Autocrine Tumor Necrosis Factor (TNF) and Lymphotoxin (LT) α Differentially Modulate Cellular Sensitivity to TNF/LT-α Cytotoxicity in L929 Cells

Els Decoster, Sigrid Cornelis, Bart Vanhaesebroeck, and Walter Fiers

Department of Molecular Biology, Flanders Interuniversity Institute for Biotechnology and University of Ghent, B-9000 Ghent, Belgium

Abstract. Tumor necrosis factor (TNF) and lymphotoxin (LT) α are structurally and functionally related cytokines. We expressed the TNF and LT-α genes in murine fibrosarcoma L929r2 cells, which can be sensitized to TNF/LT-α–dependent necrosis by inhibitors of transcription or translation. Autocrine production of murine TNF in L929r2 cells completely downmodulated the expression of the 55- and 75-kD TNF receptors, resulting in resistance to TNF/LT-α cytotoxicity. Partial downmodulation of the 55-kD receptor was observed in human TNF-producing L929r2 cells. In contrast, an unaltered TNF receptor expression was found on LT-α L929r2 transfectants. Hence, although similar cytotoxic effects are induced by extracellularly administered TNF and LT-α, endogenous expression of these cytokines fundamentally differs in the way they modulate TNF receptor expression. Unlike LT-α, secreted by the classical pathway, TNF is first formed as a membrane-bound protein, which is responsible for receptor downmodulation. To explore whether the different pathways for secretion of TNF and LT-α explain this difference, we examined the effect of membrane-bound LT-α expression. This was obtained by exchange of the classical signal sequence of LT-α for the membrane anchor of chicken hepatic lectin. Membrane retention of LT-α resulted indeed in receptor downmodulation and TNF/LT-α resistance. We conclude that membrane retention of newly synthesized TNF or LT-α is absolutely required for receptor downmodulation and TNF/LT-α resistance.

Key words: tumor necrosis factor • lymphotoxin • cytotoxicity • downmodulation • membrane

TUMOR necrosis factor (TNF)[1] and lymphotoxin (LT) α are related cytokines with 30% amino acid sequence identity (Fiers, 1992; Beyaert and Fiers, 1998). The corresponding genes are closely linked within the class III region of the major histocompatibility complex (Nedospasov et al., 1986; Spies et al., 1986). Both genes appear to be independently regulated (Sung et al., 1988; English et al., 1991). In general, TNF and LT-α exert similar biological activities, although both qualitative and quantitative differences have been found. LT-α seems less potent than TNF in proinflammatory activities, such as activation of the nuclear factor κB (Chaturvedi et al., 1994). TNF and LT-α regulate the production of interleukin (IL) 6 and macrophage colony-stimulating factor in fibroblast cells differently (Akashi et al., 1989; Mantovani et al., 1990). In contrast to TNF, LT-α acts as a growth factor for some B cell lines transformed by Epstein-Barr virus (Estrov et al., 1993). It was also reported that TNF but not LT-α could directly induce lysis of trypanosomes (Lucas et al., 1994). Macrophages are the major cellular source of TNF (Matthews et al., 1982), but several other cell types have been reported to produce TNF as well, including lymphocytes, fibroblasts, neutrophils, endothelial cells, and several tumor cell lines (Vilček and Lee, 1991). LT-α production seems to be restricted to T- and B-lymphocytes (Paul and Ruddle, 1988). Remarkably, TNF and LT-α exert their biological activities through binding to the same set of ubiquitously expressed cell surface receptors. Two types have been characterized, namely TNF-R55 and TNF-R75, named according to their relative molecular masses of 55 and 75 kD, respectively. Murine TNF (mTNF) and murine

Address correspondence to W. Fiers, Department of Molecular Biology, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium. Tel.: 32 9 264 51 31. Fax: 32 9 264 53 48.

Dr. Vanhaesebroeck's present address is Ludwig Institute for Cancer Research, London W1P 8BT, UK.

1. *Abbreviations used in this paper:* ActD, actinomycin D; CHL, chicken hepatic lectin; CHX, cycloheximide; hLT, human lymphotoxin; hTNF, human TNF; IL, interleukin; LT, lymphotoxin; mIFN, murine interferon; mTNF, murine TNF; neoʳ, neomycin-resistant; TNF, tumor necrosis factor; TNF-R55, 55-kD TNF receptor; TNF-R75, 75-kD TNF receptor.

LT-α bind both human TNF (hTNF)-R55 and hTNF-R75. In contrast, hTNF and human LT (hLT)-α bind mTNF-R55 but not mTNF-R75 (Tartaglia et al., 1992). Specific TNF-R55 triggering is known to be responsible for most of the wide variety of TNF and LT-α biological effects (Engelmann et al., 1990; Espevik et al., 1990; Tartaglia et al., 1993*b*; Fiers, 1995). The role of TNF-R75 as a direct signal transducer has so far mainly been documented in T-lymphocytes (Tartaglia et al., 1991; Vandenabeele et al., 1992; Tartaglia et al., 1993*a*).

