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Transrepression of NF-κB is not required for glucocorticoid-mediated protection of TNF-α-induced apoptosis on fibroblasts

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Abstract

The cellular resistance to tumor necrosis factor (TNF) of most cell types has been attributed to both a protective pathway induced by this cytokine and the preexistence of protective factors in the target cell. NF-κB has been postulated as one of the principal factors involved in antiapoptotic gene expression control on TNF-resistant cells. We have previously shown that glucocorticoids protect the naturally TNF-sensitive L-929 cells from apoptosis. Here we analyze the role of NF-κB and glucocorticoids on TNF-induced apoptosis in L-929 cells. We found that inhibition of NF-κB enhanced the sensitivity to TNF-induced apoptosis. Glucocorticoids inhibited NF-κB transactivation via IκB induction. Moreover, glucocorticoids protected from TNF-induced apoptosis even when NF-κB activity was inhibited by stable or transient expression of the superrepressor IκB. These results demonstrate that although glucocorticoids inhibit NF-κB transactivation in these cells, this is not required for their protection from TNF-induced apoptosis.

Keywords: Apoptosis; Glucocorticoid; Glucocorticoid receptor; Nuclear factor-κB; Tumor necrosis factor

1. Introduction

Most normal and neoplastic cell types are resistant to tumor necrosis factor (TNF) cytotoxicity unless they are sensitized with protein or RNA synthesis inhibitors, such as cycloheximide or actinomycin D [1]. The cellular resistance has been attributed to a molecular mechanism by which TNF exerts on the target cell two signals: one of them opens the door to cell death, while the other, that requires the novo synthesis of proteins, involves a protective pathway as well as the preexistence of cellular factors that can suppress the apoptotic stimulus generated by TNF [2–6].

TNF p55 is the main receptor on most cell types and after TNF binding, the activation occurs through a trimerization [7,8] and recruitment of other proteins such as TNF receptor-associated death domain (TRADD), TNF receptor-associated factor 2 (TRAF2) and receptor-interacting protein (RIP) to the p55 cytoplasmic death domain [9–11]. TRADD acts as an adapter that recruits the downstream transducer Fas-associated death domain-containing protein (FADD) and activates the apoptotic caspases, while TRAF2 has been implicated in the activation of two distinct pathways that lead to the activation of AP-1 via the c-Jun amino terminal

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kinases (JNKs) and, together with RIP, NF-κB activation, via the NF-κB-inducing kinase (NIK) [3]. Although there is some controversy about the role of these transcription factors in apoptosis, most evidence supports an antiapoptotic action for both on TNF-induced apoptosis [2]. In the case of NF-κB, some authors have shown that cells that are naturally resistant to TNF-induced apoptosis became sensitized when they are transfected with an expression vector for IκB, that keeps NF-κB inactive [12,13].

We and others have previously described a model where glucocorticoids prevent TNF-α induced cell death of mouse fibroblast L-929 cells [14,15], the opposite effect that they have in other cells, such as NK cells [16] where they induce apoptosis, having a dual role in neurons [17] or lymphocytes [18] depending on the stimulus. In addition, cells that are sensitive to glucocorticoid-induced apoptosis may be rescued from death by cytokines [17,19]. At least in part the interaction of glucocorticoids and cytokines is supported by the hypothesis of mutual antagonistic actions between the glucocorticoid receptor (GR) and NF-κB. The molecular mechanism by which glucocorticoids antagonize biological function of cytokines is now clearly explained through at least two different, but not opposite, pathways [20]. One of them involves physical interaction between GR and NF-κB or AP-1 which leaves both GR and the transcription factor inactive on the cytoplasm [21]. The other mechanism is the induction of IκB by glucocorticoids [22,23], although there are some cell lines were it does not occur [24].

In this work we analyze the role of NF-κB and its interaction with GR in the model of L-929 cells, which normally die after TNF-α treatment and are protected by glucocorticoids.

