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Original Paper

Tumour Necrosis Factor and Interferon are Selectively Cytostatic *In Vitro* for Hormone-dependent and Hormone-independent Human Breast Cancer Cells

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Since experimental studies have shown that tumour necrosis factor-α (TNF-α) has potent antitumour activity that can be potentiated with cytokines, we tested the efficacy of TNF-α with interferon-γ (IFN-γ) on different human breast cancer cell lines, particularly comparing hormone-dependent and -independent phenotypes. TNF-α inhibited the growth of hormone-dependent human MCF-7, ZR-75-1 and T47-D breast cancer cells with a half maximal concentration of 0.25 nM. In contrast, the growth of hormone-independent cells MDA-MB-231 and HS578T was not affected by TNF-α alone, but a synergistic inhibition was observed when using IFN-γ and TNF-α together. The mRNA for the proto-oncogene *C-MYC*, as an intracellular indicator of cell activation, was significantly increased in MCF-7 cells in the presence of TNF-α. In MDA-MB-231 cells this mRNA was increased only in the presence of both TNF-α and IFN-γ, without a change in the number of surface TNF receptors. These findings indicate that TNF-α treatment in combination with IFN-γ may provide a successful approach to overcome the cellular heterogeneity of advanced breast tumours.

Key words: breast cancer, hormone-dependency, tumour necrosis factor, interferon gamma

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INTRODUCTION

RECOMBINANT TUMOUR necrosis factor-α (TNF-α) has potent antitumour activity in experimental studies on immortalised tumour cell lines and on human tumour xenografts [1–5]]. Based on these observations, it was anticipated that TNF-α might be a therapeutic reagent for patients with metastatic human breast cancer (HBC). In humans, however, the administration of TNF-α induces severe systemic side-effects [6]. The maximum tolerated dose ranges from 350 to 500 mg/m^2, which is at least 10-fold less than the efficient dose in animals. In addition, TNF-α as a single agent has been relatively ineffective in the treatment of several other human malignancies [7, 8]. To overcome these difficulties, several partially successful new attempts to use TNF-α have been made during the last few years in preliminary trials in patients with different malignancies. Patients with advanced solid tumours of various types have been treated by intratumoral administration of recombinant human TNF-α [9]. Such isolated perfusions have allowed the delivery of high doses of TNF-α in a closed system with acceptable toxicity [10]. Furthermore, the effect of low doses of TNF-α can be enhanced by simultaneous administration of chemotherapeutic agents [11, 12].

In the past, several approaches were chosen to enhance the tumour necrotic activity of TNF-α in *in vitro* systems. For example, it has been reported that interferon-γ (IFN-γ) can potentiate the cytostatic/cytotoxic effects of TNF-α. IFNs may increase TNF-α binding in some cells that are sensitive to their synergistic effects by increasing the TNF receptor numbers on the cell surface [13–16]. In contrast, some findings indicate that this effect might not be the only mechanism of enhancing the cytotoxic activity of TNF-α in tumour cells [17]. Thus, the mechanisms of action of such cytokines and their synergism with each other are not yet fully understood. Several recently initiated clinical trials with TNF-α and different cytokines will help to clarify the

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clinical usefulness of the highly potent TNF-α as a cytotoxic agent [18].

Like most tumours, HBC develops with time and through different stages of malignancy. Non-metastatic human breast tumours are initially oestrogen-dependent and respond to several growth factors with accelerated growth. HBC progression results in an oestrogen-independent, growth factor non-responsive phenotype with metastatic potential. The hormone-dependent and hormone-independent cell types may be differently affected by the respective anticancer agents. In this report, we tested the efficacy of TNF-α in combination with IFN-γ on different phenotypes of immortalised HBC cells *in vitro*.

MATERIALS AND METHODS

Cell culture conditions

The mammary carcinoma cell lines MCF-7, T47-D, ZR-75-1 and MDA-MB-231 were obtained from Mason Research Institute, Rockville, Maryland, U.S.A. and the cell line HS578T from the American Type Culture Collection, Rockville, Maryland, U.S.A. Cells were grown in IMEM-ZO as described previously [19], supplemented with L-glutamine (2 mM), insulin (5 μg/ml), Hepes at pH 7.3 (10 mM), and 5% (vol/vol) fetal calf serum at 37°C in a humidified atmosphere containing 5% CO_2. In some experiments, cells were grown under serum-free conditions according to Küng and associates [20].

