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Relationship between cell kinetics and apoptotic effects in TNF-alpha and IFN-gamma-treated human tumour cell lines

Abstract

TNF-alpha is a potent inducer of apoptosis and also affects the transit of cells through the phases of the cell cycle. It is thought that the proliferation signalling pathway is related to the apoptosis pathway, the details of this cross-talk are not yet fully understood. In this report, the effects of tumour necrosis factor-alpha (TNF-alpha) and interferon-gamma (IFN-gamma) on human tumour cell

lines with respect to proliferation and apoptosis are examined. The TNF-alpha-sensitive cell lines Me-180 and MCF-7, the resistant cell line TCC-Sup, and the intermediate line 5637 were used. After a one day treatment, the transit through all phases of the cell cycle slowed down and after 3 days stopped completely, as measured with the BrdU-assay and flow cytometry. During the same time however, the levels of c-Myc and Ki-67 expression and the number of cells becoming apoptotic increased. Combined treatment with TNF-alpha and IFN-gamma augmented both the effects on the cell cycle and on apoptosis in the sensitive lines, and had only a minor additional effect on the resistant cell line as compared to single TNF-alpha treatment. The cells becoming apoptotic detached from the culture flask bottom and floated in the medium. Several apoptosis assays were used to prove that the floating cells were indeed apoptotic. As a subsidiary result of receptor measurement, we observed complexes of TNF-alpha receptors I with TNF-alpha receptors II using the related blocking antibodies and I125-TNF-alpha as ligand. The association of proliferative parameters and apoptosis became obvious by plotting the levels of c-Myc expression versus remaining live cells after apoptotic cells were detached. Our data revealed a good linear correlation indicating that high levels of c-Myc render cells sensitive to apoptosis, independent of the treatment, TNF-alpha alone or TNF-alpha in combination with IFN-gamma. The quantitative linear correlation may point to a threshold mechanism.

Full text

ABBREVIATIONS

TNF-alpha: tumour necrosis factor-alpha

IFN-gamma: interferon-gamma

PARP: poly ADP-ribose polymerase

FITC: fluorescence isothiocyanate

BrdU: bromodeoxyuridine

LIBu: labelling index after BrdU-labelling

TRAIL: TNF-alpha-related apoptosis-inducing ligand

INTRODUCTION

Tumour necrosis factor-alpha (TNF-alpha) is a pluripotent, pleiotropic cytokine which plays an important role in inflammatory, immunological and pathophysiological reactions (reviewed in [1, 2]). In tumour cells it exerts cytotoxic and cytostatic effects while normal cells are spared [3]. However, TNF-alpha is not toxic in all tumour cells, but the reason for this is still not clear. Different mechanisms can account for variation in sensitivity, including receptor numbers on the cell membrane and signalling pathways inside the cell. The dominance of TNF-alpha receptor 1 (TNF-alpha-R1) with respect to cell killing is widely accepted [4]. The receptor number is susceptible to regulation by other cytokines, *e.g.* interferon-gamma (IFN-gamma) [5]. However, the number of TNF-alpha-R1 may not be of prominent importance for sensitivity since low receptor occupancies were found to be sufficient to exert the effects [6]. TNF-alpha binding to the receptor induces a variety of intracellular signal cascades, which are interrelated to the pathways of other cytokines and cytotoxic drugs [7, 8]. In particular, interferons are able to modulate the effects of TNF-alpha [9-11]. In most reports, including our own synergistic effects with respect to toxicity were observed [12].

The simultaneous induction of proliferation and programmed cell death by cytokines has led to the hypothesis that proliferation and apoptosis are linked: Apoptosis is a normal consequence of engaging the cell's proliferative machinery [13-15], but can be prevented when life sustaining signals are available in the cell. Activation of p53 after DNA damage, for example may induce growth arrest and repair or alternatively apoptosis [14]. Expression of Myc proteins is implicated in the control of cell proliferation, and deregulated expression of Myc genes is frequent in cancer cells [16]. The pathways from Myc to apoptosis are unknown, but candidate molecules include cyclin D3 [14], reactive oxygen species (ROS) [17], NFkB [18], and Bin1 - cytochrome C [19].

It remains an open question if overexpression of c-Myc still promotes proliferation or at the same time induces an increase in sensitivity to apoptosis. In normal and tumour cells, stimulation of starved cells with serum induces elevated c-Myc expression. However, c-Myc returns to its basic level a few hours later, when the cells just begin to enter the cell cycle. A permanent elevation of

the c-Myc level after treatment with TNF-alpha and IFN-gamma, as observed in this study, may shift the balance from proliferation to apoptosis. We examined the changes of proliferative parameters, such as c-Myc, Ki 67, and the cell cycle transit in relation to apoptosis.

