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ARTICLES

Apoptosis and DNA Fragmentation Precede TNF-Induced Cytolysis in U937 Cells

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Abstract The hypothesis that activation of apoptosis and DNA fragmentation is involved in TNF-mediated cytolysis of U937 tumor cells was investigated. Morphological, biochemical, and kinetic criteria established that TNF activates apoptosis as opposed to necrosis. Within 2–3 h of exposure to TNF, U937 underwent the morphological alterations characteristic of apoptosis. This was accompanied by cleavage of DNA into multiples of nucleosome size fragments. Both of these events occurred 1–2 h prior to cell death as defined by trypan blue exclusion or ^{51}Cr release. DNA fragmentation was not a non-specific result of cell death since U937 cells lysed under hypotonic conditions did not release DNA fragments. The percentage of cells undergoing apoptosis depended on the concentration of TNF and was augmented by the addition of cycloheximide. A TNF-resistant variant derived from U937 did not undergo apoptosis in response to TNF, even in the presence of cycloheximide. Furthermore, TNF could still activate NFkB in this variant, suggesting that this pathway is not involved in TNF-mediated cytotoxicity. Two agents known to inhibit TNF-mediated cytotoxicity, $ZnSO_4$ and 3-aminobenzamide, were shown to inhibit TNF-induced apoptosis. Taken altogether, these data support the hypothesis that activation of apoptosis is at least one essential step in the TNF lytic pathway in the U937 model system.

Key words: TNF, apoptosis, U937, DNA fragmentation

Tumor necrosis factor (TNF) is a pleiotropic cytokine that was originally described as a factor mediating hemorrhagic necrosis of tumors in experimental animal models [1]. Although the mechanism by which TNF lyses some but not all tumor cells has been studied for many years, it is still not well understood [for review see 2]. Numerous studies have focused on early events in the TNF lytic pathway such as TNF-receptor interaction and signal transduction. However, little is known about the actual lethal event. In general, two different classifications of cell death have been defined based primarily on morphological criteria [for review see 3]. Necrosis is a degenerative phenomenon characterized by swelling of the whole cell and organelles, loss of membrane integrity, and irregular clumping of chromatin [3]. In contrast, cells undergoing apoptosis or programmed cell death (PCD) show cytoplasmic condensation and breaking up of the nuclei into discrete chromatin-containing fragments [3]. The most obvious morphological alterations observed by light microscopy are the numerous elongated cytoplasmic protuberances that eventually separate to produce membrane-bounded apoptotic bodies that may even contain intracellular organelles. The two types of cell death may also be distinguished by different patterns of DNA fragmentation. In necrosis, DNA fragmentation is thought to be the result of cell degeneration leading to release of lysosomal DNases that then cleave DNA into fragments of a continuous spectrum of sizes [4]. In apoptosis, the cell initiates a suicide program by activation of an endonuclease which then cleaves DNA in the internucleosomal linker regions resulting in fragments of multiples of ~ 180 base pairs [4–10]. These two types of cell death also differ with respect to the circumstances under which they occur in vivo. Thus, necrosis is generally regarded as a pathological response, whereas apoptosis appears to function in homeostatic

Abbreviations used: FCS, fetal calf serum; NK, natural killer cells; PCD, programmed cell death; TNF, tumor necrosis factor; U9-TR, TNF resistant clone of U937 cells.

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regulation [reviewed in 11] or possibly in defense against viral infection [12,13].

In this study, we have employed the human histiocytic lymphoma, U937, in an in vitro model system to determine what type of cell death TNF induces in this tumor cell line. The few studies that have examined TNF-induced changes in cell morphology and DNA content have yielded different results depending on the tumor cell line studied [14]. It has not yet been determined whether U937 cells undergo apoptosis or necrosis in response to TNF. In order to distinguish between the two responses, it is critical to analyze the sequence of events. Thus, in PCD, the morphological changes of apoptosis accompanied by DNA fragmentation occur prior to cytolysis and may be the ultimate cause of cell death. In contrast, necrotic cells do not release soluble DNA prior to cell death, although it may occur later as a result of cell death.

This study examines the mechanism of TNF cytotoxicity in the U937 model system. We tested the hypothesis that TNF activation of apoptosis contributes to target cell lysis. Kinetic studies revealed that TNF-treated U937 cells rapidly undergo the morphological alterations of apoptosis as well as DNA fragmentation prior to cell death. These findings support the hypothesis that TNF activates apoptosis in this system.