Growth experiments

A micro assay was used to study growth of cells in 96 well plates. The cells were fixed after 5–6 days in culture as previously described [21]. The absorbance (OD) of the fixed and crystal violet stained cells was measured at 590 nm in quadruplicates in an automated ELISA reader (Molecular Devices, Menlo Park, California, U.S.A.). The reagents used were purchased at Sigma (Human IFN-γ, specific activity: 1×10^6 units/mg protein), Boehringer Mannheim, Germany (human recombinant TNF-α, specific activity 1×10^8 units/mg protein) and Collaborative Biomedical Products, Bedford, Massachusetts, U.S.A. (mouse EGF).

Northern blots

Total RNA was extracted by the guanidinium thiocyanate method. Five to eight micrograms of total RNA were loaded per lane on to a 1.1% agarose gel. The RNA was transferred to Nylon membranes (GeneScreen Plus, NEN, Boston, Massachusetts, U.S.A.). Equal loading and blotting of RNA samples was verified by staining the filters with methylene blue. After fixing, the membranes were hybridised with ^{32}P-*C-MYC* DNA probe (1–5 $\times 10^6$ cpm/ml) overnight. Autoradiography was performed for 3–5 days at −70°C.

Determination of TNF receptor contents

Cells were grown in serum-free medium in the presence or absence of the reagents indicated in the text. The cells were harvested with a rubber policeman and washed twice at 4°C in serum-free medium. The cell suspension (10^6 cells/50 μl) was incubated in serum-free medium for 45 min on ice with 20 μl specific anti-TNF receptor MAb (100 μg/ml, clone utr1 for the p75 receptor and clone htr9 for the p55 receptor) [22]. Cells were washed three times as described above and resuspended in 50 μl serum-free medium. Four microlitres of FITC-labelled goat antimouse immunoglobulin was added to the suspension (Becton Dickinson, Mountain View, California, U.S.A.) and incubated in the dark for 45 min at 4°, washed twice and subsequently analysed on the flow cytometer (FACScan, Becton Dickinson) for bound FITC-fluorescence. A total of 10000 cells were analysed for each sample. Non-specific bound fluorescence was assessed with irrelevant monoclonal antibody which was specific for the cytoplasmic domain of the EGF epidermal growth factor) receptor (Ab-4, Oncogen Science, Uniondale, New York, U.S.A.) and was found to be less than 10% of total binding. Specific cell-bound FITC-fluorescence was calculated by subtracting fluorescence of cells incubated with the control antibody from the fluorescence obtained from the samples. Receptor numbers per cell were calculated by using standard beads that bind defined amounts of monoclonal antibodies (Quantum Simply Cellular microbeads, Flow Cytometry Standards, Triangle Park, North California, U.S.A.). Five sets of beads binding standardised amounts of monoclonal antibodies were incubated with either the monoclonal antibody for the p75 or the p55 TNF receptor using the same conditions as described above for the cells. After washing, the beads were incubated with the second FITC-labelled goat antimouse immunoglobulin as described above, washed again and analysed for bound fluorescence. Under the conditions used, all antibody-binding sites on the beads were assumed to be occupied by the anti-TNF receptor monoclonals. Therefore, a fluorescence standard curve could be generated using the different defined numbers of antibody sites on the five different beads. Making the assumption that one monoclonal antibody will bind to one TNF surface receptor, allowed receptor numbers on cells to be calculated by comparing bound fluorescence per cell to the standard curve generated by the beads.

Statistics

Comparison between groups was performed by a one way analysis of variance (ANOVA) using the INSTAT software (GraphPad, San Diego, California, U.S.A.). The Mann–Whitney test was used to compare differences in quantitative variables within two groups. The mean and standard errors (SE) were calculated from three to four determinations. All tests were two-tailed.