TNF-alpha is able to induce both necrotic and apoptotic forms of cell death [20]. Necrosis is characterized by an increase in cell and nuclei volume and eventual bursting of the cells, while apoptotic cells show a number of characteristics, including membrane permeabilisation, chromatin condensation, DNA degradation and formation of membrane-coated apoptotic bodies [21, 22]. The apoptotic pathway may vary in the various cells, and some of the characteristics may not appear. In MCF-7 cells, caspase-3 is missing, and these cells do not show DNA laddering, but they undergo apoptosis [23]. All cells used in this study chose the apoptotic pathway after cytokine treatment. The combination of TNF-alpha and IFN-gamma was used to produce stronger apoptotic effects, while IFN-gamma alone had, preferentially, cell cycle effects.

In the present study, the relationship between TNF-alpha- and IFN-gamma-induced cell kinetic modifications and their apoptotic effects was examined. Cell proliferation was measured by the BrdU-labelling technique and determination of the cell cycle related to Ki-67 and c-Myc expression. We expected down-regulation of c-Myc and Ki-67 in parallel with a slowing down or halting of the cell cycle. We found the opposite, elevated Ki-67 and c-Myc levels, and, at the same time an increase in apoptosis. This led us to the hypothesis that permanently elevated c-Myc may lead to sensitization of cells to apoptosis. Our data support a quantitative correlation between c-Myc level and reduction in cell number due to apoptosis.

MATERIALS AND METHODS

Cell cultures and cytokines

Four human tumour cell lines from ATCC were used, which were sensitive and resistant to tumour necrosis factor-alpha (TNF-alpha). These sensitive lines were: Me-180, a human cervix carcinoma, cultured in McCoy's 5a medium with 10% fetal bovine serum (FBS), MCF-7a, a human breast carcinoma, cultured in RPMI 1640 medium with 10% FBS. The resistant line was: TCC-Sup, a human bladder-transitional carcinoma, cultured in minimum essential medium (Eagle)

with non-essential amino acids, sodium pyruvate 1 mM, and Earle's BSS with 10% FBS, and the intermediate line 5637, human bladder carcinoma, cultured in RPMI 1640 medium with 10% FBS. Human recombinant TNF-alpha and human recombinant interferon-gamma (IFN-gamma) were purchased from Böhringer-Mannheim, Germany.

Neutral red assay

The neutral red assay was performed according to F. Kull *et al.* [24]. Briefly, the cells were cultured in 96-well plates. After treatment, the medium was removed, the cells were washed with PBS, 200 mul/well of neutral red solution (50 mul/ml in medium), were added, and the plates incubated at 37° C for 3 hours. The neutral red solution was removed, the cells washed with PBS and the dye fixed with 50% ethanol containing 1% acetic acid. The OD at 540 nm is related to the cell number.

TNF-alpha *receptors*

Binding studies were performed according to M. Grell *et al.* [25]. Cells were grown in 24-well plates, incubated at 0° C with anti-TNF-alpha receptor antibodies (10-50 mug/ml) for 30 min, then incubated with I^{125}-TNF-alpha (6 nM) for 2 hours. Cells were lysed in 1% SDS solution and measured in a Gamma counter (Packard). Non-specific binding was determined in the presence of 100 fold unlabelled TNF-alpha. For Scatchard analysis 0.5 to 6 nM I^{125} was used. The antibodies H398 (TNF-alpha receptor 55/60 kDa) and M80/7 (TNF-alpha receptor 75/80 kDa) were a gift from M. Grell, Institute for Cell Biology and Immunology, University of Stuttgart, the I^{125} TNF-alpha was purchased from Amersham, Braunschweig, Germany.

BrdU-labelling

BrdU labelling and staining was performed as previously described [26]. Briefly, cells were pulse labelled with 25 muM BrdU and 25 muM cytidin (Serva, Heidelberg, Germany) for 30 min and fixed with 70% ethanol. For staining, the cells were centrifuged, washed, and treated with 0.1 mg/ml RNase (Serva), resuspended in 2 N HCl to denature DNA, then incubated in anti-BrdU solution (DAKO, Hamburg, Germany) for 60 min, washed and incubated in FITC-conjugated antimouse IgG secondary antibody (Dianova, Hamburg, Germany)

for 30 min, and finally the DNA was stained with 10 mug/l propidium iodide (Culbiochem, Frankfurt, Germany). The cells were measured in a FACScan (Becton Dickinson, Sunnyvale, CA, USA) and the data analysed using the programme DAS, kindly provided by Wolfang Beisker, GSF, München, Germany. The transit time through S-phase Ts was calculated using the relative movement method [26].

Ki-67 staining

Staining of cells with Ki-67 was performed as previously described [27]. Briefly, the cells were fixed in acetone, resuspended in PBS, incubated in MIB1 antibody (a gift from Johannes Gerdes, Lab Molecular Immunology, Borstel, Germany) for 30 min, washed and incubated in a FITC-conjugated antimouse IgG antibody (DAKO, Hamburg, Germany) for 30 min, and stained in a 2 mul/ml PI solution, containing 0.1 mg/ml RNase. The cells were measured in a FACScan.