2. Materials and methods

2.1. Cells and reagents

The mouse fibroblast cell line L-929 was grown in Dulbecco's modified Eagle's medium (DME) (Gibco, Grand Island, NY, USA) supplemented with 10% charcoal stripped steroid-free fetal calf serum (FCS) (Gibco, Paisley, UK), penicillin (100 units/ml) and streptomycin (100 mg/ml). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. Unless stated otherwise, reagents were from Sigma (St. Louis, MO, USA) or Pharmacia (Uppsala, Sweden).

2.2. Expression vectors and reporter plasmids

κB-Luc vector (κB sites from HIV promoter) was kindly provided by Dr. M. Bell (Mayo Clinic, Rochester, MN, USA), CMV-β-galactosidase (β-gal) was kindly provided by Dr. D. Spengler (Max-Planck Institute of Psychiatry, Munich, Germany). ssIκB (a superrepressor IκB) contains a mutated site at Ser 32 and Ser 36 which impedes phosphorylation and proteolysis and was kindly provided by Dr. B. Kaltschmidt (Univ. Freiburg, Germany).

2.3. Transfections and assays of luciferase and β-galactosidase activities

Transfection and reporter determination were performed as previously described [15,25].

The L-929 cell line was transfected using lipofectamine (Gibco, Grand Island, NY, USA). Briefly, cells were cultured in plates of six wells (3×10^5 cells/well) in DME without serum and antibiotics and transfected with 2 μg of DNA. In all cases cells were co-transfected with the constitutive β-gal expression vector. After 5 h medium was removed and replaced by fresh DME with 10% charcoal stripped serum. For transient transfections and studies of κB-Luc activity, cells were stimulated with human TNF-α (Research and Diagnostic Systems, Minneapolis, MN, USA) and/or dexamethasone (DEX) 1 h after addition of new fresh medium, during 24 h. Cellular extracts for Luc and β-gal assays were made using a lysis buffer (Promega, Madison, WI, USA). Relative luminescence units (RLU) were measured in a Junior luminometer (EG and Berthold, Germany). For cytotoxicity assays, transiently transfected cells were replated on flat bottom 96-well plates and then stimulated with TNF and/or DEX as described above.

Stable ssIκB expressing clones were selected by G-418 (Gibco, Paisley, UK) 400 μg/ml and maintained with 200 μg/ml of this antibiotic. The functional role of ssIκB on stable expression was verified analyzing κB-Luc activity in response to different doses of TNF. No Luc induction was observed at any dose

of TNF, being similar to basal Luc activity, in the absence of TNF.

2.4. Determination of IκB mRNA

Northern blot was performed as previously described [15]. Briefly, total RNA, isolated by the guanidine isothiocyanate phenol-chloroform extraction method, was denatured with glyoxal, electrophoresed on a 1.2% agarose gel and transferred overnight to a nylon membrane. Filters were baked for 2 h at 80°C and stained with methylene blue. They were prehybridized for 4 h at 45°C (50% formamide, 5×SSPE, 5×Denhardt's solution, 0.1% SDS, 100 mg/ml denatured salmon sperm DNA) and then the probe was added for 12 h. Blots were washed at increasing salt and temperature stringency with a final wash of 30 min at 45°C in 0.1×SSC containing 0.1% SDS. Dried filters were exposed to Kodak XAR5 film at −70°C with intensifying screens for 2 days. The blots were reprobed after eluting the first probe with 5 mM Tris-HCl pH 8.0, 2 mM EDTA, 0.1×Denhardt's solution, at 65°C for 2 h. The 0.970 kb IκB [26] and 6.6 kb *Eco*RI fragment of a 28S and part of 18S mouse cDNA [27] cDNA probes (specific activity, 2–4×10^8 cpm/mg) were labeled with a random-priming kit using [α-^{32}P]dCTP. The control with the ribosomal 28S cDNA as probe was performed in each blot.

2.5. Cytotoxicity assay and pharmacological inhibitors

In order to determine the role of NF-κB on p55 TNF receptor-induced cell death, non-transfected mouse fibroblast L-929 cells were plated at 3×10^5 cells/well in 96-well microtiter plates in the presence of different doses of hTNF-α, which is know to bind the mouse p55 receptor [28], in the absence or the presence of different doses of pyrrolidine dithiocarbamate (PDTC), a metal chelator which inhibits ROI generation as NF-κB induction [29] or sulfasalazine [30] and DEX for 20 h. Plates were stained with 0.5% crystal violet in 70% methanol, and then washed and resuspended in acetic 33% solution; the absorbance was determined at 570 nm.