RESULTS

TNF-α inhibited several hormone-dependent HBC cell lines, whereas hormone-independent HBC cells were not affected by 1 nM TNF-α after 6 days in culture (Table 1). The growth of MCF-7, ZR-75-1 and T47-D cells was inhibited by 42, 48 and 34%, respectively. As reported, growth of these cells was significantly stimulated by EGF (10^{-8} M) [23]. In contrast, MDA-MB-231 cells and HS578T cells were neither susceptible to growth inhibition by TNF-α nor stimulated by EGF as already reported previously [23]. To investigate further the mechanisms involved in the selective inhibition by different TNF-α concentrations, two standard cell lines either from the hormone-dependent (MCF-7) or from the hormone-independent cell types (MDA-MB-231) were used. TNF-α inhibited dose-dependently the growth of non-stimulated MCF-7 cells over

Table 1. *Growth of different cell lines* in the presence of TNF-α and IFN-γ*

Cell line	Oestrogen receptor	TNF-α	IFN-γ	TNF-α + IFNγ
MDA-MB-231	–	102 ± 8†	100 ± 13†	80 ± 15‡
HS578T	–	105 ± 3†	98 ± 5†	78 ± 3‡
MCF-7	+	58 ± 3‡	136 ± 14‡	52 ± 4‡
ZR-75-1	+	52 ± 5‡	109 ± 17†	45 ± 2‡
T47-D	+	76 ± 9‡	113 ± 4†	80 ±5‡

*Cells were grown in the presence or absence of the reagents indicated for 6 days in a microassay. The concentrations used were 10^{-9} M TNF-α and 1000 U/ml IFN-γ. The data are calculated as percentage of staining of the respective cells grown in the absence of reagents. Data are expressed as the mean ± SE of at least three independent determinations. †Not significantly different from control levels ($P > 0.05$). ‡Statistically different from control levels ($P < 0.001$).

6 days under serum-free culture conditions with a half-maximal inhibition at a TNF-α concentration of 0.25 nM (Figure 1a) The differences were statistically significant ($P < 0.001$). The growth of MDA-MB-231 cells was not affected in the 6 days of the experiment. The differences between the curves was in all cases not significant ($P > 0.3$) (Figure 1b). Not only was unstimulated growth of hormone-dependent HBC cells inhibited by TNF-α, but the growth inhibitory effect of TNF-α was also evident in MCF-7 cells, which were stimulated either with IGF-I (10^{-8} M), EGF (10^{-8} M) or oestrogen (10^{-9} M) (Figure 2). The growth stimulatory effects of all three factors tested (EGF, IGF-I and oestrogen) were inhibited dose-dependently by TNF-α with a similar IC_{50} as for non-stimulated MCF-7 cells. Differences between the curves were significant ($P = 0.043$).

Synergistic effect of TNF-α and IFN-γ

The inhibitory effect of TNF-α was not significantly increased in hormone-dependent HBC cells (MCF-7, ZR-75-1 and T47-D) by the simultaneous addition of IFN-γ (Table 1). In contrast, the two tested hormone-independent HBC cell lines (MDA-MB-231 and HS578T) were significantly growth-inhibited in the presence of 1000 U/ml IFN-γ and 1 nM TNF-α (Table 1). Time courses revealed that hormone-dependent MCF-7 cells were significantly stimulated by IFN-γ and inhibited by TNF-α after 1–2 days in culture (Figure 3a). In contrast, MDA-MB-231 cells were neither inhibited by TNF-α nor stimulated by IFN-γ alone, but the combination of TNF-α and IFN-γ resulted in a significant inhibition of cell growth after 3 days (Figure 3b) ($P < 0.0001$ between cells treated with a combination of TNF-α and IFN-γ and all others). Lower concentrations of IFN-γ and TNF-α than shown in Figure 3 did not affect the growth of hormone-independent HBC cells (data not shown).