Apoptosis assays

The following methods were used to identify apoptotic cells. The methods have been described previously [21] or were used according to the manufacturer's instructions.

a) Annexin-V-FITC (Clontech, Palo Alto, CA, USA) stains phosphatidylserine translocated to the outside of the cell membrane in apoptotic cells, and is measured by FACScan.

b) Mito Tracker Green FM and Red CMX Ros (Molecular Probes, Eugene, OR, USA) detects the membrane potential of mitochondria, which is reduced in apoptotic cells.

c) Apo 2.7 (Immunotech, Marseilles, France) reacts with a mitochondrial membrane protein which is exposed in apoptotic cells. Trypsinized cells were centrifuged, resuspended in ice cold Triton X-100 (0.25%) in PBS containing 2.5% FCS and 0.01% Na_3N, incubated for 20 min, centrifuged and incubated in 100 ml Apo 2.7 (40 mug/ml) for 30 min at RT, centrifuged, incubated for 30 min in 100 mul anti mouse-FITC (DAKO, Glostrup, Denmark), stained with PI (2 mug/ml), and measured in a FACScan (Becton-Dickinson).

d) The chromatin condensation of cells was measured by assessing the sensitivity of DNA *in situ* to denaturation according to Darzynkiewicz [22], by staining with acridine orange (Polyscience Europe, Eppelheim, Germany).

e) Apoptosis-induced cleavage of DNA at the nucleosomal level was measured by the sub G_1 peak method by staining cells with PI in 30 muM phosphate buffer. In apoptotic cells, small DNA pieces diffuse out of the cells and reduce the DNA contents of apoptotic cells, which appear in the sub G_1 peak of the DNA histogram measured in the FACScan.

f) DNA strand breaks were directly measured using the TUNEL assay. Terminal deoxynucleotidyl transferase (Böhringer-Mannheim, Germany) synthesised bioinylated d UTP to the DNA breaks, which were stained with fluorescein-avidin and measured in the FACScan.

g) To detect the PARP cleavage by caspase-3, the protein of cells was isolated by standard procedures, separated by electrophoresis on SDS-polyacrylamide gels (Biorad, München, Germany), blotted on a Immobilon-P, PVDF membrane (Millipore, Bedford, UK), incubated with anti-PARP (Pharmingen, Hamburg, Germany), secondary antimouse IgG conjugated to HR peroxidase (DAKO). The ECL-kit (Amersham Pharmacia, Freiburg, Germany) was used to visualise the PARP bands with the enhanced chemiluminiscence detection system on a hybond film.

RESULTS

*Effect of TNF-*alpha*, IFN-*gamma *on cell proliferation*

The dependence of cell survival was determined using the neutral red assay. After TNF-alpha treatment the percentage of surviving cells decreased up to 1,000 U/ml and remained at the same level for higher concentrations. IFN-gamma also showed a plateau, however already at a concentration of 100 U/ml. We therefore used these concentrations in all the following experiments. In Figure 1 the results for the four cell lines tested are shown. For Me-180 and MCF-7 cells, the cell number after 3 days treatment decreased below the starting value at day 0, indicating cell loss in addition to growth reduction. The resistant cell line TCC-Sup was almost unaffected, while 5637 cells showed intermediate growth reduction. These data demonstrate the different sensitivity of the cell lines to

TNF-alpha and IFN-gamma which we studied with respect to proliferative and apoptotic parameters, see below.

*TNF-*alpha *receptors*

A possible reason for the different effects of TNF-alpha in the various cell lines might be that the cells have different numbers of TNF-alpha receptors or affinity coefficients. Indeed, as shown in Table 1, the number of TNF-alpha receptors, as well as the affinity coefficients, vary considerably. While Me-180 and MCF-7 had only type I (55/60 kDa) receptors, the BC 5637 and TCC-Sup also had type II (75/80 kDa) receptors. Moreover, these latter cell lines seem to express complexes of type I and type II receptors. This was revealed by blocking receptors I and II with specific antibodies and probing with TNF-alpha-I^{125} (see methods). After blocking for example BC 5637 receptors with anti-RI 8.5%, RII bound to TNF-alpha-I^{125}, however, blocking with anti-RII resulted in only 73% RI binding to TNF-alpha-I^{125}. The missing 18.5% can be explained by complexes of RI and RII, assuming that the binding site for TNF-alpha-I^{125} of the type I receptor will be blocked when the specific antibody is bound to the type II receptor, and *vice versa*.

Treatment of cells with IFN-gamma induced a slight increase in TNF-alpha receptor numbers with a maximum after 6 hours, see last column in Table 1. At 24 hours the receptor numbers were back to the control values (data not shown). Neutral red cytotoxicity tests with TNF-alpha after a 6 hours pre-incubation with IFN-gamma to increase the TNF-alpha receptor numbers of the cells, did not show any change in sensitivity, indicating that the number of TNF-alpha receptors is not an important characteristic for TNF-alpha effects in these tumour cells.