MATERIALS AND METHODS

Cell Lines

The human histiocytic lymphoma, U937, was obtained from the ATCC. The WEHI-164 murine fibrosarcoma clone was originally obtained from Dr. Terje Espevik. All cell lines were maintained in RPMI 1640 supplemented with 10% FCS (Irvine Scientific, Santa Ana, CA), penicillin-streptomycin, non-essential amino acids (0.1 mM), and L-glutamine (2 mM). All cell lines were routinely tested for mycoplasma and always found to be negative according to the mycotect kit (GIBCO, Grand Island, NY).

Reagents

Purified human rTNF (specific activity $=1 \times 10^7$ U/mg) was purchased from Amgen (Thousand Oaks, CA). Cycloheximide and 3-aminobenzamide were purchased from Sigma (St. Louis, MO).

U9-TR Variant Selection Protocol

U937 cells were initially cloned by limiting dilution and one clone was selected that showed NK and TNF sensitivity similar to the original cell line. This clone has been used throughout this study and is referred to as U937. This precaution was taken to insure that variant selection did not result in the outgrowth of some non-U937 tumor cell contaminating the original cell culture. The U9-TR variant was selected by culturing the parental U937 clone in the presence of increasing concentrations of TNF that ranged from 0.5 ng/ml to 10.0 ng/ml over a period of 5 months. Periodically, cells undergoing selection were tested for TNF sensitivity after being cultured in the absence of TNF for at least 5 days. After the variants started to exhibit a stable level of resistance, they were employed for the experiments in this investigation. The selection and characterization of the U9-TR variant has been described previously [15]. Karyotype analysis confirmed that U9-TR was, in fact, derived from the parental U937 cell line (data not shown).

Apoptosis Assay

U937 cells were suspended at 1×10^6/ml in RPMI-1640 plus 2.5% FCS in the presence or absence of the indicated concentrations of TNF and/or cycloheximide. All assays described in this paper were set up in medium with 2.5% FCS in order to keep conditions the same as our standard TNF toxicity assay which U937 cells are incubated for 20 h in medium with 2.5% serum. Preliminary experiments established that this decrease in FCS concentration did not cause any decrease in viability or spontaneous DNA fragmentation as compared to 10% FCS for up to 24 h of incubation. Cultures were incubated at 37°C, and at different time points aliquots were removed and examined microscopically in the presence of trypan blue. Apoptotic cells were defined as those exhibiting 2 or more prominent membrane protuberances, whereas dead cells were defined as those that could not exclude trypan blue. In most experiments, the cultures were coded and counted blindly. At least 100 cells were counted for each sample and data are reported as the percent apoptotic and the percent dead cells.

Assessment of TNF-Induced DNA Damage

In this study we have employed 3 different methods to measure TNF-induced target cell DNA damage. The standard DNA fragmentation assay was performed according to a modification of a previously described method [16].

Target cells (either U937 or WEHI-164) were labeled with 20 μCi ^{3}H-thymidine/5 × 10^6 cells by incubating for 20 h at 37°C. Cells were washed 3 times, resuspended at 1 × 10^6/ml in RPMI 1640 plus 2.5% FCS, and 100 μl added to each Eppendorf tube in triplicate. Then 100 μl of the appropriate concentrations of medium alone, TNF, or cycloheximide was added and the tubes incubated at 37°C. At the indicated time points, tubes were harvested by adding 0.8 ml ice-cold PBS and spinning at 6,000*g* for 1 min. in a microfuge. Supernatants were removed for scintillation counting and the plasma membrane of the pelleted cells was lysed by the addition of 0.4 ml of 0.2% Triton X-100, 2 mM EDTA in PBS without Ca and Mg. Intact chromatin was separated from fragmented DNA by centrifugation at 13,000*g* for 15 min. Both lysate and pellet were counted in a scintillation counter. Percent DNA fragmentation was calculated as follows:

$$\%\text{DNA release} = \frac{\text{cpm supernatant} + \text{cpm lysate}}{\text{cpm supernatant} + \text{cpm lysate} + \text{cpm pellet}} \times 100.$$

Percent specific DNA fragmentation was the difference between the experimental and spontaneous percent DNA fragmentation.

The second method to analyze DNA fragmentation employed agarose gel electrophoresis. Target cells were treated with TNF and the DNA was harvested as described previously [17] under conditions similar to cell lysis in the above DNA fragmentation assay. Triton X-100 lyses the cytoplasmic membrane, whereas chelation of divalent cations with EDTA releases nuclear DNA. Since any DNA fragments released from the cell into the supernatant during TNF treatment are not recovered by this method, it is not as quantitative as the above described DNA fragmentation assay. After lysing the cells, debris was removed by centrifugation at 13,000*g* for 10 sec. DNA in the supernatant was ethanol precipitated after phenol extraction. Equivalent amounts of material from a fixed number of cells were loaded and electrophoresed on a 1.0% agarose slab gel.

