TNF triggers mitogenic signals in NIH 3T3 cells, but induces apoptosis when the cell cycle is blocked

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ABSTRACT. Tumor necrosis factor (TNF) is known to be a mediator of a variety of cellular responses including apoptotic death or proliferation depending on the target cell and the environmental conditions. We show here that TNF triggers both growth and death signals in NIH3T3 murine fibroblasts. In cells arrested in G_0 by serum deprivation, TNF drives approximately 50% of them to enter the cell cycle, but kills the cells that remain quiescent. The presence of serum prevents toxic effects of TNF, suggesting that TNF can cooperate to drive cells through the cell cycle, but is unable to do so by itself or alternatively it triggers death signals in cells unable to proliferate. Interestingly, TNF induces a similar toxic effect in cells forced to stay at the G_1/S border, S or M phases. We have explored the TNF apoptotic pathway in arrested cells. This mechanism is not due to the loss of the anti-apoptotic capacity of NFκB, and is mediated by mitochondria since Bcl-2 overexpression partially inhibits cell death. There are, however, interesting differences in the kinetics of mitochondrial events, which indicate that this form of sensitization to TNF leads to an apoptotic mechanism different from that observed after sensitization by RNA synthesis inhibition.

Keywords: TNF, apoptosis, cell cycle, mitochondrial apoptotic pathway, NFκB

TNF can induce proliferation in several cell lines, but mainly in non-transformed cells [1], and this mitogenic effect is mediated through gene expression [2, 3]. In this context, the transcription factor nuclear factor-kappa B (NFκB) plays an important role. TNF-induced proliferation seems to be mediated by this transcription factor [4] and its inactivation can change the type of response to TNF, switching from a proliferative response to the induction of apoptosis [5]. TNF can also induce cell death by apoptosis or necrosis. However, most cell types are resistant to the toxic action of this cytokine unless protein synthesis-dependent protective mechanisms, mainly mediated by NFκB, are inhibited [6-8]. In these conditions, the caspase cascade can be activated, triggering the apoptotic response. The activation of caspases may rely or not on the activation of mitochondrial apoptotic events [9]. The action of several anti-apoptotic members of the Bcl-2 family, such as Bcl-X_L or Bcl-2, prevents the apoptotic activation of the mitochondria, avoiding the death of those cells in which the apoptotic mechanism depends on the mitochondrial pathway.

The mitogenic and cytotoxic effects of TNF seem to be closely related. Thus, in some cell lines, both types of effects can be observed simultaneously [5, 10]. Likewise, it has been found that TNF-induced cell death occurs in a particular phase of the cell cycle; however, this phase seems to change according to the cell type or the environmental conditions. In the tumor cell line WEHI-164/clon 2F, TNF inhibits DNA synthesis and induces apoptosis in the G_1/S border [11]. In some cell types, TNF induces G_1 arrest followed by apoptotic death [12]. In other cases however, the induction of G_1 protects against apoptosis [13]. In HeLa cells, TNF kills S, but not G_1 phase-arrested cells [14], meanwhile in L929 cells synchronized in the G_1/S border, TNF-induced cell death is linked to the abnormal induction of mitosis [15].

All of these studies were performed using cancer cells, which usually show deficiencies in their cell cycle checkpoints, and this fact is most likely responsible for the wide variety of responses observed. In the present work, we aimed at studying the relationship between the mitogenic and cytotoxic effects of TNF in a non-transformed cell line. We found that TNF shows both mitogenic and toxic effects in G_0-synchronized NIH 3T3 murine fibroblasts. In this model, cell death affects only that portion of cells that are unable to proliferate. Likewise, TNF induces apoptosis in cells arrested at any other point of the cell cycle (phases G1/S, S or M). This type of sensitization against TNF-induced toxicity is mediated through a mechanism not inhibitable by NFκB, and triggers a pattern of apoptotic

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mitochondrial activation different from that observed after RNA synthesis inhibition.

METHODS AND MATERIALS

Synchronization and transfection of cell cultures

NIH 3T3 cells were synchronized in the G1/S, S or M phase by treatment with 2 μg/ml aphidicolin, 200 μM hydroxyurea or 0.1 μg/mL nocodazole respectively for 24 hours. Alternatively, cells were synchronized in the G_0 phase by incubation in medium with a low percentage (0.5%) of fetal bovine serum (FBS) for 48 hours. Transfection of synchronized cells with 1 μg of Bcl-2 (a gift from Dr D.S. Ucker, together with EGFP-F plasmid), IκB-mut (a gift from Dr D. Wallace) or empty vector plasmids, or 0.25 μg of NFκB-SEAP plasmid (a gift from Dr B. Darnay), in addition to 0.2 μg of the transfection control plasmid EGFP-F, was performed using Lipofectamine Plus Reagent (Gibco BRL).

Cell cycle analysis

After treatments, cells were collected, washed with PBS and fixed in 70% ice-cold ethanol. Cells were washed twice with PBS followed by incubation in 5 μg/ml PI (Sigma) and 100 μg/mL RNase A (Sigma) for 30 min. Cell cycle analysis was performed by flow cytometry using an Ortho-Cytoron (OrthoDiagnostics, Johnsonn&Jonhson). The percentage of cells in the different phases of the cell cycle was quantified using the data processing program ModFit (Verity Software House).