TNF and LT-α are differently processed during biosynthesis. TNF is initially synthesized as a biologically active, 26-kD type-II transmembrane proform consisting of a pre-sequence, which functions as a membrane anchor, and the extracellular 17-kD TNF (Müller et al., 1986; Kriegler et al., 1988). Trimerization occurs already at the proform stage (Tang et al., 1996) and the 26-kD proform subunits are then cleaved proteolytically at the cell surface by TNF convertase, releasing mature, trimeric TNF (Black et al., 1997; Moss et al., 1997). There are two different genes in the LT system, encoding LT-α and LT-β. Secreted LT-α is presumably formed in the lumen of the endoplasmic reticulum, where nascent LT-α polypeptides form homotrimers, which then progress through the secretory pathway (Androlewicz et al., 1992). Surface-bound LT occurs as a heterotrimeric complex consisting predominantly of one LT-α monomer and two membrane-bound LT-β molecules, the latter serving as a membrane anchor (Browning et al., 1995). LT-α/LT-β complex formation presumably takes place early during biosynthesis via binding of a secretory LT-α subunit to membrane-anchored LT-β (Androlewicz et al., 1992). This heteromeric LT-$\alpha_1\beta_2$ complex does not bind to TNF-R55 or TNF-R75, but specifically interacts with a TNF receptor-related protein, the LT-β receptor (Crowe et al., 1994). Signaling via the LT-β receptor can induce cell death of some human adenocarcinomas (Browning et al., 1996). It can also activate the NF-κB transcription factor in some, but not all, LT-β receptor-positive cells (Mackay et al., 1996). This LT-α/LT-β ligand/receptor pair is especially important in lymphoid organogenesis. Mice lacking LT-α or LT-β do not develop peripheral lymph nodes, splenic germinal centers and Peyer's patches (De Togni et al., 1994; Koni et al., 1997).

Previously, we demonstrated that autocrine TNF production induced by transfection of an exogenous TNF gene rendered TNF-sensitive murine L929s fibrosarcoma cells resistant to TNF/LT-α–mediated cell lysis. In contrast, transfection of the hLT-α gene did not result in stable LT-α–producing transfectants (Vanhaesebroeck et al., 1992). In this paper, we focus on the question why the closely related LT-α can apparently not be produced by cells that are sensitive to the cytotoxic action of TNF/LT. This is not due to the inability of L929 cells to express LT-α, since transfection with a genomic hLT-α/hTNF construct resulted in stable production of both hTNF and hLT-α (Vanhaesebroeck et al., 1992). Therefore, the biological activities of autocrine LT-α production were investigated in more detail, using different types of TNF-resistant derivatives of L929s cells. Our results demonstrate that pre-existing cellular TNF resistance is essential to allow stable production of secreted LT-α. This autocrine LT-α had no effect on the phenotype of TNF resistance of the produc-

ing cells, and also there was no TNF receptor downmodulation. Next, we investigated whether these observations could be explained by the fact that LT-α is not membrane-bound in the course of its synthesis. Therefore, we examined the effect of expression of a chimera, in which the classical signal sequence of LT-α was exchanged for the membrane anchor structure of chicken hepatic lectin (CHL), a type-II trimeric protein. Expression of this CHL.hLT-α now induced receptor downmodulation and TNF/LT-α resistance. Thus, we conclude that the introduction of a membrane-anchoring step during the biosynthesis of LT-α is sufficient and necessary to mediate an effect similar to that obtained with autocrine-produced TNF.

Materials and Methods

Cell Lines and Cell Culture

Derivatives of the murine L929s fibrosarcoma cell line were used. L929sA, a TNF-sensitive subclone derived by limiting dilution, has a very low background of spontaneous TNF-resistant cells ($<4 \times 10^{-6}$ vs. 2×10^{-2} for parental L929s cells; Vanhaesebroeck et al., 1992). The TNF-resistant L929r1.1 and L929r2 derivatives of L929s were obtained by TNF selection on L929s cells (Vanhaesebroeck et al., 1991). All L929 cell lines were cultured in DME (Life Technologies, Paisley, UK), supplemented with 5% newborn calf serum (Life Technologies), 5% FCS (Life Technologies), and antibiotics. The murine WEHI 164 cl 13 fibrosarcoma cell line (Espevik and Nissen-Meyer, 1986) was cultured in RPMI 1640, supplemented with 10% FCS and antibiotics. All cell lines were repeatedly found to be *Mycoplasma*-free as judged from a DNA fluorochrome assay.

Cytokines and Antisera

Purified *E. coli*-derived recombinant mTNF, hTNF, and hLT-α were prepared in our laboratory and had a specific activity of 2×10^8, 8.4×10^7, and 3.1×10^7 IU/mg protein, respectively. International standards for TNF quantification were obtained from the National Institute for Biological Standards and Control (Potters Bar, UK). Polyclonal rabbit antiserum directed against mTNF, hTNF, or hLT-α was provided by Mr. J. Van der Heyden (Roche Research, Ghent, Belgium). Polyclonal rabbit antisera directed against mTNF-R55 and mTNF-R75 were a gift of Dr. W.A. Buurman (University of Limburg, Maastricht, The Netherlands).

Determination of TNF and LT-α Bioactivity in Cell Culture Supernatant

Supernatant of transfected cell lines was 100-fold concentrated using Centriprep-10 and Centricon-10 micro-separation devices (Amicon, Danvers, MA). TNF or LT-α were quantified in an 18-h cytotoxicity assay using WEHI 164 cl 13 cells in the presence of 1 μg/ml actinomycin D (ActD; Espevik and Nissen-Meyer, 1986). The detection limit of this assay was ∼1 pg TNF or LT-α/ml. Neutralization was performed with rabbit polyclonal antiserum specific for mTNF, hTNF, or hLT-α.

