FEBS Letters 351 (1994) 311–313

FEBS 14465

No requirement of reactive oxygen intermediates in Fas-mediated apoptosis

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Received 4 July 1994

Abstract Fas is a cell surface molecule that mediates apoptosis, but the intracellular mechanisms leading to apoptosis are not well understood. It is known that diethylmaleate (DEM)-induced cell death can be blocked by substances with antioxidant activity. Here we have studied whether antioxidants have any effect on Fas-mediated apoptosis and show that they are not able to block Fas-mediated apoptosis. Therefore, it seems that reactive oxygen intermediate (ROI)-dependent and -independent mechanisms which lead to apoptosis do exist. Fas-mediated apoptosis probably proceeds via a ROI-independent pathway.

Key words: Apoptosis; Fas; Signal transduction; Reactive oxygen intermediate

1. Introduction

Elimination of unwanted cells in multicellular organism proceeds via programmed cell death, in most cases, morphologically, recognized as apoptosis. Apoptosis can be induced by various chemicals, death factors or deprivation of growth factors [1,2].

Fas and tumor necrosis factor receptor (TNF-R) are cell surface proteins which can mediate apoptosis either by stimulation with the corresponding ligand or specific agonistic monoclonal antibodies [3,4]. However, cytoplasmic proteins and second messengers involved in the signal transduction pathway leading to apoptosis are not known.

Diethylmaleate (DEM) reduces cellular stores of glutathione by forming a thioether conjugate in a reaction catalyzed by glutathione-*S*-transferase [5], and reactive oxygen intermediates (ROIs) accumulating in the cells induce cell death [6]. A neuronal cell line overexpressing Bcl-2 was almost completely protected against DEM-induced cell death [6] suggesting that Bcl-2 and DEM act in the same signal transduction pathway leading to cell death. And it was proposed that Bcl-2 prevents cell death by decreasing the net cellular generation of ROIs [6,7]. Since Fas-mediated apoptosis is also partially inhibited by Bcl-2 [8], we address the question whether Fas-mediated apoptosis needs the action of ROIs [9–11]. Here, we have tested several antioxidants for their ability to block cell death induced by either anti-human Fas antibody or DEM in Fas-overexpressing fibroblasts. We show that only DEM-induced cell death can be blocked by the antioxidants we have tested.

2. Materials and methods

2.1. Reagents

Deferoxamine (desferrioxamine mesylate), L-γ-glutamyl-L-cysteinylglycyl-*O*-ethyl ester (GSH-OEt), glutathione (GSH), glutathione L-γ-glutamyl-L-cysteine (γ-GluCys), α-Tocopherol and catalase were purchased from Sigma Chemical Co. The monoclonal anti-human Fas IgM antibody was obtained from MBL (Japan). Recombinant murine TNF was kindly provided by Dr. D.V. Goeddel (Genentech).

2.2. Cell death assay

LB1 cells are murine L929 fibroblasts stably overexpressing human Fas [3]. Cells were treated with the antioxidants, followed by induction of apoptosis as described in the figure legends. Living cells were stained with Crystal violet as described [3]. Absorbance at 540 nm was measured using an automated micro-ELISA autoreader.

3. Results

To examine the effects of several antioxidants on Fas-mediated apoptosis we have used L929 transformants stably overexpressing human Fas (LB1) [3]. When these cells were incubated with the anti-Fas antibody, murine TNF or DEM, almost all cells were dead after approximately 6 h. TNF kills these cells only in the presence of actinomycin D or cycloheximide, whereas these substances are not required for killing mediated by Fas but they accelerated Fas-mediated apoptosis (data not shown).

Deferoxamine is an iron chelator and it prevents the formation of the hydroxyl radical (HO•) from hydrogen peroxide (H_2O_2) via the Fenton and Haber–Weiss reaction [11]. LB1 cells were incubated with several non-toxic concentrations of deferoxamine, and apoptosis was induced by an anti-Fas antibody, TNF or DEM (Fig. 1). The anti-Fas antibody completely killed the cells whereas there was a high protection against DEM-induced cell death by deferoxamine in a dose-dependent manner (Fig. 1).

The most common natural and lipophilic antioxidant α-tocopherol very likely protects against membrane lipid peroxidation. It could react with lipid peroxyl and alkoxyl radicals which are generated by the action of ROIs [11]. α-Tocopherol also showed a slight but significant protection against DEM-induced but not against anti-Fas antibody- or TNF-induced cell death (Fig. 2).