Analysis of DNA synthesis

Cells grown under coverslips were incubated in medium with 0.5% FBS for 48 hours. Cells were then treated in the presence of 20 μM 5-Bromo-2'-deoxyuridine (BrdU; Sigma) as indicated. Cells were washed with PBS and fixed with ice-cold methanol for 15 min. After washing another three times with PBS, cells were incubated with 1.5 M HCl for 30 min and washed again three times with PBS-T (PBS/0.1% BSA/0.2% Tween 20). The coverslips were incubated with anti-BrdU (Becton Dickinson) diluted 1:30 in PBS-T for 1 hour at room temperature, washed three times with PBS-T and incubated with anti-mouse IgG-FITC (Dako) diluted to 1:100 in PBS-T for 30 min at room temperature. This incubation, as well as the subsequent processing, were performed in the dark. Cells were then washed with PBS, incubated for 10 min with 5 μg/mL PI and washed again with PBS. The coverslips were mounted using Fluoromount G (Southern Biotechnic). The FITC (Ex: 490 nm; Em: 520 nm) and the PI (Ex: 590 nm; Em: 615 nm) fluorescence present in each cell was analyzed with a Confocal Laser Microscopy System (BIO RAD MCR 600). Results are the mean of the analysis of six randomly chosen fields of each sample (approximately 500 cells).

Determination of the number of cells by sulforhodamine B dye

Synchronized cells were fixed immediately after the indicated treatments using 10% trichloroacetic acid for 30 min, washed with PBS and covered with 0.4% sulforhodamine B (dissolved in 1% acetic acid) for 15 min. After that, cells were washed six times with 1% acetic acid. Finally, cell-bound sulforhodamine B was dissolved in 2 mL of 10 mM Tris-HCL pH 10.4. The sulforhodamine B fluorescence signal (Ex: 530 nm; Em: 590 nm) was measured using a Cytofluor TM 2350 fluorimeter (Millipore). A calibration curve was used to express the fluorescence registered as number of cells.

Western blot analysis

Cytosolic, mitochondria-free, extracts were prepared by suspending cells in mitochondrial buffer (80 mM KCl, 10 mM Tris HCL, 3 mM $MgCl_2$, 1 mM EDTA, 5 mM KH_2PO_4, 10 mM sodium succinate, pH 7.4) and then incubated with digitonin (at a ratio of digitonin: protein 0.2 **Please, could you check this number?**) for 5 min. Lysates were clarificated by centrifugation, and the supernatants were resolved on 12% SDS polyacrylamide gels and blotted onto nitrocellulose. Specific polyclonal antibodies were used to detect cytochrome c (Pharmingen). Immunoreactive bands were visualized by the luminol reaction (ECLplus, Amersham).

Mitochondrial membrane potential assay

Mitochondrial membrane potential was assessed, simultaneously or not with the cell cycle, as previously described [16]. In transfected cells, only EGFP-F positive cells were analyzed.

Assessment of cell shrinkage, rounding and condensation

The manifestation of typical apoptotic morphology, which includes shrinkage and cell rounding, was visualized by phase contrast microscopy. In addition, dying transfectants cells reliably displayed a condensed pattern of EGFP F fluorescence [17]. More than 500 cells in six, randomly chosen fields were counted for each condition in every experiment.

Propidium iodide uptake

Cells were harvested, washed once with PBS, and then incubated in PBS with 40 μg/mL of PI for 15 minutes at 4°C, in the dark. The percentage of PI fluorescence-positive cells was assessed by flow cytometry analysis. In transfected cells, only EGFP-F positive cells were computed for PI uptake.

Electrophoretic mobility shift assay (EMSA)

After the indicated treatment, nuclear extracts were prepared as described previously [18] and used in electrophoretic mobility shift assay cells using a double-stranded oligonucleotide probe containing the κB site from the mouse κ light chain enhancer (5'-TGCACAGAGGGGACTTTCCGAGAGG-3') as previously described [19].

Transcriptional activity of NFκB. Secreted alkaline phosphatase (SEAP) assay

Cells were co-transfected with 1 μg of the indicated plasmids and 0.5 μg of the NFκB-SEAP plasmid, containing

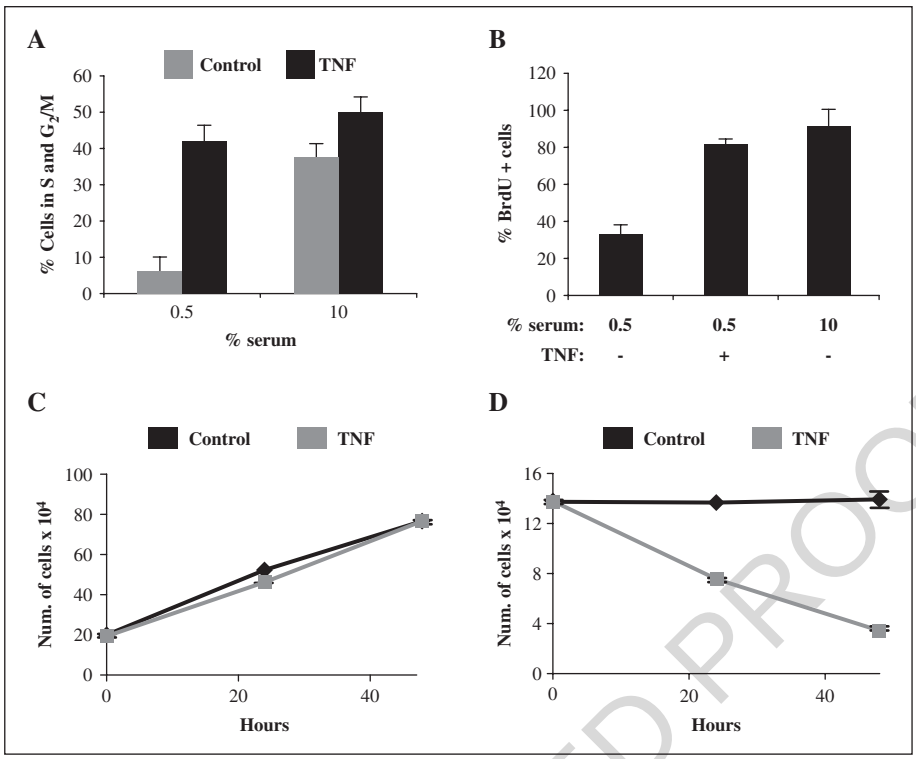

Figure 1

TNF-induced mitogenic signals and toxic effects. NIH 3T3 cells arrested in G_0 by serum deprivation for 48 hours were treated or not with 10 ng/mL TNF for 20 hours in medium containing the indicated concentrations of serum and analysed as follows. **A**) cell cycle analysis by flow cytometry. The percentage of cells in S and G_2/M phases is shown. **B**) Analysis of BrdU incorporation. Cells were treated as described in Methods and the percentage of cells positive for BrdU staining scored. **C, D**) Effect of TNF on cell growth. G_0 arrested cells were treated or not with 10 ng/ml TNF in medium with 10% (**C**) or with 0.5% of serum (**D**). At the indicated times the number of cells remaining attached to the plate was quantified after staining with sulforhodamine B.