Plasmid Constructions

The CHL.hTNF and CHL.hLT-α fusion genes containing the membrane anchor structure of CHL (a gift from Dr. K. Drickamer, Columbia University, New York, NY), followed by mature hTNF and hLT-α, respectively, were constructed by fusion PCR (Olsen, 1992) using appropriate oligonucleotide primers. The PCR products were inserted as an EcoRI-Not fragment into an EcoRI-Not-opened pSV-SPORT1 vector (Life Technologies), which contains an E-tag COOH terminally from the inserted PCR fragments. The chimeric gene sequences were verified by DNA sequencing.

Expression Vectors and Transfection

The pSV23S vector, driving the expression of a gene of interest by the constitutive SV-40 early promoter, was used in all experiments (Huylebroeck et al., 1988). Derived expression vectors were pSV23S-mTNF

(Fransen et al., 1985), pSV23S-hTNF (Müller et al., 1986), and pSV23S-hLT-α (Vanhaesebroeck et al., 1992). The pSV2neo plasmid encoding the neomycin resistance (neo^r) gene under control of the SV-40 early promoter was used as a selection marker (Southern and Berg, 1982). Plasmid DNA was purified using pZ523 columns (5 Prime → 3 Prime, Boulder, CO). Stable transfection was performed by an improved DNA calcium phosphate coprecipitation protocol as described (Vanhaesebroeck et al., 1992).

Determination of Cellular Sensitivity to TNF or LT-α

TNF/LT-α sensitivity was determined as described (Vanhaesebroeck et al., 1991). In brief, cells were seeded in 96-well plates in 0.1 ml of medium at 2×10^4 or 2,500 cells/well for 18- and 72-h assays, respectively. 12–18 h later, serial dilutions of TNF or LT-α, alone or in combination with either ActD (final concentration of 1 μg/ml), cycloheximide (CHX; final concentration of 25 μg/ml), or murine interferon (mIFN)-γ (200 IU/ml final concentration) were added in 0.1 ml of medium. After an 18-h (for ActD and CHX) or 72-h (for mIFN-γ) incubation period, the surviving cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and assayed colorimetrically (Tada et al., 1986). The percentage of surviving cells incubated in the presence of ActD, CHX, or mIFN-γ alone was never lower than 70% (vs. 100% for cells incubated with culture medium alone).

Flow Fluorocytometry

The presence of mTNF-R55 and mTNF-R75 was determined by incubation of cells with polyclonal rabbit antiserum against mTNF-R55 or mTNF-R75 (1 μg antiserum per 4×10^5 cells in 200 μl), followed by incubation with biotinylated donkey anti–rabbit IgG polyclonal antiserum (Amersham Life Science, Amersham, UK), and treatment with phycoerythrin-conjugated streptavidin. All incubations were performed for 1 h at 4°C. Analysis was achieved by flow fluorocytometry using a Coulter Epics 753 equipped with an argon-ion laser (Coulter, Hialeah, FL). Acidic treatment to remove soluble TNF or LT-α from the cell surface receptors was done by incubation of the cells for 3 min at room temperature with glycine-HCl buffer (50 mM, containing 150 mM NaCl, pH 3.0).

The presence of membrane-bound hTNF or hLT-α was analyzed using monoclonal mouse antibody directed against the E-tag fused COOH terminally to mature hTNF or hLT-α (see Fig. 1; 1 μg antibody/4×10^5/200 μl; Pharmacia Biotech, Uppsala, Sweden).

TNF-Binding Assay

TNF was iodinated by using Iodo-Gen (Pierce Chemical Co., Rockford, IL) to a specific activity of 600 Ci/mmol. Binding studies were performed on L929r2 cells grown in suspension (Vanhaesebroeck et al., 1991). Cells were harvested and either untreated or incubated for 5 min at 4°C with glycine-HCl buffer to remove putative receptor-bound, endogenously produced TNF or hLT-α. Then they were incubated for 4 h at 4°C with a saturating concentration (2 nM) of ^{125}I-hTNF in 200 μl binding buffer (DME, supplemented with 10% FCS and 0.01% NaN$_3$). Cells were pelleted through a naphthalate solution containing a mixture of 1/3 dinonyl phthalate and 2/3 dibutyl phthalate; the retained radioactivity was determined in a γ-counter. Specific binding of ^{125}I-hTNF was determined by subtracting the amount of radioactivity bound to the cells in the presence of a 500-fold excess of unlabeled TNF from the radioactivity in the presence of ^{125}I-hTNF alone. Aspecific binding of ^{125}I-hTNF was about 15% of the total binding.