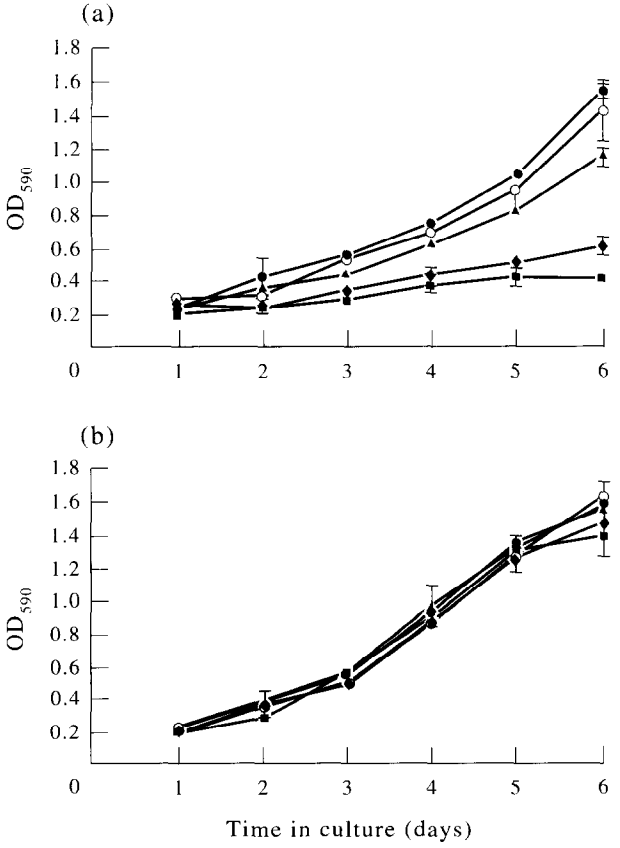

Figure 1. Growth inhibition by TNF-α in (a) MCF-7 and (b) MDA-MB-231 cells: (○) control cells; (●) 0.01 nM TNF-α; (▲) 0.1 nM TNF-α; (◆) 1 nM TNF-α; (■) 10 nM TNF-α. Data are expressed as the mean ± SE of at least three independent measurements.

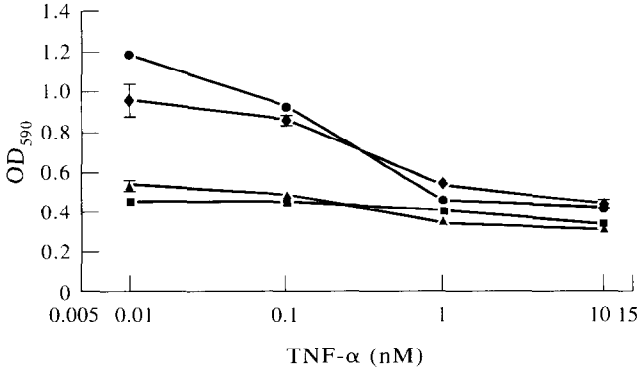

Figure 2. Inhibition of growth factor-stimulated MCF-7 cells by TNF-α. Cells were grown in 96 well plates in serum-free medium in the presence of 10^{-8} M EGF (▲), 10^{-8} M IGF-I (●) or 10^{-9} M oestrogen (◆). Non-stimulated cells (■) served as a control. Data are expressed as the mean ± SE of at least three measurements.

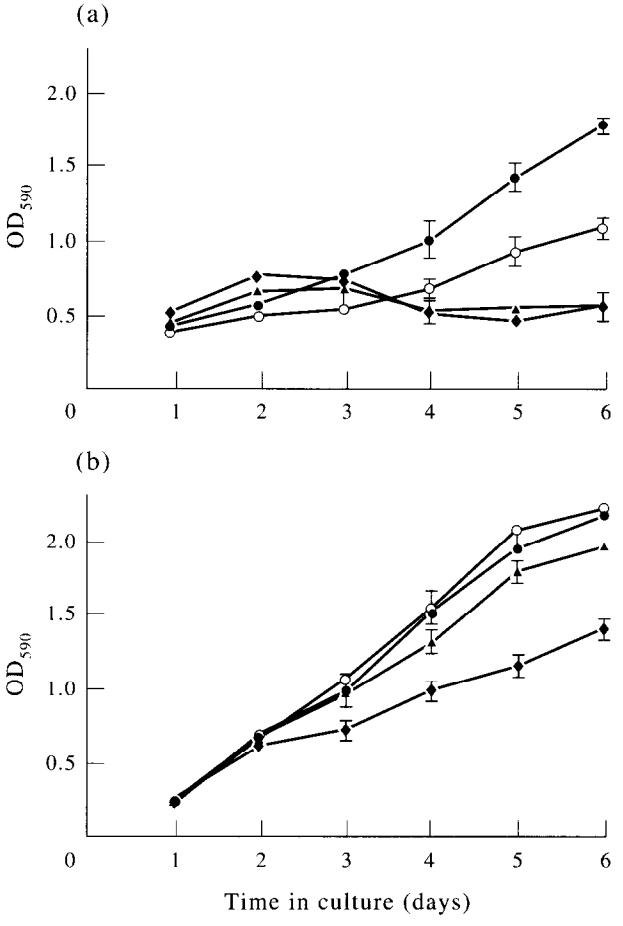

Figure 3. Inhibitory effect of TNF-α and IFN-γ in (a) MCF-7 and (b) MDA-MB-231 cells: 1000 U/ml IFN-γ (●); 10^{-9} M TNF-α (▲); and 10^{-9} M TNF-α + 1000 U/ml IFN-γ (◆). Cells incubated without the addition of reagents served as a control (○). Data are expressed as the mean ± SE of at least four determinations.

