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Cytotoxic activity of tumor necrosis factor is inhibited by amiloride derivatives without involvement of the Na^+/H^+ antiporter

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Cytotoxicity of tumor necrosis factor (TNF) on L929s cells was efficiently blocked by several amiloride analogs but not by amiloride itself. This protection did not require RNA or protein synthesis. Na^+/H^+ antiporter-negative L-M(TK^-) cells (LAP) could be killed by TNF, showing that the Na^+/H^+ exchanger is not required for TNF-cytotoxicity. Similar protection against TNF-mediated cell lysis by amiloride derivatives was found for LAP and L929s cells, excluding a blockade of the Na^+/H^+ antiporter as the cause of the protection against TNF by these agents.

Tumor necrosis factor; Cytotoxicity; Amiloride; Na^+/H^+ antiporter; Cytokine

1. INTRODUCTION

Tumor necrosis factor (TNF), primarily produced by activated macrophages, is selectively cytotoxic for many tumor cells. TNF also induces in both transformed and untransformed cells a variety of other biological effects, mostly related to inflammatory and immunomodulatory activities [1].

Signaling mechanisms involved in TNF-mediated cytotoxicity are at present largely unclear. TNF binds to its target cells via high affinity, cell surface-associated receptors [2,3]. The TNF-receptor complex is subsequently internalized by endocytosis, followed by degradation of TNF [2–4]. It is not clear whether this internalization and degradation are required for TNF-cytotoxicity [5–7]. Post-receptor mechanisms are also poorly understood. A particular G-protein might be involved [8]. TNF includes arachidonic acid release, possibly via a phospholipase A_2 activation [9,10]. TNF-mediated cytotoxicity does not depend on cellular RNA- or protein synthesis [11].

Here, we report that several amiloride analogs, but not amiloride itself, effectively protect L929s cells against TNF-cytotoxicity. Since the amiloride analogs tested are potent inhibitors of the Na^+/H^+ exchanger, a signaling target common to a number of growth factors [12], we investigated the role of this antiporter in the observed protection.

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Abbreviations: ActD, actinomycin D; CHX, cycloheximide; DMA, 5-(*N*,*N*-dimethyl)amiloride; DMSO, dimethyl sulfoxide; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; HMA, 5-(*N*,*N*-hexamethylene)-amiloride; MIBA, 5-(*N*-methyl-*N*-isobutyl)amiloride; MGCMA, 5-(*N*-methyl-*N*-(guanidinocarbonylmethyl))amiloride; MTT, 3-(4,5-dimethylthiazol-2-yl)diphenyltetrazolium bromide; OD, optical density; TNF, tumor necrosis factor; U, units/ml

2. MATERIALS AND METHODS

2.1. *Cell lines and media*

The murine L929s fibrosarcoma cell line, sensitive to TNF-cytotoxicity, was obtained from Dr Konings (Rega Institute, Leuven, Belgium). The cell lines L-M(TK^-) and its derivative LAP, which is devoid of functional Na^+/H^+ antiporter, were described previously [13]. Culture and assay medium for all cell lines was Dulbecco's modified Eagle's medium, supplemented with 5% fetal calf serum, 5% newborn calf serum, and antibiotics. All cell lines were mycoplasma-free, as judged by a DNA-fluorochrome assay [14].

2.2. *Reagents*

TNF used was recombinant murine TNF [15] (sp. act. of 1.9×10^8 international units/mg (determined as in [16]); international standard TNF (code no. 88/532) was from National Biological Standards Board, Hertfordshire, UK). Amiloride (Sigma) was dissolved in culture medium. The amiloride analogs used were described previously [17]. They were synthesized for this study as described earlier [18]. Stock solutions were prepared in dimethyl sulfoxide (DMSO) at 100 nM. For use on cells, these stock solutions were diluted in culture medium at 37°C such that the final concentration of DMSO never exceeded 0.4%. Control experiments demonstrated that such DMSO concentrations had no effect on TNF-cytotoxicity. The ability of the amiloride analogs to block Na^+/H^+ antiporter activity was checked via their protection of L929s to proton suicide [19].