the codifying sequence for the SEAP under the control of a NFκB response element. After treatment, SEAP activity present in culture medium was assayed. Ten-25 µL of these culture media were diluted in 100 µL of the assay buffer (final concentration: 100 mM Tris pH 9.0; 0.1% BSA) in 96-well plates. Plates were incubated for 1 hour at 65°C in darkness to inactivate endogenous phosphatases. Then, 50 µL of 1 mM 4-methyl-umbelipheryl-phosphate (4-MUP), freshly prepared from a 100 mM stock (kept at -70°C), was added to each well. The plate was incubated for 2-4 hours at 37°C in darkness and the fluorescence emitted (excitement: 355 nm; emission 460 nm) was measured in a Cytofluor TM 2350 (Millipore).

RESULTS

TNF induces mitogenic and cytotoxic signals in NIH 3T3 cells

TNF induces mitogenic signals in some non-transformed cell types [1]. This is the case for NIH 3T3 murine fibroblasts as indicated by the induction of DNA synthesis. Thus, a 20-hour TNF treatment of G_0-arrested cells caused a large increase in the number of cells entering the cell cycle and presenting DNA contents corresponding to S and G_2/M (*figure 1*A). This effect was better observed using low serum concentration (0.5%) but became less evident in the presence of a high serum concentration (10%). In this case, most of the TNF-induced mitogenic effect must have been masked by the serum's growth factors effect. This mitogenic effect was also confirmed by analyzing the fraction of G_0-arrested cells that incorporated bromodeoxyuridine (BrdU) to newly synthesized DNA after the treatment with TNF or 10% serum (*figure 1*B). However, in these experiments we could also observe that, despite the great increase in the percentage of BrdU-positive cells, there was a systematic decrease in the number of cells in Go-arrested cultures treated with TNF. These observations seemed to be contradictory, since TNF induces both a mitogenic signal (induction of DNA synthesis) together with a global anti-proliferative behaviour (decrease of the cell number). In order to quantify this toxic response, we used the fluorescent dye sulforhodamine B to study the kinetics of growth of G_0-synchronized NIH 3T3 cells after TNF treatment. In the presence of 10% of serum there was no significant difference between untreated and TNF-treated cultures (*figure 1*C). On the other hand, when we used 0.5% serum, control cells remained arrested and the cultures treated with TNF showed a progressive decrease in the number of cells so that after 48 hours, only 25% of the initial number of cells remained in culture (*figure 1*D). Taken together, these data suggest the coexistence of both TNF-induced mitogenic and toxic effects in cells arrested in the G_0 phase. To better understand the relationship between both types of TNF-induced signals, it became important to establish which pool of cells were sensitive to the cytokine: those that remained in the G_0 phase, those that entered the cell cycle or both types of cells equally. In order to study that phase of the cycle where cells die, we analyzed simultaneously the cell cycle and the loss of