Some cultures were stained with acridine orange and ethidium bromide and the morphology of death and surviving cells was observed at fluorescent microscopy, confirming, as previously shown [15], that the cell death induced by TNF-α is apoptotic.

Cells transiently expressing ssIκB or the empty vector were cultured and stimulated as before (with-

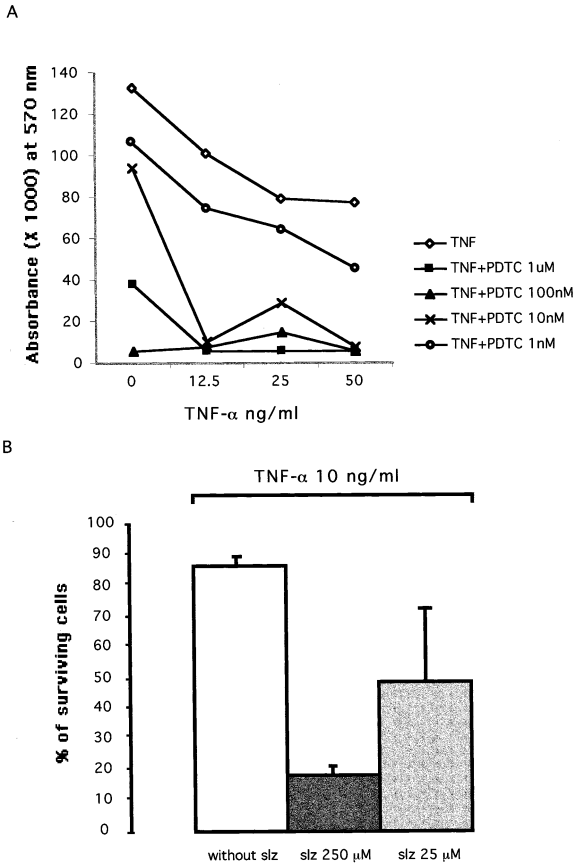

Fig. 1. (A) Effect of PDTC on TNF-induced cell death. L-929 cells cultured in 96-well microtiter plates were stimulated simultaneously with different doses of TNF and PDTC, as indicated. After 20 h monolayers were washed, fixed and stained with 70% methanol/0.5% crystal violet. Absorbance of stained surviving cells was determined at 570 nm. Each value is the average of triplicate wells. Similar results were obtained in three independent experiments. S.D.s are not shown in order to facilitate the interpretation of the figure, and they are less than 10% for each point. The same results were obtained when cultures were stained with acridine orange and ethidium bromide. (B) Effect of sulfasalazine on TNF-induced cell death. L-929 cells cultured in 96-well microtiter plates were stimulated simultaneously with different doses of TNF and sulfasalazine (slz). Cells were fixed and stained as in A. Each value of the diagram bars corresponds to the average ± S.D. of three wells, where the percentage of surviving cells was determined with respect to the basal absorbance (cells without treatment). Similar results were obtained in three independent experiments.

out PDTC or sulfasalazine) and then, non-adherent dead cells were removed and monolayers of surviving cells were fixed with 0.02% of paraformaldehyde and stained with the β-galactosidase substrate x-gal. At least two wells of blue cells were counted for each treatment. Percentage of cytotoxicity was determined with respect to the total blue cells of basal cultures (without any treatment).

