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TNF-mediated IL6 gene expression and cytotoxicity are co-inducible in TNF-resistant L929 cells

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Interleukin (IL)-6 gene induction was studied in the murine cell line, L929r2, which is resistant to the cytotoxic action of tumor necrosis factor (TNF). Increasing concentrations of TNF slightly elevated the background levels of IL6 expression as compared to non-induced cells. Under conditions where the resistant cells are sensitive to TNF by combined TNF/IFN-γ treatment, the IL6 levels were strongly induced. This induction could be further enhanced by the addition of lithium chloride, or reduced by inhibitors of cytotoxicity, such as dexamethasone. These results confirm our earlier conclusions regarding the close relationship between TNF-mediated IL6 gene expression and the pathway leading to cytotoxicity.

Tumor necrosis factor; Cytotoxicity; Interleukin-6 induction

1. INTRODUCTION

Tumor necrosis factor (TNF) is a multifunctional cytokine which mediates a variety of biological effects both in vivo and in vitro [1]. One of the most striking effects is its cytostatic/cytotoxic effect on several transformed cell lines in vitro, while leaving most normal cells unaffected [2,3]. This cellular toxicity is a 'nucleus-independent' event, the result of several (not yet fully understood) cytoplasmic steps. On the other hand TNF also induces nuclear events. Several genes are either stimulated or repressed after TNF treatment [4]. One of the genes induced by TNF in many cell types is the gene coding for the secretory protein interleukin (IL)-6 [5,6].

Previously, we studied IL6 gene expression in response to TNF in the murine fibrosarcoma cell line L929, which is very sensitive to the cytotoxic action of TNF. We found that TNF could indeed stimulate IL6 gene expression; furthermore, we could also demonstrate a close relationship between IL6 gene induction and cytotoxicity [7].

In the present study, we investigate TNF-induced IL6 gene expression in the L929r2 cell line, a variant of L929s resistant to the cytotoxic action of TNF. Our present data demonstrate that IL6 gene expression is only weakly stimulated by TNF in this resistant cell line; however, when these cells are rendered sensitive by the combined action of TNF and interferon (IFN)-γ, the IL6 gene expression could again be strongly induced and was regulated in parallel with the cel-killing effect of TNF.

Abbreviations: DEX, dexamethasone; IFN, interferon; IL, interleukin; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; TNF, tumor necrosis factor.

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2. MATERIALS AND METHODS

2.1. *Cells and reagents*

The murine fibrosarcoma L929r2 cell line is a TNF-resistant subclone derived from the original, sensitive L929 line [8]. This clone does not produce TNF by itself. These adherent cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated newborn calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.03% L-glutamine. Recombinant murine TNF from *E. coli* was purified to 99% homogeneity and stored in phosphate-buffered saline at −70°C. It had a specific activity of 1.9×10^8 IU/mg protein and contained <13 ng/mg endotoxin. Recombinant murine IFN-γ from *E. coli* was purified to 99% homogeneity and stored in phosphate-buffered saline at −70°C; it had a specific activity of 8×10^6 U/mg. Dexamethasone (DEX) was purchased from Sigma Chemical Co., Poole, UK. LiCl was from Merck, Darmstadt, Germany.

2.2. *RNA manipulations*

Total cytoplasmic RNA was isolated from L929r2 cells essentially as described [9] using an NP-40 lysis buffer. Several phenol/chloroform extractions were carried out to remove the proteins from the lysate; the cytoplasmic RNA was recovered by ethanol precipitation.

2.3. *Dot-blot hybridization*

Appropriate amounts of the isolated RNA, denatured with formaldehyde, were applied onto a nylon filter (Pall Biodyne A; Pall BioSupport, East Hills, NY, USA) in the presence of 5× standard saline citrate (SSC) and immobilized by heat treatment (1 h at 80°C). Prehybridization (at least 1 h at 42°C) and hybridization (overnight at 42°C) were carried out in a mixture containing 5× Denhardt's solution, 5× SSC, 50 mM sodium phosphate (pH 6.5), 0.1% sodium dodecyl sulfate (SDS), 250 μg/ml non-homologous DNA and 50% formamide. The final washing steps were carried out with solutions containing 2× SSC/0.1% SDS (4× 5 min) and 0.1× SSC/0.1% SDS. The hybridization probe was prepared by digestion of the murine IL6 cDNA-containing plasmid pUC8MIL6 [10] with *Eco*R1 and *Bgl*II, and labeling of the

appropriate DNA fragment (655 bp) with α-^{32}P nucleotides using random primers (Boehringer, Mannheim, Germany).