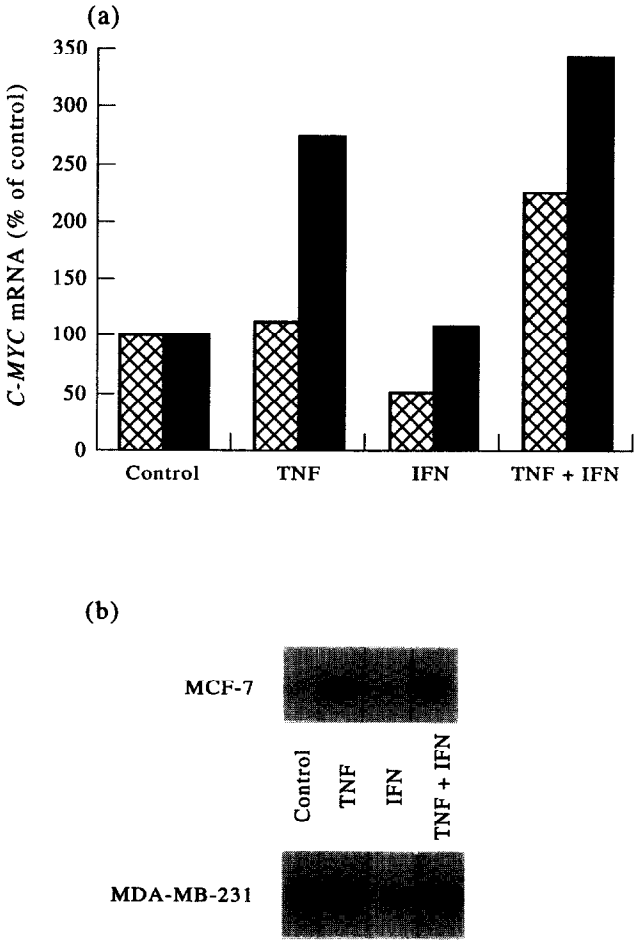

Figure 4. Expression levels of *C-MYC*. (a) Densitometry results from autoradiography: MCF-7 cells and MDA-MB-231 cells (MCF-7 cells (■); MDA-MB-231 cells (▩)). (b) Equal loading and blotting of RNA was verified by staining the blot with methylene blue. The values are expressed as percentage of control. The experiment shown is representative of three independent experiments.

Stimulation of C-MYC transcription

As an intracellular indication of the response of HBC cells to growth factors (e.g. EGF) and cytokines (TNF-α, IFN-γ), the induction of the mRNA for the immediate early gene *C-MYC* was determined (Figure 4). TNF-α increased the amount of *C-MYC* message in hormone-dependent MCF-7 cells. IFN-γ alone had only limited effects on the induction of *C-MYC* message in MCF-7 cells. Combinations of IFN-γ and TNF-α enhanced *C-MYC* expression synergistically. In contrast, *C-MYC* mRNA expression levels were increased only in the presence of both IFN-γ and TNF-α in hormone-independent MDA-MB-231 cells, while IFN-γ alone inhibited its expression. This clearly demonstrates that, only in hormone-independent cells, can the response to TNF-α be enhanced by IFN-γ. Similar results were obtained for these experiments when IFN-α was substituted for IFN-γ (data not shown).

TNF surface receptor numbers

Previously, it has been suggested that the higher sensitivity to TNF-α of IFN-treated cells is correlated with an increased number of TNF surface receptors [13]. Thus, we measured TNF receptors on two representative cell lines and flow cytometric analysis of 10000 cells for each cell type revealed that the TNF-α-sensitive MCF-7 cells responded to all reagents with an up or downregulation of both TNF surface receptors (Figure 5a), whereas the TNF receptors of the non-responsive MDA-MB-231 cells were not significantly modulated by either TNF-α and IFN-γ alone (Figure 5b). IFN-γ in combination with TNF-α down-regulated the p75 receptors in MDA-MB-231 cells. Thus, an upregulation of the TNF surface receptor densities in the presence of IFN-γ is not a major mechanism in the increased sensitivity to TNF-α of MDA-MB-231 cells. The hormone-independent MDA-MB-231 cells expressed 10-fold more TNF receptors than the hormone-dependent MCF-7 cells (Table 2). However, higher levels of TNF