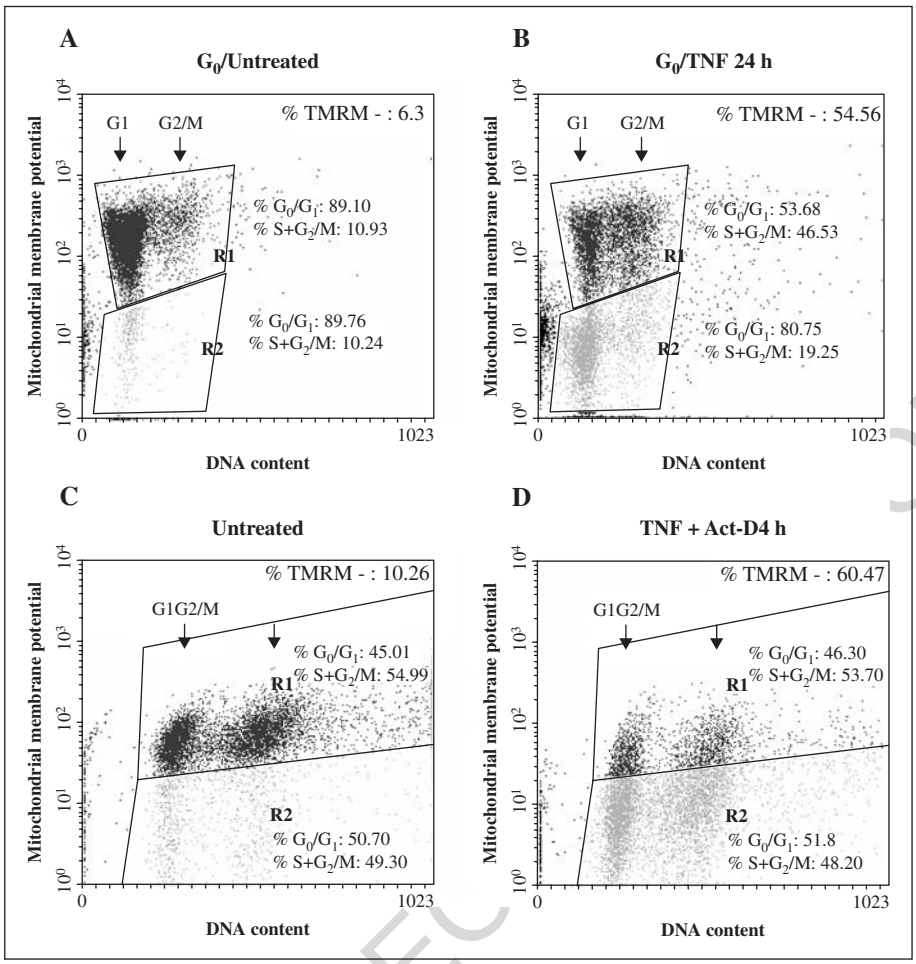

Figure 2

Preferential induction of death in G_0/G_1 cells after TNF treatment of G_0-arrested cells. NIH 3T3 cells were synchronised in G_0 (**A, B**) or left asynchronous (**C, D**) and treated with 10 ng/ml TNF for 24 hours (B), with TNF plus 0.1 µg/mL Act-D for 4 hours (**D**) or left untreated (**A, C**). Subsequently, the loss of mitochondrial potential and the DNA content were analyzed simultaneously by flow cytometry. Live cells (with high mitochondrial potential) and dying cells (with low mitochondrial potential) were gated and computed (R1 and R2 gates respectively). Likewise, the percentages of the cells in G_0/G_1 and $S+G_2/M$ phases in both gates are indicated.

mitochondrial potential by flow cytometry. TNF promoted the entry of G_0-synchronized cells into the cell cycle. Thus, in untreated cultures, about 10% of cells were in S or G2/M phases and only 6% of cells showed low mitochondrial potential (*figure 2*A). In cultures treated with TNF, 46% of cells were in S or G2/M. In agreement with the data shown above, TNF also induced a cytotoxic effect. Thus after treatment, 54% of the cells showed low mitochondrial potential. Notably, most of the cells that progressed to S or G_2 phases by effect of TNF conserved high mitochondrial potential, while most of the cells that lost mitochondrial potential remained in the G_0 phase (*figure 2*B). Untreated cells showed a similar proportion of cells with high or low mitochondrial potential, in each phase of the cycle, in both cases.

On the other hand, treatment of unsynchronized cells with TNF and actinomycin D (Act-D) for 4 hours was sufficient to induce the loss of mitochondrial potential in any phase of the cell cycle (*figure 2*C, D).

Taken together, these data suggest that TNF-induced mitogenic signals cannot be completed in the whole population of G_0-arrested cells, and this would trigger death signals in cells unable to proliferate.

TNF induces cell death in NIH 3T3 cells arrested at different points of the cell cycle

We next determined whether the ability of TNF to induce cell death associated with the impossibility to complete the mitogenic signals, is specific to the G_0 phase or, whether it is independent of the phase as long as cell growth has been blocked. To address this question we studied the toxicity of TNF in NIH 3T3 cells arrested at different points of the cell cycle. Specifically, we arrested cells in the G_1-S phase border (G_1/S) by treatment with the inhibitor of DNA polymerase, II aphidicolin; in the S phase by treatment with the inhibitor of ribonucleotide reductase, hydroxyurea or in mitosis by treatment with nocodazole, a compound that affects the assembly of microtubules (*figure 3*A). Once the arrest was achieved by treatment for 24 hours with the relevant drug, we treated the cells with TNF in the presence of the synchronicity agent for another 24 hours. After the treatment, we washed out the floating dead cells and quantified the remaining attached cells using sulforhodamine B. We found 50, 60 and 45% of TNF-induced death in the cultures arrested in the G_1/S, S and M phase respectively (*figure 3*B). The percentage of TNF-

