEX inhibits TNF-mediated cytotoxicity in a transcription-independent manner [1]. The possible role of lipocortins in mediating this response was investigated by studying the effect of DEX on intracellular and extracellular lipocortin-I and -II levels. L929 cells were labeled for 5 h with [^{35}S]methionine and 50 μM DEX was added at different times (3 h, 6 h, 8 h and 24 h) before the end of the labeling. Intracellular and extracellular lipocortin levels were then determined by immunoprecipitation and gel electrophoresis. The cell-associated amounts of lipocortin-I (fig.1) and -II (data not shown) appeared to be unchanged after DEX treatment, while these proteins could not be detected in the culture medium.

3.3. *Role of lipocortin expression in the TNF-response*

L929 cells were labeled for 4 h with [^{35}S]methionine and 1000 U TNF/ml were added at different times before the end of the labeling (2 h, 4 h, 6 h, 9 h and 21 h). The lipocortin content of the cells was then determined as in section 3.2. A TNF treatment of 21 h resulted in considerable cell death. However, the apparent amounts of lipocortin-I and -II were not chang-

Table 1

Effect of DEX on TNF-mediated cytotoxicity and TNF-induced AA release from L929 cells

	% survival[a] after 36 h in the presence of 1000 U/ml TNF	% [^{3}H]AA release after 5 h in the absence of TNF	% [^{3}H]AA release after 5 h in the presence of 5000 U/ml TNF
− DEX	25 ± 2	2.6 ± 0.3	8.3 ± 0.7
+ DEX	76 ± 4	2.4 ± 0.3	5.3 ± 1.0

[a] Cell survival in the presence or absence of DEX is shown as cell survival % under the same conditions, but in the absence of TNF. Each value represents the mean (± SD) of an experiment carried out in triplicate

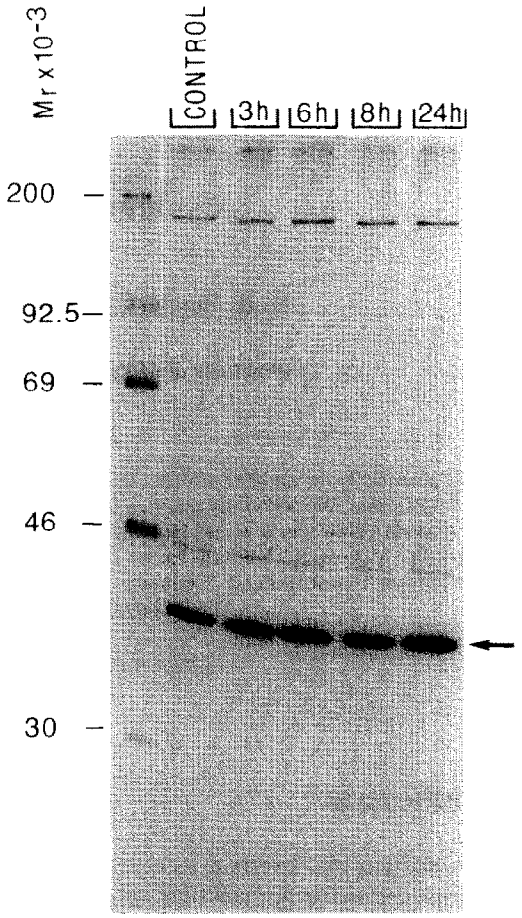

Fig.1. SDS-PAGE after immunoprecipitation with anti-lipocortin-I of [^{35}S]methionine-labeled L929 cells treated with 50 μM DEX for the times indicated. The position of lipocortin-I is indicated by an arrow.

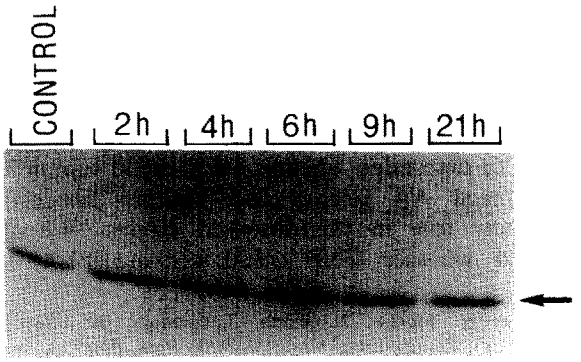

Fig.2. SDS-PAGE after immunoprecipitation with anti-lipocortin-I of [^{35}S]methionine-labeled L929 cells treated with 1000 U TNF/ml for the times indicated. The position of lipocortin-I is indicated by an arrow.