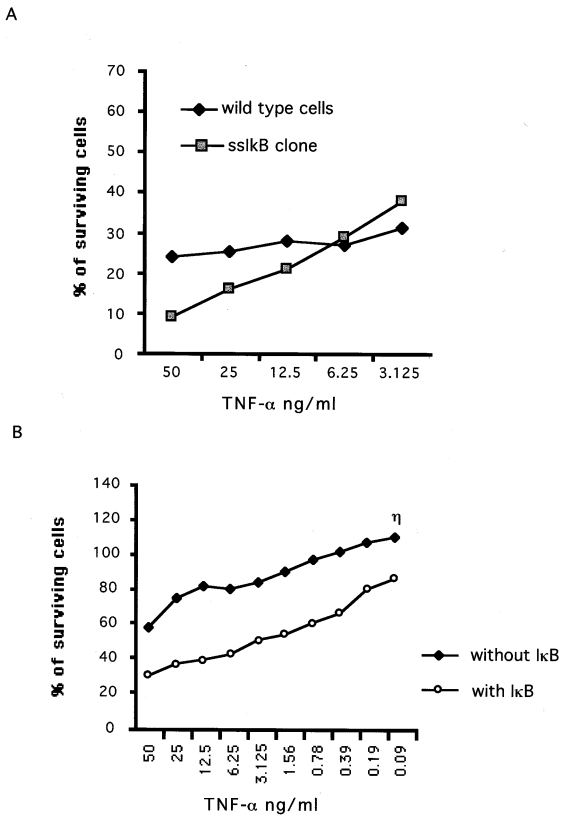

Fig. 2. (A) Effect of stable ssIκB expression on TNF-induced cell death. Wild type cells or a stably expressing ssIκB clone were cultured in 96-well microtiter plates and stimulated with different doses of TNF. Cells were fixed and stained as in Fig. 1A. Each value is the average of triplicate wells. Similar results were obtained in three independent experiments. S.D. is less than 10% for each point. (B) Effect of transient expression of ssIκB on TNF-induced cell death. Cells transfected with CMV-β-gal plus ssIκB or the empty vector were replated on 96-well microtiter plates and stimulated with different doses of TNF. After 20 h, monolayers of surviving cells where fixed and stained with x-gal. Blue cells were counted by microscopy for each dose of TNF. Percentage of surviving cells was determined with respect to the total blue cells in basal conditions. The figure shows the average for each value of four independent experiments, where each experimental point was performed at least in triplicate. S.D. is less than 10% for each point. For all conditions, at least $P<0.05$ (ANOVA with Fisher's test). η, NS.

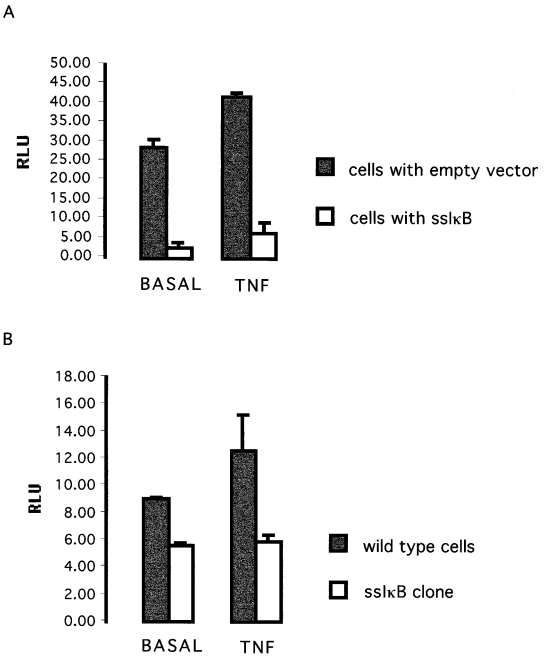

Fig. 3. Effect of transient or stable ssIκB expression on κB-Luc activity. Cells were transiently co-transfected with a κB-Luc reporter plasmid plus CMV-β-gal and the ssIκB or the empty vector (A). Wild type cells or the IκB clone were co-transfected with a κB-Luc reporter plasmid plus CMV-β-gal (B). In both cases cells were stimulated or not with 5 ng/ml of TNF-α. Relative luminescence units are the average of triplicate ±S.D. and were normalized with the corresponding β-galactosidase values.

2.6. Statistical analysis

Statistics were performed using one- and two-factor ANOVA in combination with Fisher and Scheffe's tests.