2.3. *Assay for cytotoxicity*

All assays were performed at 37°C in a humidified 10% CO_2-incubator. Cells were seeded in 96-well flat-bottom microwells (Falcon, Becton Dickinson, NJ) at 2×10^4 cells in 100 μl medium. Twelve to 16 h later, 50 μl of amiloride (analog) dilution was added. TNF (with or without metabolic blockers) was given 2 h later in a 50 μl volume. Metabolic inhibitors used were actinomycin D (ActD; 1 μg/ml) or Cycloheximide (CHX; 50 μg/ml). After 20 h of further incubation, cell viability was routinely determined via 3-(4,5-dimethylthiazol-2-yl)-biphenyltetrazolium bromide (MTT) staining

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[20]. Similar results were observed with crystal violet staining of attached cells [21] (data not shown).

3. RESULTS AND DISCUSSION

The reported [17] inhibitory activity of amiloride and its derivatives on the Na^+/H^+ antiporter is shown in table 1. Their effect on cytotoxicity, induced in L929s cells by a fixed TNF concentration, is also presented in table 1. It is clear that amiloride itself is not protective, even when tested at higher concentrations (up to 500 μM, data not shown). In contrast, several amiloride analogs showed a potent protection against TNF-cytotoxicity. The effect of EIPA, a representative protective inhibitor, at different TNF-concentrations is shown in fig.1. Only MGCMA was almost not protective at the concentrations shown, although the drug was effective in protection of L929s in a proton suicide test (data not shown). However, at 200 μM MGCMA, considerable protection was found (results not shown). It is known that amiloride and analogs penetrate the cells and at elevated concentrations directly inhibit protein synthesis [22,23]. It is unlikely that such a side-effect accounts for the protective action of some amiloride analogs, as it is well established that inhibition of protein synthesis effectively potentiates TNF-cytotoxicity [11]. Moreover, RNA or protein synthesis was not required for protection by amiloride derivatives (see table 1 for results with RNA synthesis inhibitor, ActD; similar but not shown results were found with CHX). It is clear that the reported inhibitory potential of the amiloride analogs for the Na^+/H^+ antiporter does not correlate completely with their concentration-dependent protective effect on TNF-cytotoxicity. This suggests that the Na^+/H^+ antiporter is not the target responsible for this protection. To obtain proof of this, we tested the effect of amiloride and its derivatives on TNF-cytotoxicity towards Na^+/H^+ antiporter-negative L-M(TK$^-$) (LAP) cells. Since both the parental L-M(TK$^-$) cells and LAP cells were found to be resistant to TNF alone (up to 3×10^4 IU TNF/ml; data not shown), their TNF-sensitivity was tested in the presence of ActD. Under these conditions, no difference in TNF-sensitivity was found between the L-M(TK$^-$) cell line and its Na^+/H^+ antiporter-negative derivative (50%