Cell kinetics, BrdU-labelling

Reduction in the numbers of treated cells as compared to controls may be achieved by cell loss and/or by slowing down of the cell cycle. To assess the role of cell cycle disturbances *e.g.* blockade or slowing down *versus* cell loss induced by the cytokines, the cell cycle transit was analysed using the BrdU method. Figure 2 shows as an example dot-plot of BrdU-labelled BC 5637 cells, 10 hours after pulse labelling. The labelled control cells (Figure 2A) have moved through S-phase and most of them have arrived at G1-phase. The cells treated for 16

hours with IFN-gamma before BrdU pulse labelling have only progress slightly in the cell cycle 10 hours after labelling (Figure 2B). Figure 3 shows that after 48 hours treatment of Me-180 cells with IFN-gamma the cell cycle comes to a halt. The labelling index drops to close to zero (Figure 3, bottom) and cell growth comes to a halt (Figure 3, top). This effect of IFN-gamma cannot be reversed by replacing the IFN-gamma-containing medium with fresh medium. The LI remains near zero and the cells do not resume proliferation in the following 2 days (Figure 3).

In Table 2, the BrdU measurements for all cell lines are shown. After the 24 hours treatment, only the MCF-7 cells showed a considerable decrease in LI induced by TNF-alpha. IFN-gamma was more effective in Me-180 and BC5637 cells, while TNF-alpha was more effective in MCF-7 and TCC-Sup cells. The transit time through S-phase, as measured by the relative movement method, remained more or less constant after the 24 hours treatment, only TNF-alpha + IFN-gamma induced a prolongation of t_S in TCC-Sup and IFN-gamma in BC5637. The 48 hours treatment however, induced considerable prolongation of t_S, and in most cases a complete halt to the cell cycle. The percentage of floating cells, which were shown to be apoptotic (see section Apoptosis), increased only slowly with treatment time, except for Me-180 cells where TNF-alpha, and synergistically, TNF-alpha + IFN-gamma induced apoptosis and hence detachment of cells from the culture flask bottom already after 24 hours treatment.

c-Myc and Ki-67 expression

To further characterize the status of the cells in the cell cycle, c-Myc was measured using antibodies and FACScan. An example of the c-Myc distributions in MCF-7 cells after 2 days treatment with TNF-alpha and the 2 day control is shown in Figure 4. The level of c-Myc expression was determined by calculating the mean of these distributions by subtracting the mean of the isotype and normalizing to the fluorescence of standard beads. To demonstrate that the tumour cells were able to modulate c-Myc expression, we stimulated the cells after starvation. All three cell lines responded with an increase in c-Myc to stimulation by medium with 10% FCS after serum starvation for 3 days, as shown in Figure 5, top. While Me-180 cells began to down-regulate the c-Myc after 1 hour and arrived at control levels already 5 hours after stimulation, the MCF-7 and TCC-Sup cells remained at elevated levels at least up to 5 hours. Treatment

of cells with TNF-alpha, IFN-gamma and TNF-alpha + IFN-gamma resulted in a long lasting increase of c-Myc, as shown in Figure 5, bottom. However, in the apoptosis-resistant cell line TCC-Sup, almost no increase in c-Myc was observed. It should be noted that IFN-gamma had no effect on MCF-7 cells whereas the combination of TNF-alpha with IFN-gamma increased c-Myc to the same 3-fold level as in Me-180 cells where IFN-gamma alone induced a 2-fold elevation of c-Myc. The same tendency was found for the reduction of cell number, see Figure 1. The absolute values of c-Myc in untreated cells were clearly lower in the TNF-alpha-resistant cell line TCC-Sup (0.27 ± 0.09) than in the TNF-alpha-sensitive cell lines Me-180 (1.13 ± 0.05) and MCF-7 (1.23 ± 0.65). These figures were obtained from cell nuclei as relative fluorescence values (FL1(Myc)-FL1(iso))/beads, and represent a normalization to the constant fluorescence of the beads.

Ki-67 is a marker of proliferation which is expressed only in cycling cells and not in quiescent cells. Therefore, a down-regulation of Ki-67 after treatment with TNF-alpha, IFN-gamma and the combination of both, at least after 2 days of treatment was expected. Figure 6 shows that, on the contrary, Ki-67 is up-regulated after 2 days. This is a continuous process, after 5 hours the Ki-67 was found to be only slightly higher than the controls (data not shown). Moreover, the increase in the Ki-67 level is less in TCC-Sup, where the LIBu decreased much more than in Me-180 and MCF-7 cells (Table 2). This increase in Ki-67 levels after cytokine treatment is very closely related to the increase in the c-Myc level, as reported above.

Apoptosis induction by TNF-alpha ***and IFN-***gamma

The numbers of cells remaining attached, and those released from the culture flask bottom are plotted in Figure 7. The cells treated with TNF-alpha or IFN-gamma alone grew very slowly in number, however, since the number of detached cells increased, there must have been proliferation at least up to 48 hours of treatment. Even for the combined treatment - the total number of cells increased up to 24 hours. At later times the majority of cells floated in the medium while the number of attached, viable cells further declined.