2.4. *IL6 assay*

The amount of IL6 protein in the medium of the L929 cells was quantitated by their growth-stimulating capacity on 7TD1 cells [11,12].

3. RESULTS

3.1. *IL6 gene expression in L929r2 cells upon TNF treatment*

In previous studies, we have reported on TNF-induced IL6 expression in the murine fibrosarcoma cell line L929s, which is highly sensitive to TNF cytotoxicity. Moreover, we could show a close correlation between TNF-induced IL6 gene expression and cytotoxicity [7].

In the present study we investigated the effect of TNF treatment on IL6 gene expression in a TNF-resistant subclone of L929s, namely L929r2. Cells were grown to confluency as described and treated with TNF in the same way as before with L929s cells. The isolated RNA was spotted onto a nylon filter for dot-blot hybridization with a murine IL6 cDNA probe.

As shown in Figs. 1 and 2 there is a slight increase of the IL6 mRNA levels after TNF treatment as compared to the non-induced cells, demonstrating that the IL6 gene is only marginally inducible by TNF in this TNF-resistant cell variant. The values of the biological IL6 assay (Fig. 2), measuring the amount of IL6 protein secreted in the medium, lead to the same conclusion and confirm our results published previously [13].

3.2. *IL6 gene expression in L929r2 cells under conditions of recovered sensitivity to cytotoxicity*

Next, we investigated whether the synergism between TNF and IFN-γ, which renders these cells sensitive to TNF [3,8,14], has any influence on TNF-mediated gene induction.

In a first experiment we examined the effect of different IFN-γ concentrations. After the cells had been grown to confluency 10 U/ml or 1000 U/ml IFN-γ were applied to the cells, either alone or in combination with TNF (5,600 U/ml) for 21 h. After lysis and RNA extraction the different RNA samples were transferred onto a nylon membrane in appropriate amounts. Hybridization with a radioactive IL6 cDNA probe was performed as described. The result is shown in Fig. 1. IFN-γ by itself (in concentrations of either 10 U/ml or 1,000 U/ml) causes a slight increase in the background level of IL6 mRNA, similar to the effect seen with TNF alone. However, the combined treatment of TNF together with IFN-γ resulted in a very distinct stimulation of IL6 gene expression which, furthermore, was strongest at the highest concentration of IFN-γ.

In order to investigate the effect of increasing concentrations of TNF, we set up the experiment shown in Fig. 2. From the data obtained by dot-blot hybridization, as well as from the biological activity of the IL6 secreted in the growth medium, it is clear that the levels of IL6 mRNA were increased with increasing doses of TNF. However, an appreciable gene induction effect only occurred under conditions of a combined treatment of TNF with IFN-γ. Since the combined TNF/IFN-γ treatment rendered the L929r2 cells sensitive again to TNF cytotoxicity these data clearly indicate that efficient IL6 gene induction and TNF-mediated cytotoxicity are closely linked also in L929r2 cells. This close correlation confirms the results obtained in our previous studies with the sensitive cell type of this murine fibrosarcoma cell lineage.

3.3. *IL6 gene expression under conditions of stimulation and inhibition of cytotoxicity*

LiCl is known to be a strong enhancer of TNF cytotoxicity [15], while DEX reduces the sensitivity of the cells for TNF-induced cytotoxicity [16]. We next investigated the effect of these agents on IL6 induction in the L929r2 cell line. Confluent monolayers of L929r2 cells were treated with TNF/LiCl or TNF/DEX, with or without addition of IFN-γ. After extraction of cytoplasmic RNA, the samples were fixed on a nylon filter and hybridized with a radioactive IL6 cDNA probe. The results are shown in Fig. 3. Again, TNF treatment resulted in an elevated background level of IL6 mRNA, which could be further increased by co-treatment with LiCl; similar results were obtained previously at the protein level [13]. On the other hand, DEX completely reduced the elevated IL6 mRNA level. When IFN-γ was added the TNF-produced IL6 mRNA signal was strongly increased and could be further enhanced by the potentiating effect of LiCl. Also in this case, DEX, an inhibitor of cytotoxicity, was able to completely abolish the induced IL6 mRNA signal.