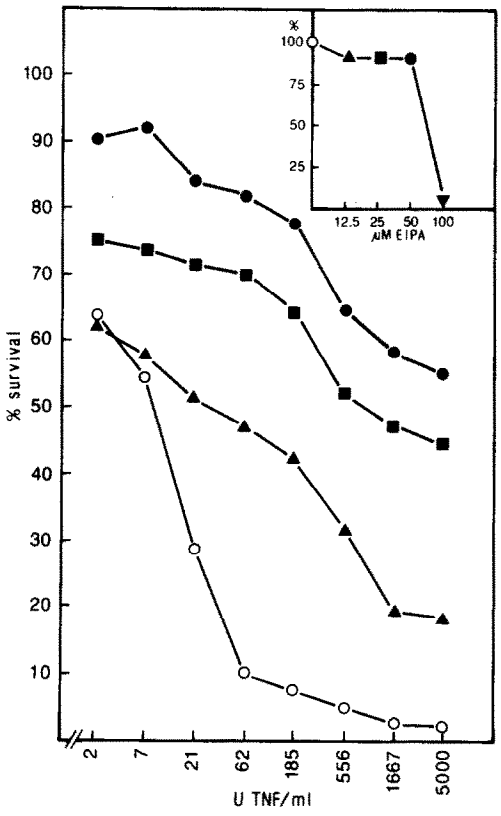

Fig. 1. Inhibition of TNF-cytotoxicity on L929c cells by EIPA. Percentage survival is plotted against increasing TNF concentrations. For each inhibitor concentration, percentage survival is calculated as follows: $100 \times OD_{cells+TNF+inhibitor}/OD_{cells}$. The insert shows the effect of different concentrations of EIPA on cell viability in the absence of TNF. (○) No inhibitor; (▲) 12 μM EIPA; (■) 25 μM EIPA; (●) 50 μM EIPA; (▼) 100 μM EIPA.

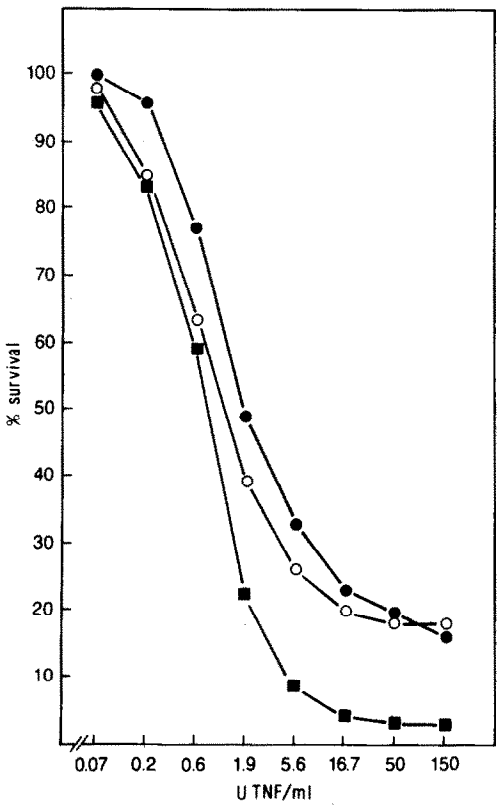

Fig. 2. TNF sensitivity of L929s (■), L-M(TK$^-$) (○) and LAP (●) cells. TNF sensitivity was determined in a 20 h assay in the presence of ActD as described in section 2. Percentage survival is calculated as follows: $100 \times OD_{cells+TNF+ActD}/OD_{cells+ActD}$. Percentage survival in the presence of ActD alone (referred to 100% = $OD_{cells\ in\ culture\ medium\ alone}$) was 89 ± 1, 90 ± 5 and 96 ± 5 for L929s, L-M(TK$^-$) and LAP cells, respectively.