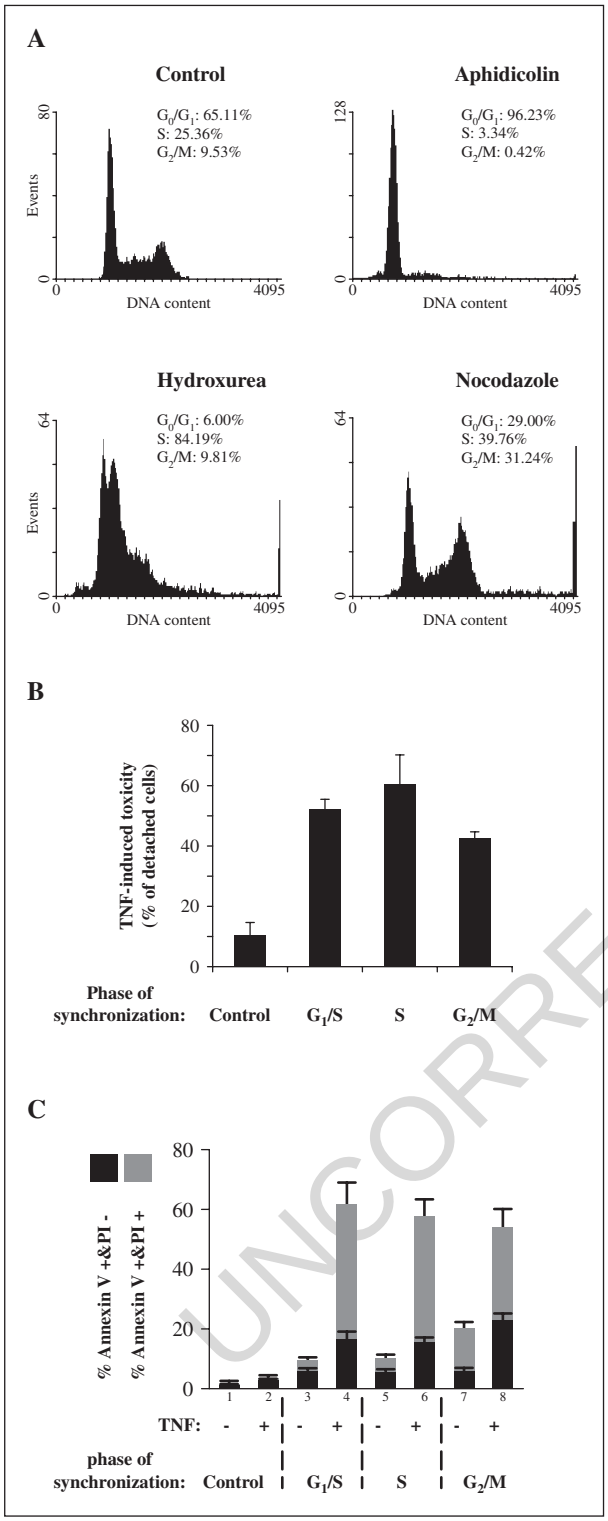

Figure 3

TNF kills cells arrested in all the phases of the cell cycle. **A**) NIH 3T3 cycling cells were left untreated (control) or synchronized in different phases of the cell cycle by treatment for 24 hours with 2 μg/mL aphidicolin (G_1/S phase), 200 μM hydroxyurea (S phase) or 0.1 μg/mL nocodazole (mitosis). Cell cycle profiles and the percentage of cells in each phase after the treatments are shown. **B, C**) Arrested and control cells were treated with 10 ng/mL TNF for 24 hours in the presence of the blocking agent. After the treatment, the number of cells remaining attached to the plate was quantified by staining with sulforhodamine B and the percentage of death compared to the corresponding controls without TNF is shown (**B**). The induction of apoptosis was also assayed by Annexin V binding. The percentages of Annexin V +/PI – (early apoptosis) or Annexin V +/PI + (late apoptosis) cells are shown (**C**).

induced cell death in cycling cells was less than 10%, and cell death due to the synchronizing agent did not exceed 10% in any case.

This TNF-induced toxicity was mediated by apoptosis as indicated by annexin V binding assays (*figure 3*C). There was a significant increase of annexin V-positive cells (both PI-positive and negative) after TNF treatment of the arrested cells. Moreover, these cells showed a characteristic apoptotic morphology: they became smaller, rounded, shrunken and, when transfected with the EGFP-F expression plasmid, showed an significant increase of the GFP fluorescence intensity due to the condensation of the cell, as previously observed [17] (a quantification of G_1/S- and S phase-arrested cells presenting this morphology is shown in *figure 4*C).

Thus, TNF induced apoptosis in NIH 3T3-arrested cells, but not in cycling cells and this cytotoxic effect is independent of the phase of the cell cycle in which cells are arrested. Therefore, it seems that TNF cytotoxic signals occurs only when the mitogenic signals cannot be completed by the blocking of the cell cycle progression at any point.

TNF-induced apoptosis in arrested cells is not due to a deficient activation of NFκB

The activation of NFκB has been revealed as one of the most important survival and proliferation signal triggered by TNF [4, 7], and its inhibition enhances TNF-induced toxicity [5, 6, 8]. We investigated whether the increase in TNF toxicity in NIH 3T3-arrested cells was due to a deficient activation of NFκB. We carried out EMSA assays using nuclear extracts of arrested or cycling cells treated or not with TNF. This cytokine induced a similar NFκB binding to DNA in cells arrested in G_0, S or M phases or in cycling cells (*figure 4*A). Moreover, this TNF-induced increase in DNA-binding activity correlated with the expression of the reporter gene SEAP under the control of NFκB, and was prevented by overexpression of a dominant-inhibitor IκBα mutant, (IκB-mut; *figure 4*B). This mutant has been reported to induce sensitivity to TNF in cycling cells [8, 20]. When we inhibited the NFκB-protective pathway in G_1/S or S phase-arrested cells by overexpression of IκB-mut, we found a further increase in the percentage of apoptotic cells observed after the TNF treatment (*figure 4*C), suggesting that this pathway was functional in arrested cells. Taken together, these data indicate that the increased toxicity of TNF in arrested cells is not due to the loss of the anti-apoptotic activity of NFκB.

TNF-induced apoptosis in arrested cells requires the mitochondrial pathway

As described above, TNF induced apoptosis in NIH 3T3 cells arrested in any phase of the cell cycle, eliminating the well-known requirement of simultaneous inhibition of protein synthesis. This type of sensitization to TNF has been described in other cell types arrested in the S phase [14], but little is known about the apoptotic signalling in these conditions. To address this issue, we analyzed the participation of the mitochondria in TNF-induced apoptosis in NIH 3T3 cells arrested in the G_1/S phase.