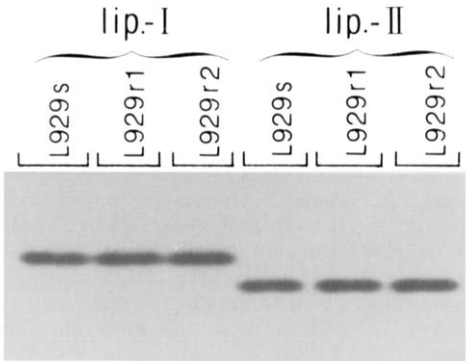

Fig.3. SDS-PAGE after immunoprecipitation with anti-lipocortin-I and anti-lipocortin-II of [^{35}S]methionine-labeled TNF-sensitive and TNF-resistant L929 cells.

ed upon TNF-treatment. Data for lipocortin-I are shown in fig.2. Similar experiments were performed with some other TNF-sensitive cells (HeLaD98/AH2 and MCF7, which lacked lipocortin-I), leading to the same conclusions. In addition, we examined whether lipocortin levels differed between TNF-sensitive L929 cells and their resistant subclones L929r1 and L929r2, which are fully or partially TNF-resistant, respectively (B. Vanhaesebroeck et al., in preparation). However,

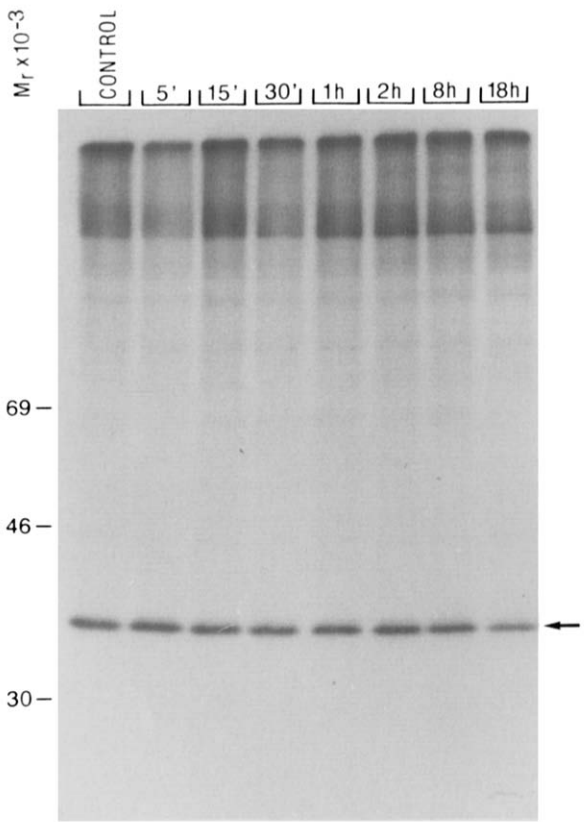

Fig.4. SDS-PAGE after immunoprecipitation with anti-lipocortin-II of $^{32}P_i$-labeled L929 cells treated with 1000 U TNF/ml for the times indicated. The position of lipocortin II is indicated by an arrow.

there was no clear difference in expression of lipocortin-I or -II between the different cell types (fig.3).

3.4. *Role of lipocortin phosphorylation in the TNF-response*

L929 cell membranes were isolated and treated with TNF in the presence of [γ-^{32}P]ATP. Lipocortin-I phosphorylation was then determined after immunoprecipitation and gel electrophoresis. As a positive control we used EGF-induced phosphorylation of lipocortin-I in A431 membranes [10]. We could not observe a TNF-induced phosphorylation of lipocortin-I in L929 cell membranes (data not shown). Phosphorylation of lipocortin-I and -II was also studied in viable L929 cells which were labeled with $^{32}P_i$ for 3 h and treated with TNF for increasing time periods (5 min, 15 min, 30 min, 1 h, 2 h, 8 h and 18 h) before the end of the labeling. In contrast to lipocortin-II, lipocortin-I was only very weakly phosphorylated in these cells. Again no difference in phosphorylation of lipocortins could be seen upon TNF treatment. Data for lipocortin-II are shown in fig.4.

4. DISCUSSION

In this report we demonstrate that, although both TNF-mediated cytotocity and TNF-induced AA release are inhibited by DEX, the latter drug has no effect on the synthesis of lipocortins. We could also not detect constitutive or DEX-induced secretion of lipocortins from L929 cells, although there are several reports about an extracellular location of these proteins [12,13]. Inhibition of cellular PLA2 by extracellular lipocortins has been reported by two different groups [14,15]. However, our preliminary results show that addition of purified lipocortin-II to L929 cells does not block TNF-mediated cytotoxicity or TNF-induced AA release. Our results are consistent with a recent report which documents the failure to correlate the action of lipocortin-I with inhibition by glucocorticoids of zymosan-stimulated AA release from macrophages [16]. Others also showed that inhibition of thromboxane A synthesis in U937 cells by glucocorticoids is not mediated by lipocortin-I [17]. The possibility that some other recently identified lipocortins [18] do play a role in TNF action is unlikely as well, since two-dimensional gel analysis revealed no induction by DEX of the known lipocortins in L929 cell lysates or supernatants, although we were able to detect an induction of some other proteins by DEX in the same cell lysates (data not shown). Experiments to further characterize these proteins are now in progress.

The hypothesis that TNF would down-regulate or phosphorylate lipocortin-I and -II, in order to activate PLA2, is doubtful since we could not detect any effect of TNF on the synthesis or phosphorylation of lipocortin-I and -II in L929 cells. Furthermore, there

was no difference in the constitutive or TNF-induced expression of lipocortin-I and -II between TNF-sensitive and TNF-resistant L929 cells. The activation of PLA2 is believed to be responsible for the increase in prostaglandin E_2 synthesis observed in dermal fibroblasts [19]. However, our preliminary results do not show any role of lipocortins in these cells either.

The results reported above do not support the suggestion that lipocortins are involved in the regulation of TNF-mediated cytotoxicity.

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