Results

Expression of hLT-α in L929 Derivatives with Different TNF-resistant Phenotype

Previously, we demonstrated that in contrast to TNF, transfection of LT-α in TNF/LT-α–sensitive L929sA cells does not result in stable LT-α–producing cells (Vanhaesebroeck et al., 1992). However, transfection of genomic hLT-α/hTNF in L929s cells led to the production of both hTNF and hLT-α, excluding the possibility that this cell
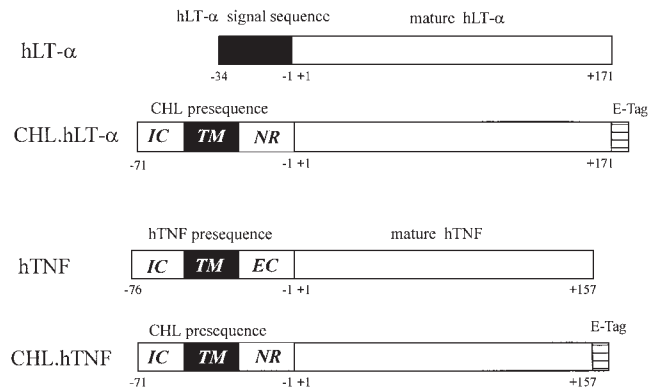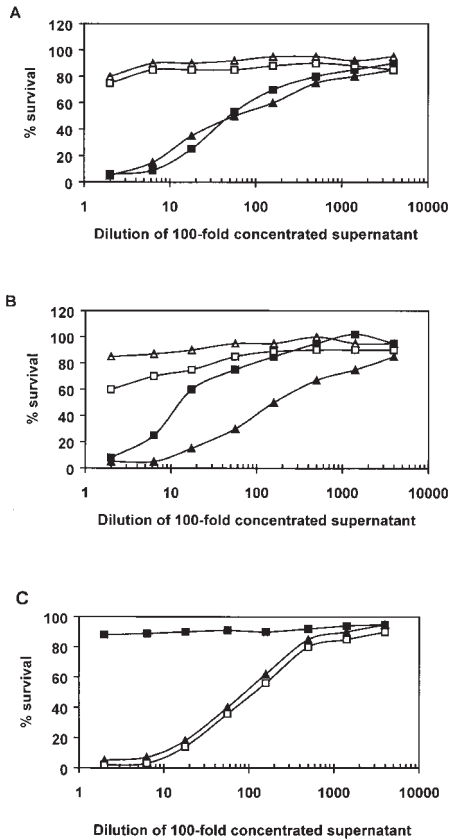

Figure 1. Overview of chimeric constructs. *IC*, intracellular domain; *TM*, transmembrane domain; *EC*, extracellular domain; *NR*, neck region.

type is intrinsically unable to produce functional LT-α (Vanhaesebroeck et al., 1992). To determine whether co-expression of TNF or, more likely, TNF/LT-α resistance is necessary to efficiently express LT-α, we transfected hLT-α cDNA in TNF-resistant L929s derivatives. L929r1 cells constitutively produce low levels of TNF and are completely TNF-resistant, whereas non-TNF-producing L929r2 cells can be sensitized to TNF cytotoxicity by inhibition of transcription or translation with ActD or CHX, respectively (Vanhaesebroeck et al., 1991). After transfection of hLT-α cDNA in combination with the neo^r gene in L929r1 and in L929r2 cells, G418-resistant colonies were obtained (Table I), which were tested for the production of mTNF and hLT-α. In L929r1 cells, the cytotoxic activity in the supernatants of cells transfected with neo^r alone could be completely neutralized by antiserum against mTNF (Fig. 2 *A*), whereas antiserum against hLT-α was also required to completely block the cytotoxic activity of supernatants of clones obtained after cotransfection with the hLT-α gene (Fig. 2 *B*). The cytotoxic activity in supernatants of L929r2 transfectants was completely neutralized by antiserum directed against hLT-α (Fig. 2 *C*). These results indicate that both types of TNF-resistant cells can produce hLT-α. Clearly, efficient hLT-α production does not require TNF gene expression; a preexisting TNF/LT-α resistance is sufficient for L929 cells to become LT-α producers after transfection of the LT-α gene.

Table I. Number of L929 Colonies Surviving G418 Selection after Transfection with TNF or LT-α Genes

Cell line	Genes transfected	Number of G418-resistant colonies/2×10^5 transfected cells
L929r1.1	neo^r	115 ± 15 (100)*
	neo^r + hLT-α	108 ± 20 (94)
L929r2	neo^r	100 ± 20 (100)
	neo^r + mTNF	125 ± 25 (125)
	neo^r + hTNF	55 ± 15 (55)
	neo^r + hLT-α	80 ± 10 (80)

*Expressed as mean ± SD of triplicate culture flasks of four representative experiments. The number of surviving colonies, expressed as percentage of the number of colonies obtained after transfection with the neo^r gene alone, is shown in brackets.

A
(graph: % survival vs Dilution of 100-fold concentrated supernatant)

B
(graph: % survival vs Dilution of 100-fold concentrated supernatant)

C
(graph: % survival vs Dilution of 100-fold concentrated supernatant)

Figure 2. Cytotoxic activity present in the supernatant of L929r1 cells transfected with neo[r] alone (*A*), or with neo[r] and hLT-α (*B*), as well as in L929r2 cells transfected with neo[r] and hLT-α (*C*). Cytotoxicity was tested in the absence (▲) or presence of antiserum to hLT-α (■), mTNF (□), or hLT-α and mTNF (△).

Autocrine LT-α Production Does Not Alter the TNF-resistant Phenotype

We further analyzed the effect of autocrine production of TNF and/or LT-α on TNF/LT-α sensitivity. As shown in Table I, transfection of TNF or hLT-α cDNA did not result in counterselection, neither in L929r1 nor in L929r2 cells. The TNF/LT-α–resistant phenotype of the L929r1

Table III. Cytokine Production in the Supernatant of L929r2 Transfectants

L929r2 cells transfected with	Amount of mTNF/hTNF/hLT-α produced after treatment for 18 h with		
	—	1 µg/ml ActD	25 µg/ml CHX
neo[r]	0*	0	0
neo[r] + mTNF	3,797	2,400 (63%)	84 (2%)
neo[r] + hTNF	2,400	1,410 (58%)	96 (4%)
neo[r] + hLT-α	1,700	854 (50%)	84 (5%)