3. Results

3.1. Inhibition of NF-κB activity increases the sensitivity to TNF-α-induced cell death

A pharmacological approach was performed in order to determine the role on NF-κB on TNF-induced cell death in L-929 cells. As shown in Fig. 1A, all the doses employed of the NF-κB inhibitor PDTC increased TNF-induced cell death at all the TNF-α doses employed (Fig. 1A). PDTC alone resulted

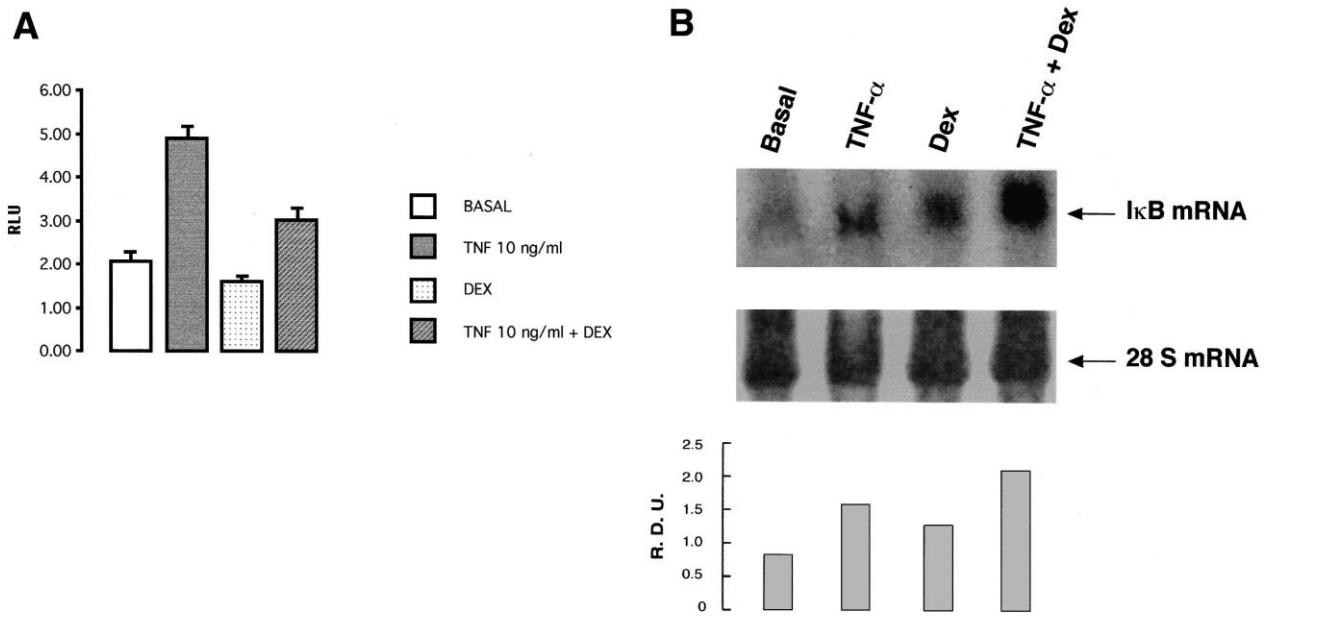

Fig. 4. (A) Effect of glucocorticoids on TNF-induced NF-κB activity in L-929 cells. Transiently κB-Luc and β-gal co-transfected cells were stimulated with TNF and/or DEX during 20 h. Relative luminescence units are the average of triplicate ±S.D. and were normalized with the corresponding β-galactosidase values. The conditions BASAL and DEX are not significantly different from each other. Similar results were obtained in three independent experiments. (B) Glucocorticoid action on IκB mRNA levels. Cells were stimulated for 3 h with 5 μg of TNF and/or 20 nM of DEX as indicated. Each lane contains 16 μg of total RNA. Membrane hybridized with IκB probe was reprobed with the ribosomal 28S probe. Densitometric units relativized (RDU) to 28S ribosomal RNA signal are indicated at the bottom. Similar results were obtained in three independent experiments and also with 10 μg of TNF and/or 50 nM of DEX.

proapoptotic for this cell line, even at lower doses than those used by others to inhibit NF-κB [29] (not shown). Since this effect of PDTC may not only be due to the inhibition of NF-κB, in similar experiments cells were treated with different doses of sulfasalazine, a more specific inhibitor of NF-κB activation [30]. It also increased TNF-induced cytotoxicity, the action being dose-dependent (Fig. 1B).