In the following set of experiments the involvement of glutathione (GSH) [12] in cell death was determined. It has been shown that GSH-OEt can be transported into cells and thereafter is split intracellularly into glutathione [13]. γ-GluCys is a precursor of glutathione [12]. GSH itself, GSH-OEt (Fig. 3) or

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Abbreviations: ROI, reactive oxygen intermediates; TNF, tumor necrosis factor; TNF-R, tumor necrosis factor receptor; DEM, diethylmaleate; HO•, hydroxyl radical; H_2O_2, hydrogen peroxide; GSH, glutathione; GSH-OEt, L-γ-glutamyl-L-cysteinylglycyl-*O*-ethyl ester; γ-GluCys, L-γ-glutamyl-L-cysteine.

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SSDI 0014-5793(94)00852-3

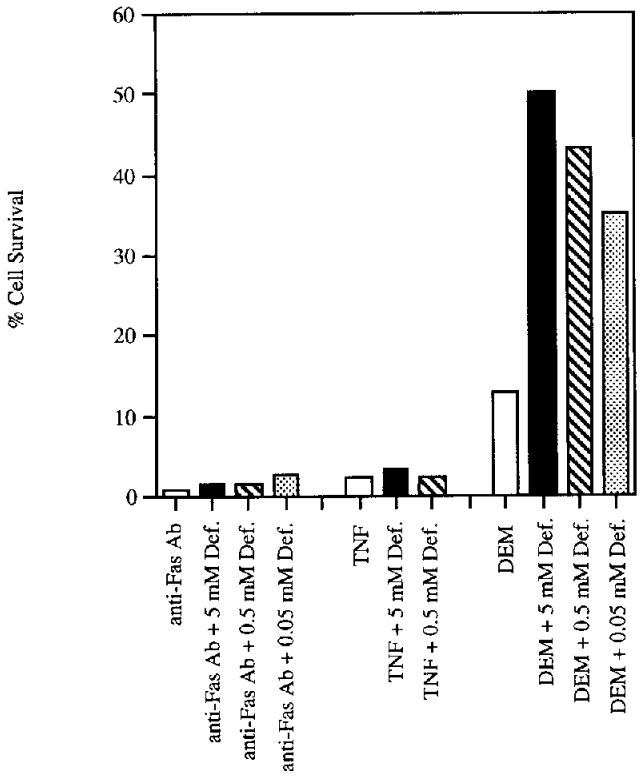

Fig. 1. LB1 cells were preincubated for 2 h with the indicated concentrations of deferoxamine (Def.). Cell death was induced by treating the cells overnight with either 0.1 μg/ml anti-Fas antibody, 0.01 μg/ml murine TNF, or 0.0037% DEM. Anti-Fas antibody- and TNF-treatment was in the presence of 50 μg/ml actinomycin D. Cells were stained and counted as described in section 2. The assays were done in quadruplicate, and mean values are expressed as the percentage of surviving cells incubated with the anti-oxidant alone. The standard deviation was less than 15%.

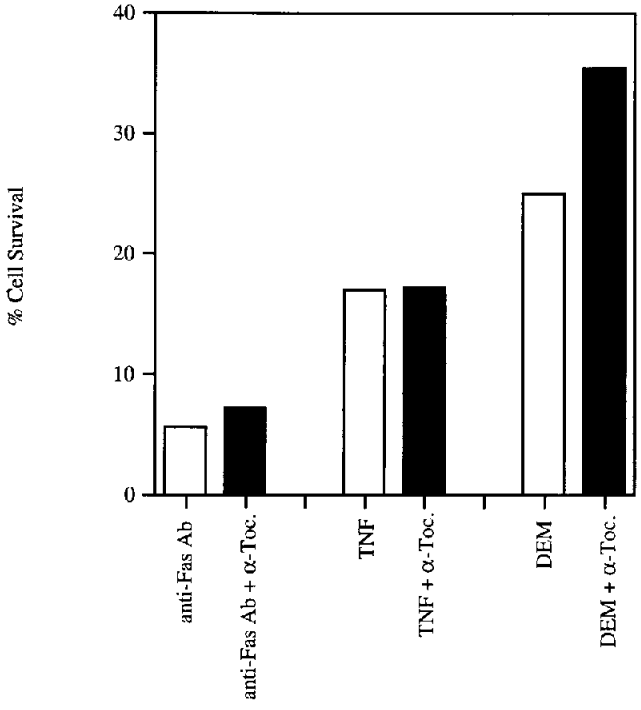

Fig. 2. LB1 cells were preincubated for 2 h with 0.01% α-tocopherol (α-Toc) and cell death was induced and determined as described for Fig. 1.

γ-GluCys (Fig. 4) in the indicated non-toxic concentrations did not show any effect on Fas- or TNF-R-mediated apoptosis, whereas they were able to block DEM-induced cell death. This indicates that GSH works as protective agent against DEM-induced but not Fas- or TNF-R-mediated apoptosis.