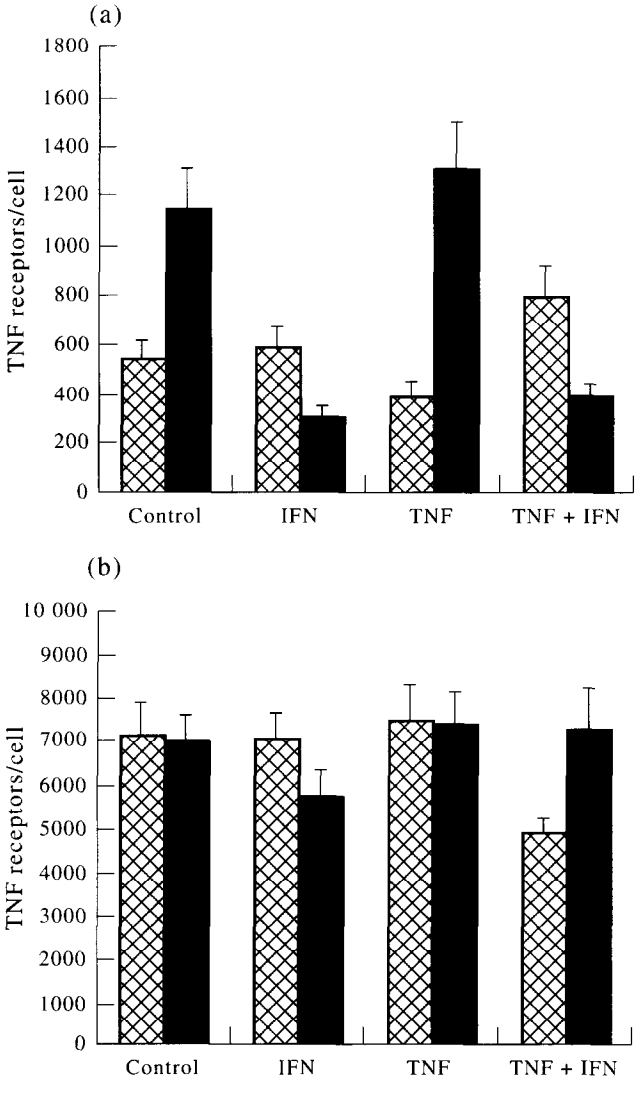

Figure 5. TNF receptors on (a) MCF-7 and (b) MDA-MB-231 cells (p75 receptor (▩); p55 receptor (■)). Data are expressed as the mean of the calculations of the TNF receptors per cell of 3–4 different samples ± SE. The differences between the p75 receptor numbers were highly significant between control and TNF-α-treated ($P = 0.0289$) and between control and IFN-γ + TNF-α-treated ($P = 0.0005$) MCF-7 cells, but not significant for IFN-γ-treated and control cells ($P = 0.5215$). Differences from control values were only significant for MDA-MB-231 cells treated with a combination of both reagents ($P = 0.006$). p55 values were highly significantly different between all groups in MCF-7 cells ($P < 0.0001$), but not significantly different in MDA-MB-231 cells ($P > 0.5$). Only IFN-γ decreased the surface receptor concentration on MDA-MB-231 cells significantly ($P = 0.016$).

receptor expression were not correlated with insensitivity to TNF-α. The hormone-dependent and TNF-α-sensitive T47-D cells expressed a TNF receptor density comparable to the TNF-α-insensitive cell lines (Table 2).

DISCUSSION

Based on the observation that the cell number did not decrease in the presence of TNF-α (Figures 1–3), we showed that TNF-α has a dose- and time-dependent antimitogenic effect on all the HBC cells tested without the cytotoxicity seen in other transformed and normal mammalian cells [24]. The half-maximal antimitogenic effect of TNF-α on MCF-7 cells was 0.25 nM and well within the range observed by others [24]. The inhibitory effect of TNF-α on cell growth was observed only on hormone-dependent MCF-7, ZR-75-1 and T47-D cells whereas the hormone-independent MDA-MB-231 and HS578T cells were TNF-α resistant (Table 1).