*IU/ml found in 100-fold concentrated supernatant of L929r2 transfectants. The supernatants were harvested, concentrated and tested in a cytotoxicity assay on WEHI 164 cl 13 cells (percentages in brackets indicate cytokine production as compared to the amount produced by untreated cells).

cell line was not influenced by autocrine hLT-α production (data not shown). Expression of mTNF levels (even minimal ones) in L929r2 cells conferred complete resistance to TNF/LT-α cytotoxicity in the presence of ActD, CHX, or mIFN-γ (Table II; data for CHX and mIFN-γ are not shown). Expression of hTNF induced only partial TNF/LT-α resistance (Table II), with a level of resistance proportional to the amount of hTNF produced in the supernatant, a phenomenon also observed in hTNF L929sA transfectants (Vanhaesebroeck et al., 1992). In contrast, no alteration in TNF/LT-α susceptibility was observed after LT-α production, not even by high LT-α production levels (Table II). So it is clear that the absence of TNF/LT-α resistance in LT-α–producing cells is not due to lower cytokine expression levels as compared with TNF-producing cells. This different effect of autocrine TNF vs. LT-α cannot be explained by a differential sensitivity of production of these cytokines to a treatment with metabolic blockers (Table III). ActD or CHX treatment for 18 h was found to have a similar effect on LT-α and TNF secretion in L929r2 transfectants. ActD treatment (at a concentration used in standard cytotoxicity assays) resulted in a twofold drop in cytokine expression levels, whereas treatment with CHX reduced the cytokine production levels by a factor of 20–50 (Table III). These observations show that autocrine LT-α expression, in contrast to TNF, is not capable of modulating the TNF-resistant/susceptible phenotype of L929r2 cells.

Table II. TNF/LT-α Sensitivity of L929r2 Cells Transfected with neo[r] Alone, or Combined with TNF or LT-α Genes

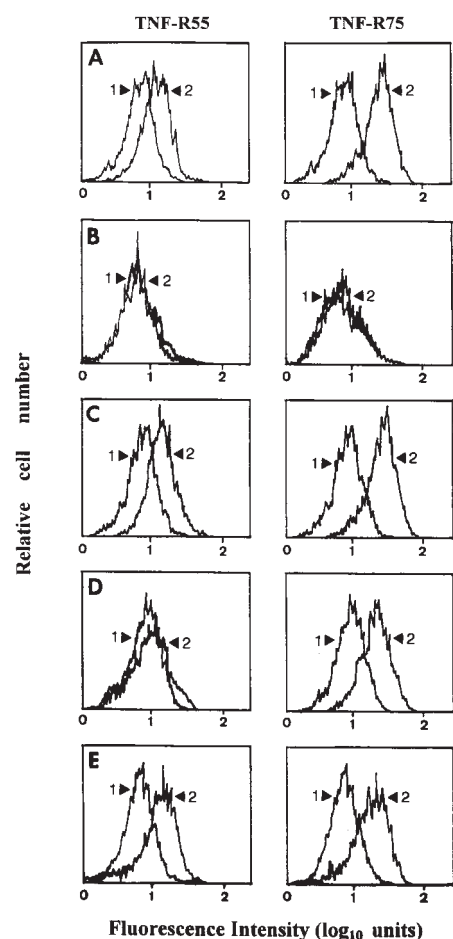| Genes transfected	Cells analyzed	TNF or LT-α in supernatant	Sensitivity to[‡]					
			mTNF	hTNF (72-h assay)	hLT-α	mTNF	hTNF (18-h assay + ActD)	hLT-α
		*pg/ml**						
neo[r]	Clone B1	0	>40,000[§]	>40,000	>40,000	18	54	54
	Clone B2	0	>40,000	>40,000	>40,000	54	164	164
neo[r] + mTNF	Clone C1	170	>40,000	>40,000	>40,000	>40,000	>40,000	>40,000
	Clone C2	3,700	>40,000	>40,000	>40,000	>40,000	>40,000	>40,000
neo[r] + hTNF	Clone D1	94	>40,000	>40,000	>40,000	493	4,444	4,444
	Clone D2	4,700	>40,000	>40,000	>40,000	20,000	>40,000	>40,000
neo[r] + hLT-α	Clone E1	300	>40,000	>40,000	>40,000	18	54	54
	Clone E2	1,700	>40,000	>40,000	>40,000	54	265	240

*Cytokine production found in 100-fold concentrated supernatant.
‡Expressed in U/ml TNF or LT-α needed to obtain 50% cytotoxicity.
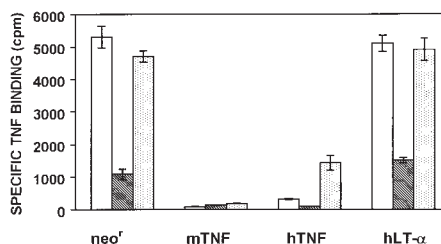§>40,000 means that 50% killing was not observed using the indicated TNF/LT-α concentration.