In brief, the regulation of the IL6 gene under conditions of enhanced or reduced cytotoxicity, as observed in the L929s cells, is also true for the L929r2 variant after it had been rendered sensitive to TNF by co-treatment with IFN-γ. However, when the cells were kept fully resistant to TNF only marginal amounts of IL6 induction could be visualized following induction with TNF.

4. DISCUSSION

IL6 is a multifunctional cytokine which is inducible by various low-molecular weight compounds or by cytokines, such as IL1 or TNF [17]. The induction of IL6 is not a general phenomenon but is dependent on the cell type used. We have already found that, as far as the murine L929 cell line is concerned (which is very sensitive to the cell killing effect of TNF) IL6 gene induction by TNF is closely related to TNF-mediated cytotoxicity. From this we concluded that the signaling path-

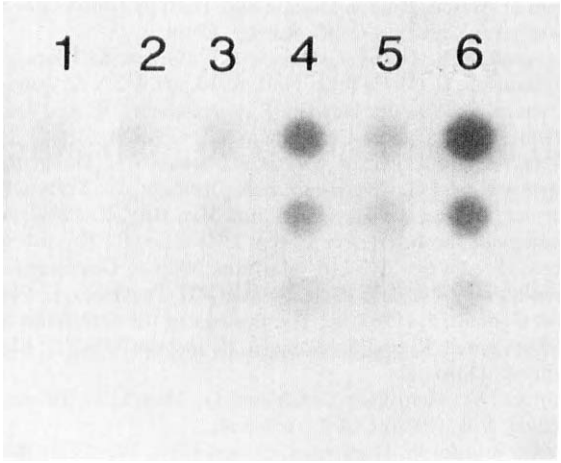

Fig. 1. Induction of IL6 mRNA with TNF and different concentrations of IFN-γ. Total cytoplasmic RNA was isolated from non-induced L929r2 cells (lane 1), or L929r2 cells treated for 21 h with TNF (5,600 U/ml; lane 2), or IFN-γ (10 U/ml; lane 3), IFN-γ (1,000 U/ml; lane 5), or with the combination of TNF (5,600 U/ml) and IFN-γ (10 U/ml; lane 4), or TNF (5,600 U/ml) and IFN-γ (1,000 U/ml; lane 6). 30 μg of the isolated cytoplasmic RNA from each experiment was serially diluted (3-fold dilution steps) and immobilized on Pall Biodyne A nylon membranes; hence, the consecutive lanes correspond to 20, 6.7, 2.2 and 0.7 μg, respectively. The filter was hybridized with a radioactive murine IL6 cDNA probe and, after several washing steps, exposed to an X-ray film.

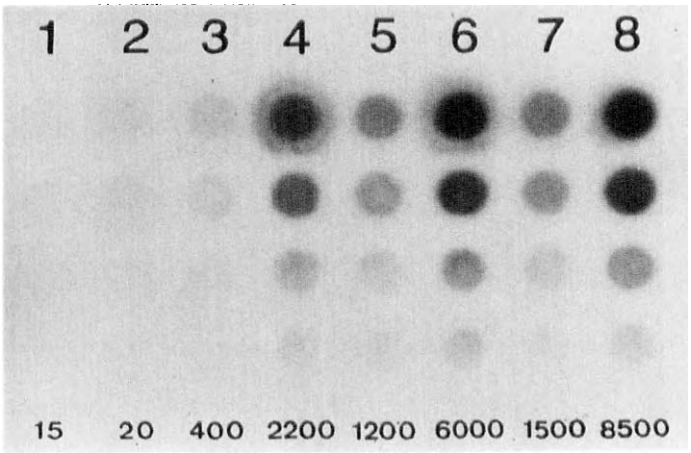

Fig. 2. Induction of IL6 mRNA with increasing concentrations of TNF. L929r2 cells were grown to confluency and treated for 24 h with 1,000 U/ml (lane 3), 5,600 U/ml (lane 5) or 12,500 U/ml (lane 7) of TNF, either alone or in combination with 1,000 U/ml IFN-γ (lanes 4, 6 and 8, respectively). 30 μg of total cytoplasmic RNA from untreated cells (lane 1), cells treated with 1,000 U/ml IFN-γ (lane 2), or from cells treated as indicated above, was isolated and used for dot-blot hybridization as described above. The values underneath represent the amount of IL6 protein in the medium, as measured in an IL6 bioassay.

way giving rise to IL6 gene induction in response to TNF, coincides, at least partially, with the reaction mechanisms taking place in the cytoplasm and finally leads to cell death [7].