To assess the viability status of attached and floating cells, we applied a variety of methods to determine the percentage of cells with apoptotic characteristics. The majority of cells which remained attached to the bottom of the culture flask

after treatment were viable, only about 10%, as compared to the control, showed all the characteristics of apoptotic cells. The detached floating cells, on the other hand, were close to 100% apoptotic (Figure 8). However, some parameters showed a deviant behaviour in the various cell lines, *e.g.* DNA cleavage (sub G_1) was less abundant in Me-180 cells (Figure 8, top), and PARP cleavage was virtually absent in IFN-gamma-treated as well as control MCF-7 cells (Figure 8, bottom).

Discussion

Treatment of human tumour cell lines with the cytokines TNF-alpha and IFN-gamma results in reduced cell numbers (Figure 1). The various cell lines show different sensitivity to TNF-alpha and IFN-gamma, *e.g.* Me-180 is sensitive to both while MCF-7 is only sensitive to TNF-alpha and not to IFN-gamma. The combination of both cytokines, however, is more than additive in sensitivity. The reason for the different sensitivities could be (a) different numbers and affinity of receptors (b) down-regulation of proliferation and © induction of apoptosis.

The TNF-alpha receptor number is indeed lower on the resistant TCC-Sup cells than on the other three lines (Table 1). On the other hand, the BC 5637 cells are as sensitive as the MCF-7 cells to TNF-alpha despite their nearly 50% lower receptor number. When a low receptor occupancy is sufficient to induce the effects as observed by Coffman *et al.* [6], the number of receptors per cell should not be crucial for sensitivity. As a corollary, we found in two cell lines 17 and 20% complexes of TNF-alpha-R1 and TND-R2. This was revealed by the fact that a TNF-alpha-R1 blocking antibody also blocked a fraction of the TNF-alpha-R2 and vice versa (Table 1). Such hetero-complexes were also observed by J.K. Pinckard, *et al.* [28] after TNF-alpha ligand binding using a direct assay. In contrast, Chan *et al.* [29] found no evidence of hetero-complexes, only homo trimers of TNF-alpha-R1 and TNF-alpha-R2, respectively. The expression of more TNF-alpha-receptors after IFN-gamma treatment is well known [30]), in our cells the increase of receptor numbers was only slight and cannot explain the drastic reduction in surviving cells.

The decrease of cell growth after treatment with TNF-alpha and IFN-gamma may depend on prolongation of the cell cycle time T_C or blockage of cycling, and in addition, cell death. The growth curves of TNF-alpha- and IFN-gamma-treated cells were nearly identical (Figure 7), however, the numbers of detached cells

after TNF-alpha treatment were three times higher than those of IFN-gamma treated cells. Since the detached (floating) cells are apoptotic as will be shown and discussed below, this indicates that TNF-alpha acts more *via* programmed cell death and IFN-gamma more *via* interference with the cell cycle. This is also illustrated by Figure 3, where, after IFN-gamma treatment, the S-phase labelling index drops between 24 hours and 48 hours to nearly 0, and at the same time cell growth stops. The increase of S-phase transit times T_S after 24 hours of IFN-gamma and not after TNF-alpha also supports this explanation (Table 2). After longer treatment times, and in particular after combined TNF-alpha + IFN-gamma treatment, the cell cycle slows down or stops. The down-regulation of the cell cycle was not accompanied by a blockage in any of the phases. Growth arrest was also found in other cell lines: HL-60 [31], breast cancer line TD 47 [32], and glioblastoma SF-188 and LN-382 [33] after TNF-alpha treatment. However, the authors observed only a G_1 prolongation or blockage and did not look at S-phase transit times T_S. In conclusion, our data indicate that TNF-alpha, and more so IFN-gamma, slow down cell cycle transit times, rather than inducing accumulation in one of the phases.

Growth arrest, as found after treatment with TNF-alpha and IFN-gamma should be associated with low levels of c-Myc and Ki-67. We obtained, on the contrary, elevated levels of both c-my and Ki-67, see Figure 5 bottom and Figure 6. The most prominent increases were found after TNF-alpha + IFN-gamma, where the halting of the cell cycle was most complete. This contradiction cannot be explained by a dysfunctional c-Myc in these tumour cell lines. In Figure 5, top, we show that stimulation of starved cells by medium with 10% serum increases the c-Myc level for several hours, then it is down-regulated to its basic level, which is a normal behaviour. After TNF-alpha and IFN-gamma treatment the c-Myc and Ki-67 levels rose continuously up to 3 days, and in parallel, the number of cells becoming apoptotic also increased. A correlation between c-Myc level and apoptosis is discussed below. The cells that were resistant to apoptosis (TCC-SUP) were also resistant to cell kinetic changes after treatment with TNF-alpha. IFN-gamma treatment induced a blockade in the cell cycle in TNF-alpha-resistant as well as sensitive cells. The combined treatment, however, increased apoptosis only in the sensitive cell lines.