Effect of TNF or LT-α Transfection on TNF-R55 and TNF-R75 Expression Levels

Previously, we demonstrated a correlation between the induction of TNF resistance and the downmodulation of TNF receptors (Vanhaesebroeck et al., 1992). TNF-R55 and TNF-R75 expression in the different L929r2 transfectants was first measured by flow fluorocytometric analysis. In the fully resistant mTNF-producing L929r2 transfectants, no TNF-R55 or TNF-R75 expression could be detected (Fig. 3 B). On hTNF L929r2 transfectants, a downmodulation of TNF-R55 but not of TNF-R75 was observed (Fig. 3, C–D). This downmodulation was proportional to the amount of hTNF production (Fig. 3 C, low producer; D, high producer). In contrast, hLT-α–producing L929r2 cells showed no downmodulation of TNF-R55 or TNF-R75 (Fig. 3 E). Despite the apparent absence of detectable TNF-R55 on some hTNF transfectants, TNF could still induce cytotoxicity, which is mediated via TNF-R55 in L929r2 cells (Vercammen et al., 1995). This suggests that low numbers of functional TNF-R55 must be

Figure 4. Binding of ^{125}I-hTNF by representative colonies of L929r2 transfectants. Transfected genes are shown below. Cells were either untreated (*white bars*) or treated with acidic glycine-HCl buffer (*black bars*), or with glycine-HCl buffer followed by addition of fresh ^{125}I-hTNF for 4 h at 4°C (*gray bars*).

Figure 3. Flow cytometric analysis of TNF-R55 and TNF-R75 expression on L929r2 cells transfected with neo^r alone (*A*) or neo^r combined with genes encoding mTNF (*B*), hTNF (*C*, low producer; *D*, high producer) or hLT-α (*E*). Cells were treated either with secondary antibody alone (curve 1) or with antiserum directed against TNF-R55 or TNF-R75 and secondary antibody (curve 2).

present on the hTNF-producing cells. To detect these and to confirm the data obtained with flow cytometric analysis on the expression levels of TNF-R55, we examined the presence of specific binding sites for ^{125}I-hTNF (TNF-R55) on mTNF-, hTNF-, or hLT-α–producing L929r2 transfectants. Specific hTNF binding could be detected on neo^r transfectants. This binding disappeared after treatment with glycine-HCl at pH 3 (Fig. 4), indicating that a low pH leads to the dissociation of TNF from its receptor. Pretreatment with glycine-HCl had no effect on subsequent hTNF binding, demonstrating that acid pretreatment did not remove the TNF-R55 molecules from the plasma membrane or had any adverse side effects. No specific ^{125}I-hTNF binding could be detected on mTNF transfectants, even not after pretreatment with glycine-HCl (Fig. 4). This is in agreement with the absence of receptor molecules found on mTNF-producing L929sA cells (Vanhaesebroeck et al., 1992). L929r2 transfectants producing high amounts of hTNF showed considerably reduced hTNF binding, which completely disappeared by glycine-HCl treatment (Fig. 4). Pretreatment of hTNF transfectants with glycine-HCl could increase ^{125}I-hTNF binding (Fig. 4), indicating that a fraction of the TNF-R55 molecules on the cell surface was occupied by secreted hTNF. It may be noted that ^{125}I-hTNF binding on hLT-α transfectants was completely similar to that of neo^r transfectants (Fig. 4), indicating that autocrine hLT-α production did not downmodulate TNF-R55 on the plasma membrane of L929r2 cells. In conclusion, induction of unresponsiveness to TNF/LT-α–mediated cytotoxicity is correlated with downmodulation of the TNF/LT-α receptor on the cell surface. Moreover, in contrast to mTNF and hTNF, hLT-α is not able to mediate such TNF-R55 downmodulation.

Introduction of a Membrane-anchoring Step in the Biosynthesis Pathway of hLT-α Induces TNF/LT-α Resistance and Downmodulation of TNF/LT-α Receptor

Since membrane retention of TNF is crucial for downmodulation of TNF receptors and induction of TNF/LT-α resistance (Decoster et al., 1998), the differential effect of autocrine-produced TNF as compared with LT-α can be explained by a different pathway of producing mature TNF and LT-α. Hence, it is possible that if LT-α would be produced as a membrane-bound form like TNF, it would

A

B

C

Fluorescense Intensity (log)
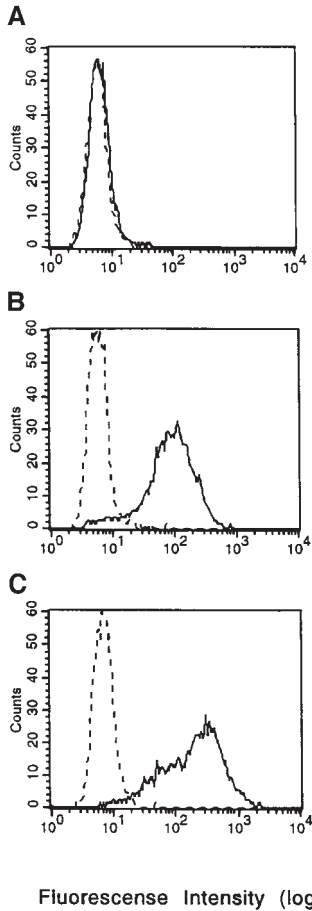
Figure 5. Flow cytometric analysis of expression of membrane-bound fusion protein on L929r2 transfectants. Cells were transfected with neo^r alone (*A*), or with neo^r combined with genes encoding CHL.hTNF (*B*) or CHL.hLT-α (*C*). Cells were treated with secondary antibody alone (*dotted line*) or with antibody directed against COOH-terminal E-tag and secondary antibody (*solid line*).