Therefore, it was of particular interest to investigate how IL6 gene expression is affected in a related, TNF-resistant cell line. Because the L929r2 cell variant is derived from the L929 TNF-sensitive cell line studied previously we have chosen this resistant cell type for the present studies. When these L929r2 cells were treated with increasing concentrations of TNF, the IL6 expression levels were enhanced as compared to the background level in non-induced cells, showing that the acquired resistance mechanisms to TNF lysis had not completely abolished the signaling pathway for IL6 gene induction. However, when L929r2 cells were subjected to the combined treatment of TNF and IFN-γ, a treatment by which the resistant cells become sensitive again to the lytic action of TNF, the IL6 gene became strongly induced. Furthermore, and analogous to the effects observed with the original L929-sensitive cells, this induction of IL6 expression could still be further enhanced by the addition of LiCl, or, on the other hand, reduced by DEX treatment to a level below the one present in these cels treated with TNF but in the absence of IFN-γ.

The present data extend and further support our previous findings concerning the interconnection between IL6 gene induction and cytotoxicity in response to TNF. They suggest that the ongoing cytotoxicity reactions are largely identical to those leading to abundant IL6 expression.

The signaling pathway(s) going to the nucleus for gene induction is (are), as far as the L929 cell system is concerned, at least in part determined by cytoplasmic events. Nevertheless, the signaling system for IL6 gene expression is not completely blocked or overruled in the resistant state, and thus may represent a basal state of TNF-inducible (and regulatable) gene expression. Only when cytoplasmic reactions amenable to cell killing are operative does the IL6 gene expression become fully induced. In agreement with this conclusion is the observation that agents which increase the toxic effect of TNF in L929 cells, such as LiCl, in parallel enhance IL6 expression both in L929 and L929r2 cells, while compounds that restrict the killing effect of TNF also diminish IL6 expression level. It appears as if there exists, besides the pathway leading to basal TNF-mediated expression, another additional signaling system for superimposed, abundant gene expression, proceeding in parallel with and determined by the cytoplasmic reactions for cellular toxicity. This scheme of a dual signaling system for TNF-induced IL6 gene expression is now being further explored.

As already mentioned previously, NF-κB is an important factor for TNF-mediated gene induction. Although different studies have shown rapid activation of NF-κB after TNF treatment [18,19], and although this is also true for L929 (N. Patestos, G. Haegeman, V. Vandevoorde and W. Fiers, in preparation), it is not known whether NF-κB activation also plays a role in the onset of cellular toxicity, which is a much later

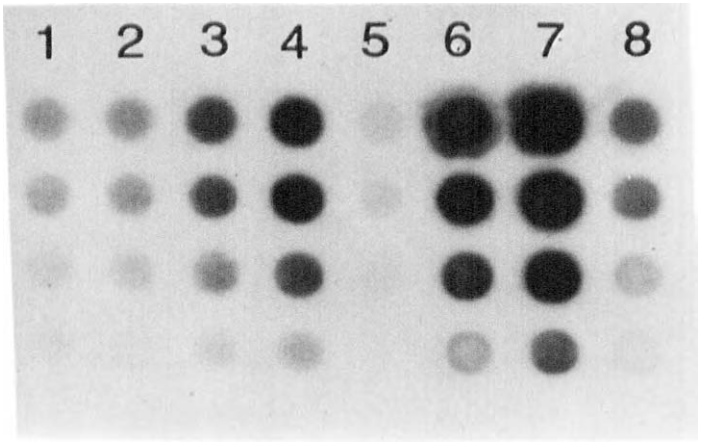

Fig. 3. Regulation of the IL6 mRNA level in the TNF-resistant and TNF-sensitive state of the L929r2 cells. L929r2 cells were grown to confluency and treated for 5 h with 1,000 U/ml IFN-γ (lane 2), 5,600 U/ml TNF (lane 3), 5,600 U/ml TNF and 10 mM LiCl (lane 4), 5,600 U/ml TNF and 0.1 mM DEX (lane 5), a combination of 5,600 U/ml TNF and 1,000 U/ml IFN-γ (lane 6), a combination of 5,600 U/ml TNF, 1,000 U/ml IFN-γ and 10 mM LiCl (lane 7), or a combination of 5,600 U/ml TNF, 1,000 U/ml IFN-γ and 0.1 mM DEX (lane 8). LiCl and IFN-γ were applied to the cells at the same time as TNF; DEX was added 2 h before. Total cytoplasmic RNA was isolated and 30 μg was taken for a dot-blot hybridization as described in the legend to Fig. 1. Lane 1 contains the RNA of non-induced cells.

event. Certainly there is good evidence that NF-κB is important for activation of the IL6 gene transcription [20–22], but it remains an open question whether the close relationship between IL6 expression and TNF-induced cytotoxicity can be explained by this single factor. This aspect is now under further investigation.