The characteristics of apoptotic cells are manifold and we have used several methods to reveal how many of the cells were apoptotic. The main finding was that only few of the cells attached to the culture flask bottom became apoptotic

after treatment with TNF-alpha and IFN-gamma alone while 30 to 70%, depending on the method, showed apoptotic traits after combined TNF-alpha + IFN-gamma-treatment. On the other hand, the detached, floating cells proved to be apoptotic using most of the methods. One of the exceptions, the missing cleavage of PARP after IFN-gamma and in the control could be explained by the fact that MCF-7 cells do not express caspase-3. In the TNF-alpha treated cells, however, PARP was degraded, probably by caspase-6 or -7, which are able to replace caspase-3 in a compensatory pathway [34]. These findings indicate that cells in an early apoptotic stage detach and become floating.

To demonstrate the correlation between c-Myc level and apoptosis we plotted Myc levels of treated cells, normalized to the value of the control at the same time, *versus* percentage of remaining cells, also related to the respective control in Figure 9. The data used for this correlation are those shown in Figures 1, 5, and 7. The plots include the data of all three cell lines where the different experiments were normalized to their common mean value, which did not change the steepness of the regression line. As can be seen from the figure, a good correlation between c-Myc level and percentage of remaining viable cells is obtained. The strong correlation shows that c-Myc levels and apoptosis are related: high c-Myc levels are related to extended apoptosis. This correlation indicates, that the c-Myc level is crucial, independent of the way it has been generated, since data for three different treatments (TNF-alpha, IFN-gamma, TNF-alpha + IFN-gamma) are included. A similar relationship was found in HeLa cells treated with TRAIL or staurosposine by Stoneley *et al.* [35]. In transgenic mice, where c-Myc was activated by hydroxytamoxifen, thymocytes became increasingly apoptotic after 48 hours [36]. Our results and those of the cited authors may be explained by the "dual signal model for c-Myc action" of Evans [13]. It suggests that any mechanism promoting cell proliferation also activates the potential for cell suicide. A cell activated by c-Myc needs survival signals to avoid apoptosis. In our experiments, the cells were treated with TNF-alpha which activates apoptotic pathways rather than survival signals. The simultaneous, high Ki-67 levels indicate that the proliferation signals also remain active. The mechanisms involved in how c-Myc promotes apoptosis are not completely understood. A putative participant of the pathway is Bin 1 which is able to bind c-Myc [37] and induces cytochrome C release from mitochondria to the cytoplasm and thus sensitizes cells to TNF-alpha since cytochrome C is also involved in a TNF-alpha-induced pathway to apoptosis [38]. The quantitative correlation between c-Myc level and the number of cells remaining attached after apoptotic

cells were released, is a novel finding. Permanently elevated c-Myc obviously leads to sensitization of cells to apoptosis, according to our hypothesis. Moreover, the quantitative correlation may indicate a threshold mechanism; a higher c-Myc mean value implies more cells of the population above a threshold of sensitivity to apoptosis and hence more cells becoming apoptotic.

CONCLUSION

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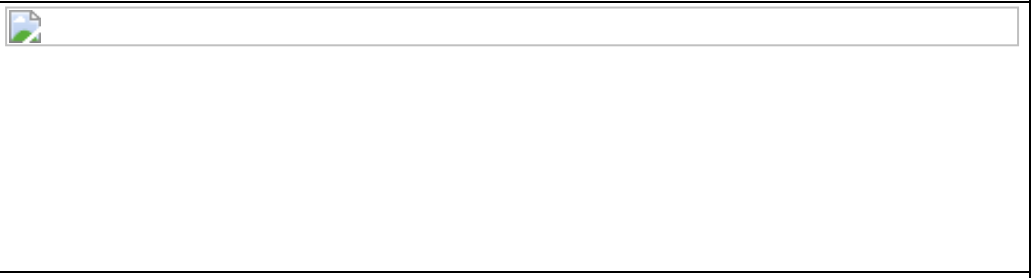
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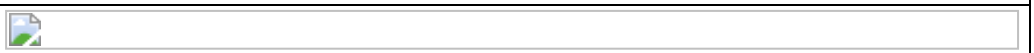
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Figures



































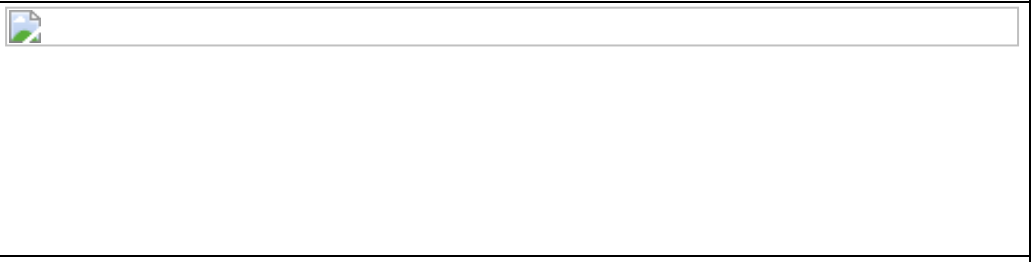


Table 1

TNF-receptor numbers and affinity coefficients Kd measured with iodinated TNF. These numbers include both receptor types, blocked with an antibody and *vice versa*. The vast majority are of a type I (TR 55/60). The column "% complexes" represents presumed complexes of both receptors, see text. In the last column the increase in receptor numbers (both types, % of untreated) after 6 hours treatment of cells with 100 U/ml IFN are given