Table IV. TNF/LT-α Sensitivity of L929r2 Transfectants

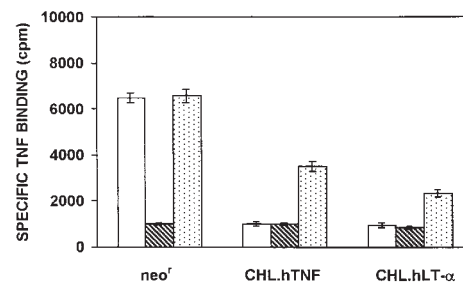| Genes transfected	Cells analyzed	Sensitivity to*		
		mTNF	hTNF	hLT-α
neo^r	Clone B1	18	54	54
	Clone B2	6	18	18
neo^r + CHL.hTNF	Clone E1	1,481	4,444	5,000
	Clone E2	13,333	>40,000§	>40,000
neo^r + CHL.hLT-α	Clone F1	4,444	13,333	13,333
	Clone F2	20,000	>40,000	>40,000

*Expressed in U/ml TNF or LT-α needed to obtain 50% cytotoxicity (assay in combination with 1 μg/ml ActD for 18 h).
‡>40,000 means that 50% killing was not observed using the indicated TNF/LT-α concentration.

effect of autocrine LT-α production and is comparable with the effect of autocrine-produced hTNF (Table II). Since induction of TNF/LT-α resistance is correlated with downmodulation of TNF receptors on the cell surface, we examined the expression level of receptors on the plasma membrane of L929r2 transfectants expressing CHL.hTNF or CHL.hLT-α fusion protein. These transfectants showed considerably reduced binding of ^{125}I-hTNF (TNF-R55) compared with neo^r transfectants (Fig. 6). Pretreatment with glycine-HCl buffer could increase ^{125}I-hTNF binding (Fig. 6), indicating that a fraction of the TNF-R55 molecules on the cell surface was occupied by secreted hTNF or hLT-α. This phenomenon was also observed in hTNF-producing L929r2 cells (Fig. 4). As expected, no downregulation of TNF-R75, determined via flow cytometric analysis, could be revealed on CHL.hTNF or CHL.hLT-α transfectants (data not shown). Taken together, we showed that membrane retention of hLT-α is crucial and sufficient for the induction of TNF/LT-α resistance and downmodulation of TNF-R55 on the cell surface. Furthermore, these results strongly indicate that the differential effect of autocrine-produced TNF and LT-α on TNF/LT-α sensitivity and downmodulation of TNF/LT-α receptors can be explained by their different way of processing for secretion.

Discussion

Previously, we have demonstrated that after transfection in TNF/LT-α–sensitive L929s cells, TNF production induces resistance to the cytotoxic effect of both autocrine

also confer receptor downmodulation and TNF/LT-α resistance. To test this hypothesis, we exchanged the classical signal sequence of LT-α for the membrane anchor structure of CHL (Mellow et al., 1988); by choosing the latter instead of the TNF presequence we avoided any possible specific functional contribution of the TNF presequence. CHL is a trimeric, type II transmembrane liver glycoprotein (Chiacchia et al., 1984; Steer et al., 1990), which contains a membrane anchor allowing trimerization of the extracellular domain. Transfection of L929r2 cells with the neo^r gene, combined with the CHL.hLT-α chimeric gene (Fig. 1) or with the CHL.hTNF chimeric gene (used as a control), yielded normal numbers of G418-resistant colonies (data not shown). L929r2 cells transfected with CHL.hLT-α secreted between 1.25 and 30 IU hLT-α/ml; in the culture supernatant of CHL.hTNF transfectants between 1.5 and 14 IU/ml hTNF were detected. Expression of membrane-bound fusion protein could be revealed via flow fluorocytometric analysis in CHL.hLT-α as well as in CHL.hTNF transfectants (Fig. 5). These results clearly demonstrate that expression of either the CHL.hTNF or the CHL.hLT-α chimeric gene gives rise to production of a membrane-bound and secreted form comparable to the biosynthesis of wild-type TNF. We further analyzed the effect of autocrine-produced CHL.hLT-α or CHL.hTNF on TNF/LT-α sensitivity. As shown in Table IV, expression of these chimeric proteins induced unresponsiveness to the cytotoxic effect of TNF or hLT-α. This is in contrast to the

Figure 6. Binding of ^{125}I-hTNF by representative colonies of L929r2 transfectants. Transfected genes are shown below. Cells were either untreated (*open bars*), or treated with acidic glycine-HCl buffer (*hatched bars*), or with glycine-HCl buffer followed by addition of fresh ^{125}I-hTNF for 4 h at 4°C (*stippled bars*).