Table 1

Drug[a]	K_i[a] (μM)	Drug conc.[c] (μM)	% Survival[d] L929s no TNF −ActD	% Survival[d] L929s no TNF +ActD	% Survival[d] L929s 62 U TNF[e] −ActD	% Survival[d] L929s 0.6 U TNF[e] +ActD	% Survival[d] LAP no TNF +ActD	% Survival[d] LAP 150 U TNF[e] +ActD	Relative survival[f] L929s −ActD	Relative survival[f] L929s +ActD	Relative survival[f] LAP +ActD
none	–	–	100 ± 7	90 ± 3	21 ± 4	17 ± 3	90 ± 8	18 ± 4	0.21	0.19	0.20
Amil	83.3	100	100 ± 6	93 ± 5	18 ± 2	20 ± 5	80 ± 3	15 ± 3	0.18	0.22	0.19
DMA	6.9	100	83 ± 5	84 ± 5	56 ± 4	58 ± 6	73 ± 5	33 ± 3	0.67	0.69	0.45
		50	95 ± 4	85 ± 3	49 ± 5	39 ± 5	86 ± 5	34 ± 3	0.52	0.46	0.40
		25	95 ± 4	86 ± 7	41 ± 2	23 ± 5	93 ± 4	29 ± 3	0.43	0.27	0.31
		12	98 ± 7	93 ± 8	30 ± 5	18 ± 3	98 ± 2	31 ± 5	0.31	0.19	0.32
MGCMA	1.4	100	96 ± 3	86 ± 5	27 ± 3	30 ± 3	89 ± 2	48 ± 2	0.28	0.35	0.54
		50	97 ± 6	86 ± 3	27 ± 2	24 ± 3	91 ± 3	39 ± 5	0.28	0.28	0.43
		25	99 ± 5	90 ± 8	27 ± 4	20 ± 2	92 ± 5	29 ± 3	0.27	0.22	0.32
		12	104 ± 5	95 ± 8	23 ± 3	20 ± 5	100 ± 3	23 ± 6	0.22	0.21	0.23
MIBA	0.44	50	69 ± 3	68 ± 6	45 ± 5	69 ± 5	T	T	0.65	1.01	T
		25	94 ± 2	99 ± 6	75 ± 2	81 ± 5	63 ± 6	58 ± 5	0.80	0.82	0.92
		12	81 ± 2	86 ± 5	45 ± 6	33 ± 2	79 ± 5	55 ± 4	0.56	0.38	0.70
EIPA	0.38	50	90 ± 6	95 ± 9	82 ± 4	95 ± 4	39 ± 3	42 ± 5	0.91	1.00	1.08
		25	88 ± 9	90 ± 6	73 ± 3	60 ± 5	67 ± 3	51 ± 3	0.83	0.67	0.76
		12	90 ± 6	92 ± 3	49 ± 6	24 ± 3	75 ± 2	46 ± 6	0.54	0.26	0.61
HMA	0.16	25	80 ± 5	81 ± 9	75 ± 2	77 ± 5	35 ± 6	38 ± 4	0.94	0.95	1.09
		12	88 ± 2	90 ± 6	67 ± 7	77 ± 5	63 ± 5	54 ± 5	0.76	0.86	0.86

[a] Amil = amiloride

[b] Apparent K_i values of inhibition of the Na^+/H^+-antiporter, evaluated in the presence of 140 mM extracellular Na^+ [24]

[c] Only data obtained with drug concentrations resulting in cell survival higher than 35% in the absence of TNF are shown. Highest concentration of drugs tested was 100 μM

[d] OD of cells incubated in culture medium alone was taken as 100%. T = toxicity, due to drug alone, gives survival < 35%

[e] In the absence of ActD, higher concentrations of TNF were applied on L929s cells in order to obtain a similar percent survival as in the presence of ActD. As LAP cells were found to be less TNF sensitive than the L929s cells (especially at higher TNF concentrations, see fig.2), TNF concentrations were chosen so that both cell lines showed a similar % survival

[f] For each inhibitor concentration, relative survival is defined as follows:

$$OD_{cells \vdash TNF \vdash inhibitor} / OD_{cells \vdash inhibitor}$$

value: ~2 IU TNF/ml; fig.2), showing that the Na^+/H^+-antiporter is not essential for TNF-cytotoxicity. The relative potency of the amiloride analogs for protection of LAP cells against TNF-lysis was similar to that for protection of L929s cells, and again not related to their reported inhibition efficiency of the Na^+/H^+ antiporter (table 1). These results indicate that the effective protection against TNF-cytotoxicity by the amiloride analogs is not related to their activity on the Na^+/H^+ exchanger.

In summary, our results show that the Na^+/H^+-antiporter is not needed for signaling in the cytotoxic mechanism of TNF. Protection by amiloride analogs against TNF-cytotoxicity almost certainly does not act via this antiporter. Experiments are currently under way to find the target recognized by these amiloride analogs in their effective protection against TNF.

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