According to previous findings, TNF-α inhibits only a minority (approximately 30%) of tumour cell lines. However, it has been reported that interferons act synergistically with TNF-α by exerting an antitumour effect on a number of cell lines, such as hormone-dependent HBC cells (T47-D, ZR-75-1, SKBR-III and MCF-7) [1, 25]. In our study, a combination of TNF-α and IFN-γ was antimitogenic for both types of cells, although the hormone-independent cells were significantly less inhibited than the hormone-dependent cells (Table 1). Hormone-independent MDA-MB-231 and HS578T cells were only inhibited 20 and 22%, respectively, by a combination of both agents. These effects of TNF-α and IFN-γ also correlated well with the transcription of the early gene *C-MYC*. TNF-α has been shown to stimulate the transcription of *C-MYC* in several cell lines [26]. In hormone-independent HBC cells, however, TNF-α increased the message for *C-MYC* only in the presence of IFN-γ (Figure 4). This is consistent with the observation that only both IFN-γ and TNF-α had an impact on the cellular behaviour of hormone-independent HBC cells. Furthermore, the observation that the growth inhibitory reagents stimulated the expression of *C-MYC* in both cell types confirm observations that modulation of *C-MYC* expression does not reflect the growth behaviour of HBC cells [27]. Messages for other early genes, such as *C-FOS* and *C-JUN*, which have been demonstrated to be activated by TNF-α [28], were not measured since MDA-MB-231 cells do not express a significant increase in the amounts of these two mRNA levels upon stimulation (Mueller and colleagues, unpublished observations).

To date, many attempts have been made to understand the mechanisms underlying the increased sensitivity of cells towards TNF-α in the presence of IFN-γ [13–16]. Some reports have shown that the expression of the surface receptors for TNF-α can be enhanced by pretreating the cells with IFN-γ. The IFN-γ-induced increase of TNF receptor numbers was observed on different cell types regardless of their resulting sensitivity to TNF-α, suggesting that the increase in the number of TNF surface receptors is not sufficient to account for the enhanced cytostatic/cytotoxic response [17]. Since the p55 TNF receptor is reported to be responsible for the cytostatic/cytotoxic effects, we examined the two TNF receptors separately [29, 30]. However, on MDA-MB-231 cells, we could not demonstrate a significant change in the number of either the p55 or the p75 TNF surface receptor upon IFN-γ treatment (Figure 5). Thus, a change in the numbers of TNF surface receptor upon IFN-γ treatment seems not to be involved in the increased sensitivity for TNF-α in hormone-independent HBC cells.

Due to the fact that most tumours such as HBC become heterogeneous in their cellular composition during tumour progression, a different response of these cell populations to

Table 2. TNF-receptor numbers on hormone-dependent and hormone-independent cell lines*

Cell line	ER	TNF sensitivity	TNF receptors/cell p75	p55	Total
MDA-MB-231	–	–	7105 ± 1477	6995 ± 566	14100 ± 693
HS578T	–	–	5735 ± 1293	5803 ± 85	11538 ± 856
MCF-7	+	+	556 ± 55	1140 ± 157	1696 ± 198
ZR-75-1	+	+	4015 ± 510	2647 ± 485	6662 ± 1234
T47-D	+	+	6746 ± 707	6197 ± 669	12943 ± 985

*TNF receptors were determined for each type separately using monoclonal antibodies specific for the two receptor types. TNF-α sensitivities were deduced from Table 1. Receptor numbers were calculated by using standard beads containing defined amounts of bound fluorescence. Data are expressed as the mean ± SE of at least three independent determinations of 10 000 cells each. OR, oestrogen receptor. p75 and p55, p75 and p55 TNF receptors, respectively.

factors such as growth inhibitory cytokines should be assumed. Since, in our study, TNF-α only inhibited the growth of hormone-dependent cells, without having any effect on hormone-independent cells (Table 1), this hypothesis seems to be valid. Our findings could indicate that *in vivo* therapy with TNF-α alone could enhance the selection for rapidly proliferating hormone-independent cells, pushing tumour progression towards a hormone-independent, more malignant and potentially metastatic state. With such a treatment, the probability of a cure would be inversely correlated to the degree of clonal dominance by hormone-independent cell variants in the primary tumour. Therefore, treatment with TNF-α alone would only be beneficial for patients with tumours consisting exclusively of oestrogen-dependent (ER-positive) cells. Consequently, as a treatment for patients with breast cancer, at least both TNF-α and IFN-γ would have to be involved.

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