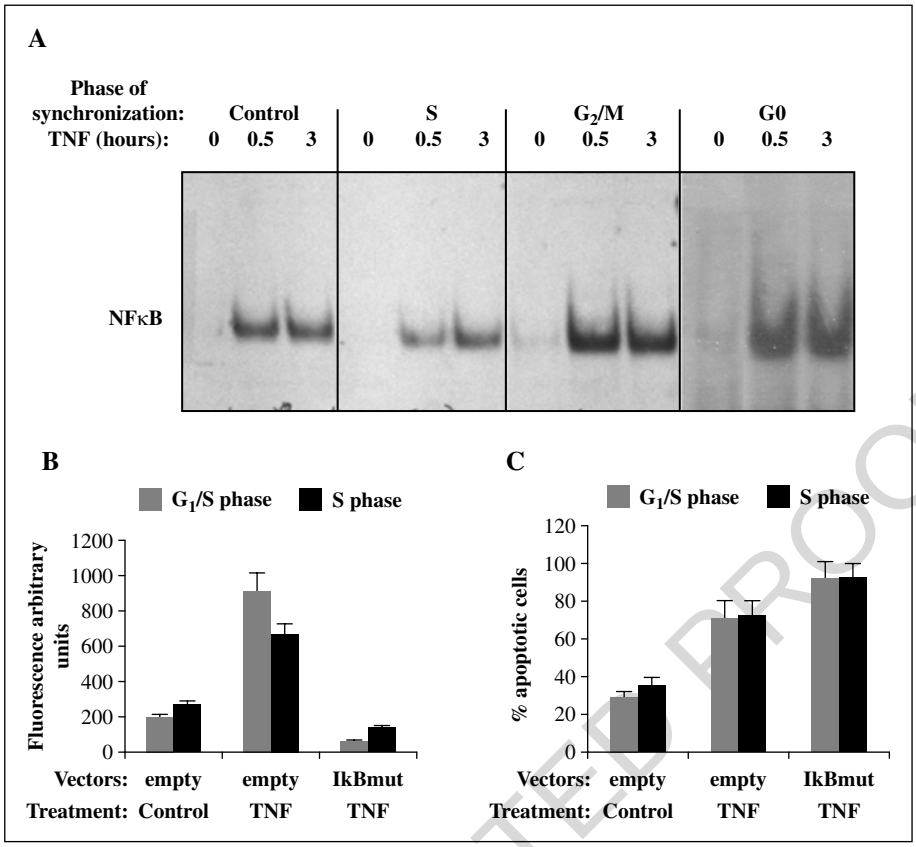

Figure 4

TNF-induced apoptosis in arrested cells is not due to the lack of NFκB activation. **A**) NIH 3T3 cycling cells (control) or cells arrested in S (S), mitosis (M) or G_0 phases (G0) as described in methods and materials were treated with 10 ng/mL TNF for the indicated times in the presence of the synchronizing agent. Subsequently, nuclear extracts were assayed for NFκB binding to DNA by EMSA. **B**) G_1/S or S phase synchronized cells were transfected with 0.25 μg of the NFκB-SEAP plasmid and with 1 μg of the indicated plasmids. Then, transfected cells were treated or not with TNF for 24 hours in the presence of the synchronizing agent. After treatment, aliquots of the culture medium were collected and assayed for SEAP activity. This activity expresses NFκB-mediated gene expression. **C**) G_1/S or S phase synchronized cells were transfected with 0.2 μg of the EGFP-F plasmid and with 1 μg of the indicated plasmids and treated as in **B**. Subsequently, EGFP-F positive cells were visualized by fluorescence microscopy and the percentage of them showing apoptotic morphology was estimated by counting more than 500 cells in six randomly chosen fields of each condition.

The mitochondrial potential of the G_1/S-arrested cells remained unaffected in most cells after seven hours of TNF treatment, although after 24 hours more than 50% of cells showed low mitochondrial potential (*figure 5*A). The treatment of cycling cells with TNF for 24 hours did not affect the mitochondrial potential. This behaviour of the mitochondrial potential contrasted with that observed for cycling cells treated with TNF/Act-D. In this case, after four hours of treatment, more than 50% of cells showed low mitochondrial potential and after six hours more than 90% of cells showed uncoupled mitochondria (*figure 5*B). Cytochrome c can be detected in the cytosol in G_1/S-arrested cells after one hour of treatment with TNF, reaching the maximum level after eight hours (*figure 5*C), well in advance of the loss of mitochondrial potential. The synchronization process itself did not induce the release of cytochrome c. On the other hand, the release of cytochrome c from the mitochondria and the loss of mitochondrial potential after TNF/Act-D treatment of cycling cells followed similar kinetics (*figure 5*D).

The activation of the mitochondrial pathway might not be essential for the apoptotic mechanism triggered by death receptors [9]. To study the relevance of this pathway in the apoptotic mechanism induced by TNF in G_1/S arrested cells or cycling cells co-treated with Act-D, we ectopically expressed the anti-apoptotic protein Bcl-2, a well-known inhibitor of the mitochondrial pathway. The co-transfection with a green fluorescent protein (GFP) variant allowed us to detect the transfected cells either by flow cytometry or fluorescence microscopy. This variant, a membrane-targeted GFP (EGFP-F; [21]), is retained in dying cells, avoiding the leaking shown when soluble GFP is used [17]. In the experiments carried out with arrested cells, we found a high level of apoptotic cells (approximately 30%) in the control conditions (arrested cells transfected with an empty vector and left untreated). This percentage of abnormally high toxicity is probably due to the process of transfection of arrested cells.

We first checked the ability of Bcl-2 to prevent the mitochondrial apoptotic events. We found that Bcl-2 overexpression prevented significantly TNF-induced loss of mitochondrial potential in arrested cells (*figure 6*A). To see if Bcl-2-mediated inhibition of the mitochondrial pathway corresponds with an effective inhibition of the cell death, we analyzed two late apoptotic events. We found that overexpression of Bcl-2 prevented both the appearance of cells with apoptotic morphology (*figure 6*B) and the loss of the cell membrane integrity (*figure 6*C) in cells arrested in the G_1/S border and treated with TNF for 24 hours. Similar results were obtained after TNF/Act-D treatment of unsyn-