A clone of L-929 cells stably expressing ssIκB also resulted more sensitive to TNF-induced cytotoxicity than wild type cells, although this was not observed at the lower doses of TNF (Fig. 2A). The same was observed with cells transiently transfected with IκB (Fig. 2B). In addition, in order to prove the effectiveness of IκB we co-transfected the cells with κB-Luc. Cells transiently expressing ssIκB and the reporter plasmid κB-Luc did not show NF-κB activity in the presence or the absence of TNF treatment (Fig. 3A), but the stable ssIκB clone transiently transfected with the reporter shows a small but detectable basal NF-κB activity (Fig. 3B).

All together, these experiments indicate that TNF-induced NF-κB has a protective role on TNF-induced L-929 cell death.

3.2. Glucocorticoids antagonize NF-κB transactivation via IκB induction

As shown in Fig. 4A, DEX, at doses that protect from TNF-induced cell death [15], significantly inhibited basal and TNF-induced NF-κB activity.

Fig. 4B shows the effect of DEX on mRNA expression for IκB after 3 h of treatment. TNF induced IκB mRNA and DEX increased IκB mRNA both in the absence and presence of a cytotoxic dose of TNF. According to these data, IκB induction by glucocorticoids may be one mechanism by which transrepression can occur in these cells.

3.3. Glucocorticoid protection of TNF-induced cell death does not require NF-κB transrepression

Since glucocorticoids had a protective effect on TNF-induced cytotoxicity in these cells, they inhib-

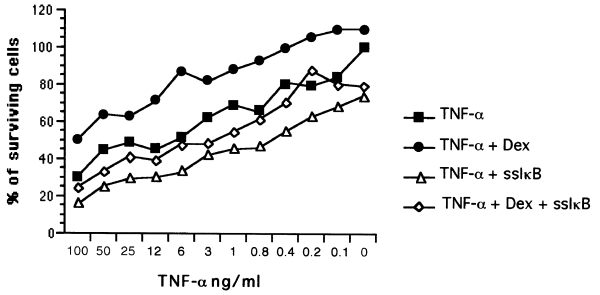

Fig. 5. Effect of glucocorticoids on TNF-induced cell death in ssIκB transiently transfected cells. Transiently IκB expressing vector (empty symbols) or empty vector (full symbols), and CMV-β-gal co-transfected cells were replated in 96-well microtiter plates and stimulated with different doses of TNF and DEX 20 nM. After 20 h, monolayers of surviving cell were fixed and stained with x-gal. Percentage of surviving cells was calculated as in Fig. 2B. Similar results were obtained in three independent experiments. S.D.s are not shown in order to facilitate the interpretation of the figure, and they are less than 10% for each point. Statistical analysis of the results was performed by two-way ANOVA with post-hoc Scheffe's test. Effect of DEX treatment with ssIκB, $F(23,47) = 20.701$, $P = 0.0001$; without ssIκB, $F(23, 47) = 11.865$, $P = 0.0001$. The values without TNF, between equivalent conditions (with or without ssIκB), are not different in the presence or the absence of DEX.

ited NF-κB transactivation and NF-κB inhibition increased cell death, we designed experiments to analyze the role of NF-κB in the glucocorticoid cytoprotection, using cells stably or transiently expressing ssIκB. As shown in Fig. 5, DEX (20 nM) significantly protected (in the same range) from different doses of TNF-induced cell death in both normal cells (transiently transfected with empty vector) and cells without induction of NF-κB (transiently transfected with ssIκB). Similar results were obtained with cells treated with sulfasalazine and the stable clone ssIκB (data not shown). These results indicate that the signal by which glucocorticoids protect from TNF-induced apoptosis does not require NF-κB transrepression.