Catalase which catalyzes the dismutation of H_2O_2 also showed an approximately 2-fold protection against DEM-induced cell death but no significant protection against anti-Fas antibody- or TNF-induced apoptosis (Fig. 4). Vitamin C had no effect on Fas- and TNF-R-mediated apoptosis but showed a weak inhibition on DEM-induced cell death (data not shown). In addition, ω-nitro-L-arginine, an inhibitor of nitric oxide synthase, the antioxidants thioredoxin, dimethyl sulfoxide, thiourea, or butylated hydroxyanisol could not block Fas-mediated apoptosis (data not shown).

4. Discussion

It has been proposed that redox modulation may play a role in the regulation of cell death [6,7]. That is, substances that increase the oxidation state of a cell (like H_2O_2 or DEM) induce apoptosis and at higher concentrations they lead to necrosis. In these systems antioxidants can be used to inhibit apoptosis. Here, we could not detect any protective effect of antioxidants

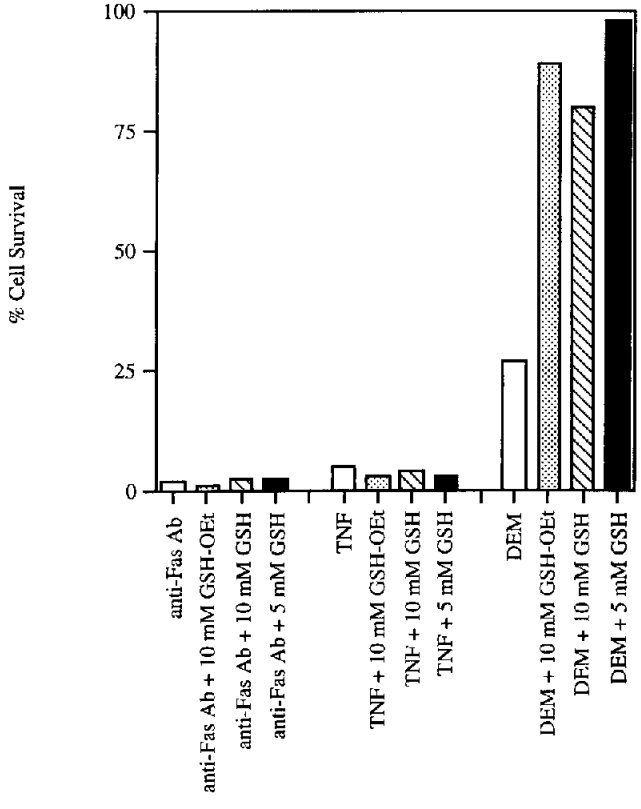

Fig. 3. LB1 cells were preincubated for 2 h with the indicated concentrations of GSH or GSH-OEt and cell death was induced and determined as described for Fig. 1.

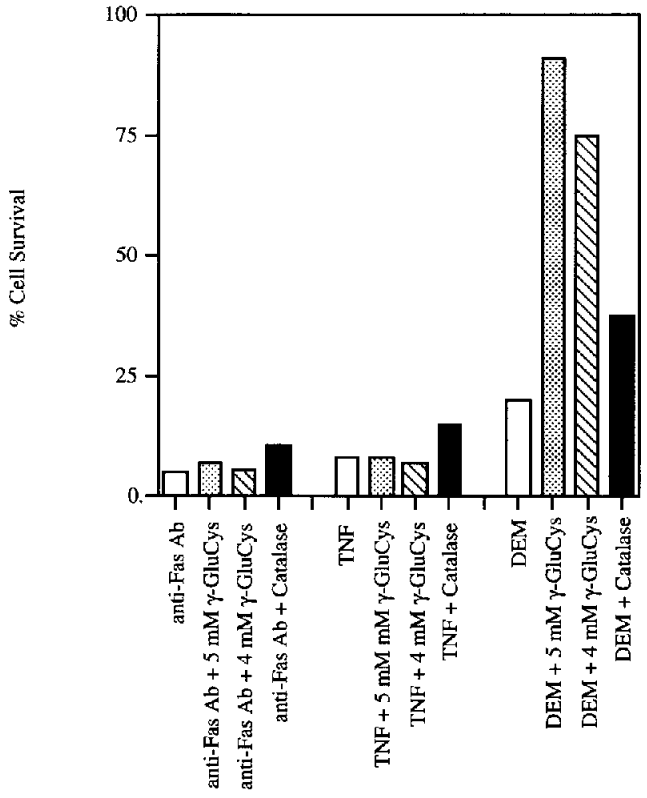

Fig. 4. LB1 cells were preincubated for 2 h with the indicated concentrations or γ-GluCys or 10 μg/ml catalase and cell death was induced and determined as described for Fig. 1 except that 0.1 μg/ml murine TNF was used.

on Fas-mediated apoptosis, which indicates that Fas-mediated apoptosis does not proceed via an increase of the cellular oxidation level.