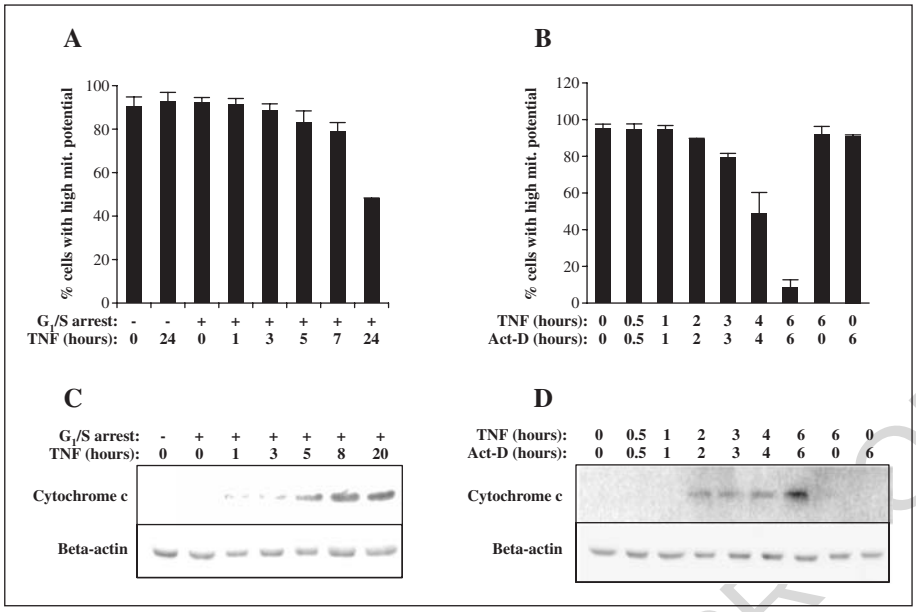

Figure 5

TNF-mediated activation of the mitochondrial apoptotic pathway. NIH 3T3 cells were arrested in the G_1/S phase and treated with 10 ng/mL TNF in the presence of the blocking agent (**A, C**) or left asynchronous and treated with TNF and 0.1 µg/mL Act-D (**B, D**) for the indicated times. Subsequently, changes in mitochondrial membrane potential were assayed (**A, B**) and the percentages of the cells presenting high mitochondrial potential after the treatment are shown. Alternatively, cytosolic extracts were prepared for every condition and the presence of cytochrome c was detected by western blot analysis using a monoclonal anti-cytochrome c antibody (**C, D**). β-actin levels in the extracts were included as loading controls.

chronized cells overexpressing Bcl-2 (*figure 6*D, E). These data indicate that the mitochondrial pathway is an essential step in TNF-induced apoptosis in NIH 3T3 sensitized either by cell cycle arrest or RNA synthesis inhibition, even though the kinetics of cytochrome c release and mitochondrial collapse appear to be quite different in both conditions.

DISCUSSION

TNF is a pleiotropic cytokine that is at the origin many different types of responses including proliferation or cell death. This variety of effects depends strongly on the cell type. Thus in many tumoral cells, TNF can induce cell death while stimulating the growth of normal cell types

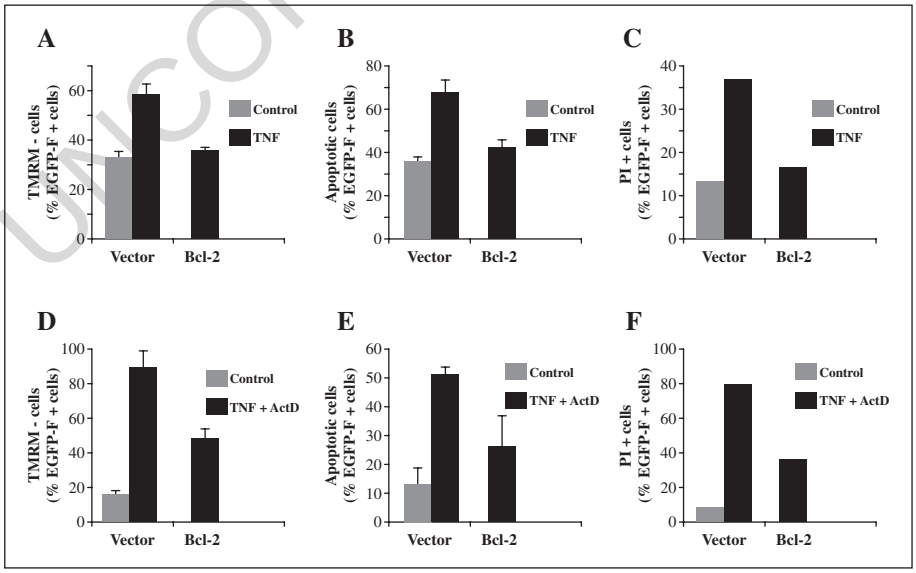

Figure 6

Bcl-2 overexpression prevents TNF-induced death. NIH 3T3 cells arrested in the G_1/S border (**A-C**) or left asynchronous (**D-F**) were transfected with 0.2 µg EGFP-F and with 1 µg of the indicated plasmids. Subsequently, G_1/S arrested were treated or not (control) with 10 ng/mL TNF for 24 hours in the presence of the blocking agent (**A-C**) and asynchronous cells were treated or not with TNF and 0.1 µg/mL Act-D for seven hours (**D-F**). After the treatment, transfected cells were assayed for the loss of mitochondrial potential by simultaneous analysis of TMRM and EGFP-F fluorescence intensities by flow cytometry (**A, D**). Alternatively, EGFP-F positive cells were visualized by fluorescence microscopy and the percentage of them presenting apoptotic morphology was estimated by counting six randomly chosen fields of each condition (**B, E**) or the loss of plasma membrane integrity in transfected cells was determined by analysis of PI incorporation to the EGFP-F positive cells by flow cytometry (**C, F**).

[1]. TNF can also induce both effects in the same cell type [5, 10] indicating the influence of other factors in the type of response to TNF. Moreover in some cases, a relationship between the anti-proliferative effects of TNF and progression through the cell cycle has been observed [11]. It is already known that RNA synthesis inhibition or the repression of NFκB activation alters the response of cells to TNF, so that cells become sensitive to the toxic action of TNF [5-7]. In the present study, we show that the TNF-induced mitogenic effect can be superseded by the apoptotic signals when cell growth is inhibited at different points of the cell cycle.