4. Discussion

There is evidence showing the pro- or antiapoptotic role of NF-κB in different cell lines and its action is controversial [12,13,31]. In different cell lines which are resistant to TNF, some authors have shown that cells with both overexpression of IκB and downregulation of Rel-A (or double mutants cells Rel-$A^{-/-}$) become susceptible to TNF-induced cell death [4,12,13]. However, it has not been described previously which is the role of NF-κB in cells naturally sensitive to TNF-induced cell death.

In this work we show that overexpression of ssIκB or downregulation of NF-κB on fibroblast L-929 enhanced the sensitivity to TNF-induced cell death. These results showing an enhanced TNF-induced apoptosis by inhibition of NF-κB activity indicate that in this cell line, naturally sensitive to TNF-induced cell death, this transcription factor is not involved in induction of proapoptotic genes as it is in other models [32]; moreover, it has a protective role on signaling to death by the p55 receptor.

Interestingly, the transient expression of ssIκB resulted partially lethal per se, in the absence of TNF, but we were able to generate a stable clone expressing ssIκB and it was not lethal for this cell line. These observations indicate that either the basal levels of NF-κB activity (Fig. 2B) are sufficient or some unknown mechanisms that compensate the loss of normal levels of NF-κB may take place on cells stably downregulating this transcription factor.

Binding of TNF to the p55 receptor results in the activation of caspase cascades mainly through the caspase 8 pathway [33]. This mechanism may bypass the mitochondrial and caspase 9 pathway of cell death induction. On the other hand, it has been shown previously that, in L-929 cells, both the generation of free radicals and mitochondrial damage play an important role on TNF-induced cell death [34]. Taking together these observations and our data, we could hypothesize that NF-κB might have a protective role only in the caspase 8 pathway, and thus explain the behavior of these cells in response to TNF. In other words, L-929 cells might die in response to TNF mainly by the mitochondrial pathway and caspase 9 activation, where NF-κB is not protective. The increase on cell death by ssIκB could probably be due to an increase on TNF sensitivity, because the caspase 8 pathway becomes relevant when NF-κB is inhibited.

At physiological and molecular levels, almost always cytokines and glucocorticoids antagonize each other. The molecular mechanism by which glucocorticoids and cytokines mutually antagonize their function is explained through at least two different but

not opposite ways [20]: one of them involves physical interaction between the glucocorticoid receptor (GR) and NF-κB [21], and the other the induction of IκB by glucocorticoids [22,23], although there are some cell lines were it does not occur. According to our data, in L-929 cells glucocorticoids antagonize NF-κB transactivation trough the induction of IκB mRNA, although we cannot exclude an additional inhibitory mechanism of interaction between GR and NF-κB. We have previously shown that in L-929 cells glucocorticoids do not physically interact with NF-κB at least until 1 h after treatment [15]. Moreover, priming with low non-cytotoxic doses of TNF enhances the transcriptional activity of GR via the GRE [15]. In accordance with this, the same priming condition enhances the GR-mediated protection of TNF-induced apoptosis [15], suggesting that GRE-responsive genes are involved in the protective action. Here we show that glucocorticoids, 3 h after treatment, both alone and in combination with a low cytotoxic dose of TNF enhanced the expression of IκB mRNA which correlated with glucocorticoid inhibition of NF-κB transactivation. It is well known that glucocorticoids induce apoptosis in different cell types whereas cytokines rescue from it [19]. However, L-929 cells are at present one of the few models where glucocorticoids protect from TNF-induced apoptosis. In addition, this is one of the few cell lines which are naturally sensitive to TNF. If NF-κB could have an apoptotic role in this cell line, the glucocorticoid transrepression of this transcription factor would be a plausible model to explain its protective action on TNF-induced cell death. However, according to our results, glucocorticoid protection from TNF-induced apoptosis occurs at the same ratio at either normal or low levels of NF-κB induction. These observations suggest that although GR and NF-κB mutually antagonize, both trigger surviving signals in L-929 cells. Thus, glucocorticoids turn off NF-κB target genes, which protect from a major death but also induce the expression of GRE target protective genes.

Acknowledgements

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