Reduced GSH is used intracellularly to reduce numerous oxidizing compounds including ROIs. It also protects proteins with sulfhydryl groups from oxidation. DEM inactivates these functions of GSH by binding to its reactive sulfhydryl group [5]. The resulting increase of the intracellular oxidation state then leads to cell death as already mentioned. Accordingly, all antioxidants we have tested showed some protective effect on DEM-induced death, while they were not able to block Fas-mediated apoptosis. Therefore, it is very likely that ROIs are not necessary for Fas- or TNF-R-mediated apoptosis and that Fas- or TNF-R use a mechanism different from that induced by DEM leading to cell death. Bcl-2 may inhibit apoptosis by blocking several signal transduction pathways including ROI-dependent and ROI-independent ones. The partial inhibition of Fas-mediated apoptosis by Bcl-2 [8] could therefore be due to an inhibition of a ROI-independent step.

Previously, Schulze-Osthoff et al. [14] have observed an inhibitory effect of antioxidants on TNF-R-mediated cytotoxicity, and suggested that ROIs generated from the mitochondrial respiratory chain serve as second messengers for TNF-mediated cytotoxicity. Here, we could not detect any effects of antioxidants on TNF-R- as well as Fas-mediated apoptosis. Although the TNF-R-mediated apoptotic pathways may be different among different cell types, these results suggest that ROIs may not be essential for TNF-R-mediated apoptosis. The p55 TNF-R and Fas carry a similar domain (a death domain), which is essential for TNF-R or Fas-mediated apoptosis [15,16]. The fact that apoptosis induced by Fas or TNF-R cannot be blocked by antioxidants suggests a similar pathway for TNF-R-mediated and Fas-mediated apoptosis. However, it seems that TNF-R and Fas use distinct pathways to induce apoptosis, because manganous superoxide dismutase induced by pretreatment with TNF, can protect TNF-R-mediated apoptosis but not Fas-mediated apoptosis [17]. The inability of Fas to activate the transcription factor NF-κB (unpublished results), which can be activated by TNF [18], agrees with the above notion. Identification of molecules which associate with the cytoplasmic region of TNF-R or Fas would be necessary to understand the apoptotic mechanism mediated by those receptors.

Acknowledgements: We thank Dr. N. Itoh for his contribution during the initial phase of this work, and H.H. gratefully acknowledges a fellowship from the German Cancer Research Center (AIDS-Stipendienprogramm). This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan.

References

[1] Raff, M.C. (1992) Nature 356, 397–400.
[2] Nagata, S. (1994) Adv. Immunol., in press.
[3] Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S., Sameshima, M., Hase, A., Seto, Y. and Nagata, S. (1991) Cell 66, 233–243.
[4] Suda, T., Takahashi, T., Golstein, P. and Nagata, S. (1994) Cell 75, 1169–1178.
[5] Harlan, J.M., Levine, J.D., Callahan, K.S., Schwartz, B.R. and Harker, L.A. (1994) J. Clin. Invest. 73, 706 713.
[6] Kane, J.K., Sarafian, T.A., Anton, R., Hahn, H., Gralla, E.B., Valentine, J.S., Örd, T. and Bredesen, D.E. (1993) Science 262, 1274–1277.
[7] Hockenbery, D.M., Oltvai, Z.N., Yin, X., Milliman, C.L. and Korsmeyer, S.J. (1993) Cell 75, 241–251.
[8] Itoh, N., Tsujimoto, Y. and Nagata, S. (1993) J. Immunol. 151, 621–627.
[9] Schreck, R. and Baeuerle, P. (1991) Trends Cell Biol. 1, 39–42.
[10] Buttke, T.M. and Sandstrom, P.A. (1994) Immunol. Today 15, 7–10.
[11] Yu, B.P. (1994) Physiol. Rev. 74, 139–162.
[12] Meister, A. (1994) Cancer Res. (Suppl.) 54, 1969s–1975s.
[13] Meister, A. (1991) in: Glutathione Centennial: Molecular and Clinical Implications (Taniguchi, N., Higashi, T., Sakamoto, Y. and Meister, A. Eds.) pp. 3-21, Academic Press, New York.
[14] Schulze-Osthoff, K., Beyaert, R., Vandevoorde, V., Haegeman, G. and Fiers, W. (1993) EMBO J. 12, 3095–3104.
[15] Itoh, N. and Nagata, S. (1993) J. Biol. Chem. 268, 10932–10938.
[16] Tartaglia, L.A., Ayres, T.M., Wong, G.H.W. and Goeddel, D.V. (1993) Cell 74, 845–853.
[17] Wong, G.H.W. and Goeddel, D.V. (1994) J. Immunol. 152, 1751–1755.
[18] Baeuerle, P. and Henkel, T. (1994) Annu. Rev. Immunol. 12, 141–179.