	Number of TNF receptors Kd (pM)		% TR 55/60	% TR 75/80	% complexes	% increase receptor numbers Treatment IFN-γ 100 U/ml 6 hours
Me180	10,754 ± 1,409	1,630 ± 200	96.6 ± 8.4	1.3 ± 5.3	0	115 ± 11
MCF-7	9,080 ± 1,240	70 ± 9	103.5 ± 4.3	0.8 ± 1.0	0	127 ± 35
BC5637	5,090 ± 1,711	1,436 ± 503	73.0 ± 4.6	8,5 ± 4.0	17.3 ± 5.3	180 ± 10
TCC-SUP	721 ± 80	300 ± 34	70.5 ± 6.7	7.5 ± 3.1	22.0 ± 7.3	120 ± 7

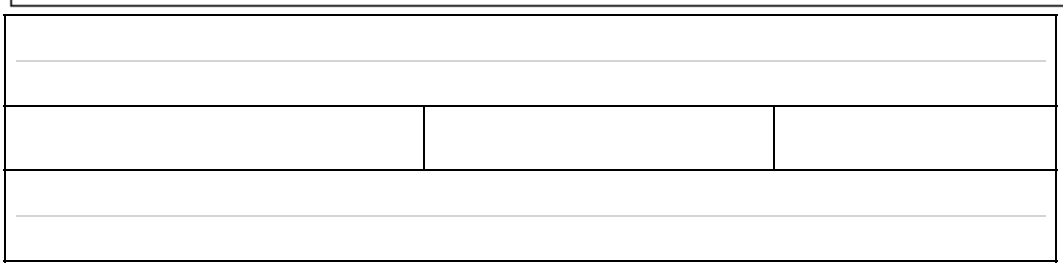


Table 2
Cell kinetic parameters measured using the BrdU-labelling method after treatment with TNF-α, IFN-γ and, TFN-α + IFN-γ

	LI (Bu) %		T_s (h)		% floating cells (apoptosis)	
	24 hours	48 hours	24 hours	48 hours	24 hours	48 hours
Me 180						
Contr.	33.2 ± 9.4	24.2 ± 6.4	9.2 ± 0.7	10.5	2.8 ± 0.8	3.5 ± 0.7
TNF	34.7 ± 5.3	36.3 ± 8.2	8.9 ± 0.8	nd	33.3 ± 17.9	40.5 ± 13.5
IFN	33.4 ± 1.5	15.2 ± 4.8	11.0 ± 1.8	nd	7.1 ± 3.8	25.6 ± 5.8
T + I	19.9 ± 2.8	14.3	12.5 ± 2.0	nd	93.1 ± 2.2	97.8 ± 1.0
MCF-7						
Contr.	38.0 ± 3.6	33.1 ± 8.1	16.0 ± 1.6	14.1 ± 3.4	1.0 ± 0.4	0.7 ± 0.3
TNF	21.5 ± 6.7	15.7 ± 5.2	9.1 ± 1.8	csc	3.6	13.3
INF	31.8 ± 7.1	22.7 ± 6.6	n.d.	38 ± 7.5	1.6 ± 0.8	1.6 ± 1.0
T + I	8.5 ± 3.6	7.9 ± 2.1	7.9 ± 1.3	csc	2.7 ± 0.8	11.4 ± 3.1
TCC-SUP						
Contr.	38.0 ± 2.9	10.5 ± 7.3 (a)	9.4 ± 0.8	11.8 ± 3.4 (b)	4.3 ± 1.9	9.0 ± 3.0
TNF	33.5 ± 0.7	3.0 ± 1.7 (a)	9.2 ± 0.8	9.3 ± 1.5 (b)	4.5 ± 1.7	20.8 ± 2.6
IFN	36.5 ± 5.0	7.3 ± 4.7 (a)	10.4 (c)	csc (b)	5.7 ± 3.3	13.7 ± 1.3
T + I	29.5 ± 1.5	3.2 ± 2.3 (a)	18.2 (c)	csc (b)	6.0 ± 1.1	23.3 ± 4.6
BC5637						
Contr.	52.3 ± 7.6	17.5 ± 3.8 (b)	13.2 ± 1.7	14.0 ± 5.0	3.2	17.1
TNF	45.7 ± 5.2	12.6 ± 1.4 (b)	16.2 ± 2.2	n.d.	3.6	23.1
IFN	48.3 ± 1.8	5.2 ± 0.8 (b)	33	csc	nd	nd
T + I	nd	nd	nd	nd	nd	nd

(a) 72 hours treatment; (b) the percentage of unlabelled S-phase cells remained high, indicating that cells in all phases of the cell cycle became quiescent; (c) calculated from doubling time, csc = complete stop cycling, nd = not done.