TNF treatment of NIH 3T3 cells arrested in the G_0 phase induces a significant increase in the percentage of cells that reach the S phase of the cell cycle *(figure 1)*. However, rather than triggering cell proliferation, the final effect of the TNF is a significant decrease of the number of cells *(figure 2)*. Moreover, TNF induces cell death of G_0 phase-arrested in a dose-dependent manner (data not shown). These data agree with those previously observed in 3T3-L1 pre-adipocites [22]. Based on these observations, three models of the relationship between mitogenic and cytotoxic signals induced by TNF in G_0-arrested cells may be proposed: 1) TNF-induced mitogenic signals could be part of the toxic effect. In this model, TNF would favour entry into the S phase and would subsequently induce cell death at some point of the S, G_2 or M phases. In this case, cell death would affect those cells that had incorporated BrdU, while cells remaining at the G_0 phase would be protected. 2) TNF would induce mitogenic signals. However, the shortage of some essential growth factors required for TNF-induced cell proliferation in the low serum condition, would not allow the whole population to enter into the cell cycle. The inability of cells that have received TNF mitogenic signals to grow would trigger the cytotoxic signal. In this case, death would affect non-proliferating cells, i.e. those that remain in the G_0 phase. 3) The mitogenic and the cytotoxic signals would be completely independent. In this case, death would affect equally proliferating and non-proliferating cells.

By the simultaneous analysis of the loss of mitochondrial potential and the DNA content *(figure 3)*, we found that death occurs specifically in non-proliferating cells, i.e. those that remained in the G_0 phase, while for those cells presenting at the S phase, DNA content was protected. These data agree with the second of the proposed models. The fact that the G_0-arrested cells treated with TNF in the presence of serum are resistant to the toxic action of TNF *(figure 1)* is also in agreement with this model. In a rich medium, TNF mitogenic signals would not be blocked by the lack of a given growth factor and the toxic effect would not take place. Also in agreement with this hypothesis is the fact that it has been shown that cells can be protected against TNF-induced toxicity by the expression of several growth factors, such as PDGF-B or bFGF [23, 24]. Moreover, TNF-induced proliferation requires the synthesis of GM-CSF [25].

NIH 3T3 cells also became sensitive to TNF when arrested in other phases of the cell cycle (G_1/S, S or M) *(figure 4)*. On the other hand, exponentially growing cells treated with TNF/Act-D lost mitochondrial potential in a similar proportion in all phases of the cell cycle (*figure 2*B, C, D). Therefore, TNF-induced toxicity is more likely related to the inability to grow rather than a particular phase of the cell cycle. However, there are some contradictory data in this regard. Human cervix carcinoma HeLa cells arrested in the S phase, but not in G_1, are more sensitive to the toxic effect of TNF [14]. On the other hand, S phase-block does not sensitize the murine fibrosarcoma L929 cell line to TNF [26]. These differences could be explained by the fact that cell cycle checkpoints are frequently disrupted in cancer cells and this could lead to a lack of sensitivity of cells arrested in a particular phase of the cell cycle to TNF. Non-transformed cells, such as NIH 3T3 on the other hand, became sensitive to TNF after any kind of arrest as they retained all the cell cycle controls.

In the present work, we describe for the first time, different aspects of the TNF apoptotic pathway in cells sensitized by cell cycle-arrest. In particular, we have studied cells arrested in the G_1/S border by treatment with aphidicolin. We found interesting differences in the kinetics of the activation of the mitochondrial apoptotic events depending of the type of sensitization to TNF. In cells treated with TNF/Act-D, the loss of mitochondrial potential and the release of cytochrome c are both completed during the first six hours of treatment. On the other hand, in arrested cells, the loss of mitochondrial potential shows an significant delay compared to the release of cytochrome c *(figure 5)*. These results suggest that both forms of sensitization lead to TNF cytotoxicity, but by different mechanisms. This observation is also supported by the fact that the TNF-induced cell death in G_0-arrested NIH 3T3 cells is significantly accelerated when transcription is inhibited by Act-D (data not shown). Different types of relationship between the loss of mitochondrial potential and the release of cytochrome c, depending on the apoptotic system, have been reported. In some cases, the loss of mitochondrial potential is an early event and coincides with cytochrome c release, as we observed after TNF/Act-D treatment. However in other models, is only a later sign of apoptosis, similar to the case of arrested cells treated with TNF (see [27] for a review). Moreover, the overexpression of Bcl-2 both in G_1/S-arrested cells and in cycling cells treated with TNF/Act-D prevents not only the loss of mitochondrial potential induced by TNF but also other apoptotic hallmarks such as the appearance of apoptotic morphology and the loss of plasma membrane integrity. This suggests that in these cells, the activation of the mitochondrial pathway is indispensable for TNF-mediated induction of apoptosis after both types of sensitization [9].

Similarly, it has been previously reported that the inhibition of RNA synthesis and the repression of NFκB activation also caused sensitivity to TNF by different pathways [6]. We checked if the TNF-induced toxicity in arrested cells is due to a defective activation of NFκB protective pathway. We found that NFκB is equally activated by TNF in cycling cells and in cells arrested in the different phases of the cell cycle *(figure 5)*, in agreement with previous reports [28]. Moreover, the inhibition of NFκB in arrested cells further increases the extent of TNF-induced apoptosis, suggesting that the blockage of cell cycle progression and the inhibition of NFκB are two independent mechanisms for sensitization to TNF.

In summary, we show here that TNF induces proliferation signals in non-transformed NIH 3T3 cells, which can lead to apoptosis when the cell growth is impaired and at any phase of the cell cycle. These findings should be taken in account when investigating the ability of TNF to enhance

the toxicity of cancer chemotherapeutic agents such as doxorubicin [29, 30]. This, and other drugs, can induce cell cycle-arrest in non-transformed cells [31, 32], which in turn could increase the risk of unwanted toxicity in normal cells after TNF treatment. We have also found that the apoptotic mechanism triggered by TNF in the arrested cell is different from that previously described for other known models of sensitization, inhibition of RNA synthesis or repression of NFκB activation.

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