and exogenous TNF (Vanhaesebroeck et al., 1992). In contrast, transfection with the hLT-α cDNA gene did not allow to isolate clones that produced hLT-α or had become TNF/LT-α resistant. Normally, LT-α expression is restricted to lymphoid cell types (Paul and Ruddle, 1988). In the case of human tumor cell lines only those of lymphoid origin have been demonstrated to express and secrete LT-α after stimulation, whereas TNF can be produced by tumor cell lines of both lymphoid and non-lymphoid origin (Krönke et al., 1987). In this report, we demonstrate that nonlymphoid L929 murine fibrosarcoma cells can produce LT-α, but only when these cells are resistant to TNF/LT-α cytotoxicity. Remarkable differences were found between the effects of autocrine TNF and LT-α in TNF-resistant L929r2 cells. The latter cells can be sensitized to the cytotoxic action of TNF/LT-α by addition of ActD, CHX or mIFN-γ. But when tested under these conditions, autocrine mTNF production prevented the L929r2 cells to become sensitive. Autocrine hTNF production resulted in a partial resistance to TNF and LT-α under these conditions. In contrast, even high levels of autocrine hLT-α production did not alter the TNF/LT-α–resistant/sensitive phenotype after addition of ActD, CHX or mIFN-γ. Since we demonstrated a correlation between induction of resistance and downmodulation of cell surface receptors after TNF production in L929s cells (Vanhaesebroeck et al., 1992; Decoster et al., 1998), we investigated whether TNF and LT-α differ in their capacity to downmodulate these receptors on the cell surface. As documented previously for L929s cells, both receptor types were completely downmodulated on L929r2 cells after autocrine mTNF production. hTNF and hLT-α L929r2 transfectants showed an unaltered expression level of TNF-R75 on their cell surface. This is in agreement with data indicating that hTNF and hLT-α only interact with mTNF-R55 (Lewis et al., 1991). Whereas hTNF transfectants showed considerable downmodulation of TNF-R55, autocrine hLT-α production had no effect on TNF-R55 expression levels. A possible lower binding affinity of hLT-α for mTNF-R55 might explain the inability of hLT-α to downregulate TNF-R55. However, this is very unlikely, since hLT-α seems to compete better than hTNF with ^{125}I-hTNF for binding on L929 cells (Browning and Ribolini, 1989). Moreover, hLT-α shows even a higher binding affinity than hTNF for binding on L929 cells (Hass et al., 1985; Vanhaesebroeck et al., 1992). A more likely hypothesis is that the different biosynthesis pathways of TNF and LT-α are at the basis of our observation. TNF is initially synthesized as a transmembrane TNF proform, which is then proteolytically cleaved at the cell surface, releasing the secreted, trimeric TNF form. In contrast, LT-α polypeptides are secreted in the lumen of the endoplasmic reticulum concomitantly with cleavage of the signal sequence. Membrane retention of TNF was shown to be crucial for the induction of TNF/LT-α resistance and downmodulation of the cell-surface TNF receptor (Decoster et al., 1998). Moreover, we had already demonstrated previously that a functional interaction between TNF and TNF receptor is required to that end; expression of a biological inactive mutein with strongly reduced TNF receptor-binding capacity could indeed not induce TNF receptor downmodulation nor TNF resistance (Vanhaesebroeck et al., 1992).

To confirm the hypothesis that membrane retention of hLT-α is sufficient to downmodulate TNF/LT-α receptor expression and to induce resistance to the cytotoxic effect of exogenous TNF or LT-α, we analyzed the effect of expression of a CHL.hLT-α fusion gene. Like TNF, CHL is a type-II, trimeric transmembrane protein and the CHL membrane anchor allows trimerization of the extracellular domains. Expression of this CHL.hLT-α gene gave rise to both a membrane-bound and a secreted form of hLT-α, a feature characteristic of TNF synthesis. CHL.hLT-α transfectants exhibited TNF/LT-α resistance and showed downmodulation of TNF-R55, but not of TNF-R75. This is consistent with the fact that hLT-α can only interact with mTNF-R55 and not with mTNF-R75 (Lewis et al., 1991). The data obtained with CHL.hLT-α are in contrast with the results for hLT-α, expressed as a secreted protein, and are analogous to the results obtained with hTNF. Thus the inability of LT-α to induce TNF/LT-α resistance and downmodulation of the TNF/LT-α receptor is unambiguously explained by the absence of membrane retention of LT-α during processing for secretion.

It is remarkable that the mammalian genome codes for two closely related cytokines, i.e., TNF and LT-α, which have different biological functions and which are synthesized and processed by a different mechanism. The results here described illuminate some implications of this difference. First, our data suggest a possible dual biological role of membrane-bound TNF. The latter, produced by different cell types, such as macrophages, some T cells and some tumor cells, leads to primarily local inflammatory and/or immune reactions; but it is also involved in downmodulation of the TNF receptor, hence avoiding that TNF-producing cells become a target of their own product (Fiers, 1993). Second, the different effect observed for autocrine-produced TNF and LT-α might possibly explain why some cell types, such as HL-60, produce both TNF and LT-α; indeed, autocrine TNF downregulates the TNF/LT-α receptors and avoids counterselection. Third, tumor cells expressing TNF may have been selected to do so in order to avoid negative effects by TNF (or LT-α) released as an inflammatory/immune response of the host. Fourth, our observations might also partially explain why LT-α is mainly produced by cells of lymphoid origin, since such cells either also express TNF or are devoid of TNF-R55 receptors (Fiers et al., 1986; Beyaert and Fiers, 1998). Finally, it is quite possible that the production of LT-α during development corresponds mainly to formation of membrane-bound LT-α/LT-β complexes.

In summary, the observation that in contrast to LT-α, TNF expression protects L929 against TNF/LT-α–mediated cytotoxicity, is explained by the different pathway of processing of TNF and LT-α for secretion. Production of TNF or, as shown in this paper, of hLT-α as a membrane-bound form allows the producer cells to downmodulate TNF-R55 (and TNF-R75, depending on the ligand), such that they become unresponsive to their own product. This essential role of membrane anchoring is further supported by the fact that production of secreted TNF after substitution of the presequence for the signal sequence of IL-6 did not induce TNF resistance and downmodulation of the TNF receptors on the cell surface (Decoster et al., 1998), while membrane anchoring of LT-α in a similar construct

as TNF did result in receptor downmodulation and resistance.

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