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In this issue

Monocyte chemotactic protein-3

Patricia Menten, Anja Wuyts, Jo Van Damme
MCP-3, induction, diseases, structure, receptors.

Adenoviral supply of active transforming growth factor-beta1 (TGF-beta1) did not prevent lethality in transforming

growth factor-beta1-knockout embryos

Hedia Chagraoui, Stéphane Giraudier, William Vainchenker, Françoise Wendling
TGF-beta1, cytokine, knockout mice, gene therapy, adenovirus.

Absence of endogeneous interleukin-10 enhances the evolution of murine type-II collagen-induced arthritis

Salvatore Cuzzocrea, Emanuela Mazzon, Laura Dugo, Ivana Serraino, Domenico Britti, Massimo De Maio, Achille P. Caputi
collagen, inflammation, nitric oxide, peroxynitrite, superoxide dismutase, IL-10.

Reduced adrenal response and increased mortality after systemic Klebsiella pneumoniae infection in interleukin-6-deficient mice

Frank H.J. van Enckevort, C.G.J. Sweep, Paul N. Span, Mihai G. Netea, Ad R.M.M. Hermus, Bart Jan Kullberg
corticosterone, cytokine, IL-6-deficient mice, granulocyte recruitment, survival.

Gamma-interferon induces apoptosis of the B lymphoma WEHI-279 cell line through a CD95/CD95L-independent mechanism

K.-E. Ben Jilani, K. Akarid, D. Arnoult, F. Petit, E. Baert, J.-P. Gaillard, J.-C. Ameisen, J. Estaquier
IFN-g, B cells, apoptosis, CD95, caspases, cytokines.

IFN-beta stimulates the production of beta-chemokines in human peripheral blood monocytes. Importance of macrophage differentiation

Laura Fantuzzi, Irene Canini, Filippo Belardelli, Sandra Gessani
MCP-1, MIP-1alpha, MIP-1beta, IFN-beta, monocytes/macrophages, differentiation.

Relationship between cell kinetics and apoptotic effects in TNF-alpha and IFN-gamma-treated human tumour cell lines

Heinz Baisch
apoptosis, c-Myc, cell cycle, cytokines, dual pathways.

L-Buthionine-(S,R)-sulfoximine, an irreversible inhibitor of gamma-glutamylcysteine synthetase, augments LPS-

mediated pro-inflammatory cytokine biosynthesis: evidence for the implication of an IkappaB-alpha/NF-kappaB insensitive pathway

John J. Haddad
antioxidant, chemioxyexcitation, cytokines, glutathione disequilibrium, pharmacotherapy, reactive oxygen species, redox/thiol regulation.

Endotoxin regulates the maturation of sterol regulatory element binding protein-1 through the induction of cytokines

Luisa Diomede, Diego Albani, Marco Bianchi, Mario Salmona
SREBP-1, LPS, HepG2, liver, mice.

Regulation of Staphylococcus aureus-mediated activation of interleukin-18 in peripheral blood mononuclear cells

Ulrich Böcker, Tobias Manigold, Joanna M. Watson, Manfred V. Singer, Siegbert Rossol
human, cytokine, bacterial antigen, CD14, signal transduction.

Interleukine-6 (IL-6) synthesis and gp130 expression by human pericardium

Emmanuelle Menet, Pierre Corbi, Cécile Ancey, Franck Morel, Adriana Delwail, Martine Garcia, Anne-Marie Osta, John Wijdenes, Daniel Potreau, Jean-Claude Lecron
IL-6, gp130, pericardium.

Plasmodium falciparum liver-stage antigen-1 peptide-specific interferon-gamma responses are not suppressed during uncomplicated malaria in African children

Adrian J.F. Luty, Martina Bongartz, Philipp Rezbach, Jean-Francois Faucher, Michael R. Hollingdale, Peter G. Kremsner
malaria, cytokine, interferon-gamma, liver.

Immunoexpressions of p21, Rb, mcl-1 and bad gene products in normal, hyperplastic and carcinomatous human prostates

Mar Royuela, María Isabel Arenas, Fermín R. Bethencourt, Manuel Sánchez-Chapado, Benito Fraile, Ricardo Paniagua

p21, Rb, mcl-1, bad, human prostate, benign prostatic hyperplasia, prostatic carcinoma.

Recombinant human interleukin-1 beta: new possibilities for the prophylaxis and correction of toxic myelodepression in patients with malignant tumors I. Phase I-II clinical trials of recombinant human interleukin-1 beta as a leukopoiesis stimulator in cancer patients receiving combination chemotherapy

M.L. Gershanovich, L.V. Filatova, S.A. Ketlinsky, A.S. Simbirtsev
human recombinant interleukin-1 beta, myelopoiesis, myelodepression, cancer chemotherapy.

Recombinant human interleukin-1 beta: new possibilities for the prophylaxis and correction of toxic myelodepression in patients with malignant tumors II. Phase II study of the protective effect of recombinant human interleukin-1 beta on myelodepression induced by chemotherapy in cancer patients

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