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REFERENCES

[1] Old, L.J. (1990) in: Tumor Necrosis Factor: Structure, Mechanism of Action, Role in Disease and Therapy (Bonavida, B. and Granger, G. eds) pp. 1–30, Karger, Basel.
[2] Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N. and Williamson, B. (1975) Proc. Natl. Acad. Sci. USA 72, 3666–3670.
[3] Fransen, L., Van der Heyden, J., Ruysschaert, R. and Fiers, W. (1986) Eur. J. Cancer Clin. Oncol. 22, 419–426.
[4] Fiers, W., Brouckaert, P., Devos, R., Fransen, L., Haegeman, G., Leroux-Roels, G., Marmenout, A., Remaut, E., Suffys, P., Tavernier, J., Van der Heyden, J. and Van Roy, F. (1987) in: The Biology of the Interferon System 1986 (Cantell, K. and Schellekens, H. eds) pp. 205–216, Martinus Nijhoff, Dordrecht.
[5] Defilippi, P., Poupart, P., Haegeman, G., Tavernier, J., Fiers, W. and Content, J. (1987) in: The Biology of the Interferon System 1986 (Cantell, K. and Schellekens, H. eds) pp. 217–222, Martinus Nijhoff, Dordrecht.
[6] Kohase, M., Henriksen-DeStefano, D., May, L.T., Vilček, J. and Sehgal, P.B. (1986) Cell 45, 659–666.
[7] Vandevoorde, V., Haegeman, G. and Fiers, W. (1991) Biochem. Biophys. Res. Commun. 178, 993–1001.
[8] Vanhaesebroeck, B., Van Bladel, S., Lenaerts, A., Suffys, P., Beyaert, R., Lucas, R., Van Roy, F. and Fiers, W. (1991) Cancer Res. 51, 2469–2477.
[9] White, B.A. and Bancroft, F.C. (1982) J. Biol. Chem. 257, 8569–8572.
[10] Van Snick, J., Cayphas, S., Szikora, J.-P., Renauld, J.-C., Van Roost, E., Boon, T. and Simpson, R.J. (1988) Eur. J. Immunol. 18, 193–197.
[11] Van Snick, J., Cayphas, S., Vink, A., Uyttenhove, C., Coulie, P.G., Rubira, M.R. and Simpson, R.J. (1986) Proc. Natl. Acad. Sci. USA 83, 9679–9683.
[12] Landegren, U. (1984) J. Immunol. Methods 67, 379–388.
[13] Beyaert, R., Schulze-Osthoff, K., Van Roy, F. and Fiers, W. (1991) Cytokine 3, 284–291.
[14] Williamson, B.D., Carswell, E.A., Rubin, B.Y., Prendergast, J.S. and Old, L.J. (1983) Proc. Natl. Acad. Sci. USA 80, 5397–5401.
[15] Beyaert, R., Vanhaesebroeck, B., Suffys, P., Van Roy, F. and Fiers, W. (1989) Proc. Natl. Acad. Sci. USA 86, 9494–9498.
[16] Suffys, P., Beyaert, R., Van Roy, F. and Fiers, W. (1987) Biochem. Biophys. Res. Commun. 149, 735–743.
[17] Billiau, A. (1987) Immunol. Today 8, 84–87.
[18] Osborn, L., Kunkel, S. and Nabel, G.J. (1989) Proc. Natl. Acad. Sci. USA 86, 2336–2340.
[19] Hohmann, H.-P., Remy, R., Pöschl, B. and Van Loon, A.P.G.M. (1990) J. Biol. Chem. 265, 15183–15188.
[20] Zhang, Y., Lin, J.-X. and Vilček, J. (1990) Mol. Cell Biol. 10, 3818–3823.
[21] Libermann, T.A. and Baltimore, D. (1990) Mol. Cell Biol. 10, 2327–2334.
[22] Shimizu, H., Mitomo, K., Watanabe, T., Okamoto, S. and Yamamoto, K. (1990) Mol. Cell Biol. 10, 561–568.