Although one advantage of the first DNA fragmentation method is that it is quantitative, a major disadvantage is that it is inconvenient to assay a large number of samples. To overcome this limitation, we developed a DNA release assay, which is similar to a technique used previously to assess cytotoxic T lymphocyte-induced nuclear damage by release of labeled DNA from target cells [18]. U937 cells were labeled with ^{3}H-thymidine as in the standard DNA fragmentation assay. The assay was set up in triplicate in a flat bottom 96 well microtiter plate in RPMI 1640 with 2.5% FCS in a total volume of 0.05 ml. Target cells were suspended at 1 × 10^6/ml and 0.025 ml was added to each well. Wells for spontaneous release and total counts received an additional 0.025 ml of medium, whereas experimental wells received 0.025 ml of the appropriate concentrations of TNF or cycloheximide. Plates were incubated for the indicated length of time (not to exceed 5 h) at 37°C. The assay was harvested by addition of 100 μl 10 mM Tris, 10 mM EDTA, 0.3% Triton X-100 to each well except the total. Plates were harvested using a Skatron harvester and samples were counted in a scintillation counter. The percent DNA release was calculated as follows:

$$\%\text{DNA release} = \frac{\text{total cpm} - \text{experimental cpm}}{\text{total cpm}} \times 100.$$

Using this technique, the % spontaneous DNA release varied from 0 to 5% over a 5 h incubation period. Due to space limitations in some of the graphs, standard deviations were omitted. However, for every data point they were always less than 15% of the mean value for this assay. Preliminary experiments were performed to determine how the results of this assay compare to the standard DNA fragmentation assay described above. When we compared the kinetics of DNA damage induced by TNF 1.0 ng/ml plus cycloheximide (0.5 μg/ml) over a 1 to 5 h incubation period, we observed no significant differences in the two assays. Therefore, we conclude that the DNA release assay closely correlates with the standard DNA fragmentation assay, at least during a 5 h incubation period.

^{51}Cr Release Assay

U937 target cells were prepared by labeling overnight at 5 × 10^5/ml in the presence of ^{51}Cr at 0.02 mCi/ml. Target cells were washed 3 times and plated at 5 × 10^3 cells/well in round bottom microtiter plates. The medium used for

this assay was the same as that used to culture the cells except that the concentration of FCS was 2.5%. Wells for spontaneous and total ^{51}Cr release contained target cells and medium only, whereas test wells contained different concentrations of cytokines or drugs. After incubating at 37°C for the indicated length of time, the ^{51}Cr released into the supernatant was determined. The percent specific ^{51}Cr release was calculated as follows:

[(Test cpm − spontaneous cpm) divided by

(Total cpm − spontaneous cpm)] × 100.

Nuclear Extracts and Electrophoretic Mobility-Shift Assay

Mini nuclear extracts were prepared using a modified protocol of Schreiber et al. [19]. One million cells were washed once in PBS, resuspended in ice-cold 10 mM Hepes, 10 mM KCL, 0.1 mM EDTA, 1.0 mM DTT, and 0.5 mM PMSF, and lysed by addition of 0.4% NP-40. Nuclei were pelleted, resuspended in 0.1 ml cold 20 mM Hepes, 0.4 MNaCl, 1.0 mM EDTA, 1.0 mM DTT, and 1.0 mM PMSF, and rocked for 15 min at 4°C. Nuclear extracts were clarified by centrifugation and incubated with an end ^{32}P-labeled NFkB fragment or an appropriate control. DNA-protein complexes were analyzed by electrophoresis on a native 4% polycrylamide gel.

RESULTS

TNF Induces Apoptosis in the U937 Cell Line

Initial experiments were performed to determine if we could visualize TNF-induced apoptosis in U937 cells by light microscopy. U937 cells were cultured under identical conditions in the presence and absence of TNF at 37°C. At various time intervals aliquots were removed and examined microscopically in the presence of trypan blue. Untreated U937 cells exhibit their typical non-adherent fairly round morphology as shown in Figure 1A. However, after only 2 h of treatment with TNF, some of the cells still appear normal, whereas others exhibit dramatic morphological alterations characteristic of apoptosis (Fig. 1B). The most obvious change is the exaggerated cytoplasmic protuberances characteristic of apoptotic cells. Numerous apoptotic bodies, which are membrane enclosed vesicles that have budded off the cytoplasmic extensions, are also visible. Using time-lapse microcinematography we have actually observed these vesicles budding off the cytoplasmic protuberences of TNF-treated U937 cells (data not shown). Several of the cells are already dead as evidenced by their inability to exclude trypan blue, in contrast to the apoptotic cells which are still viable by this criterion. These findings do not rule out the possibility that some of the dead cells may

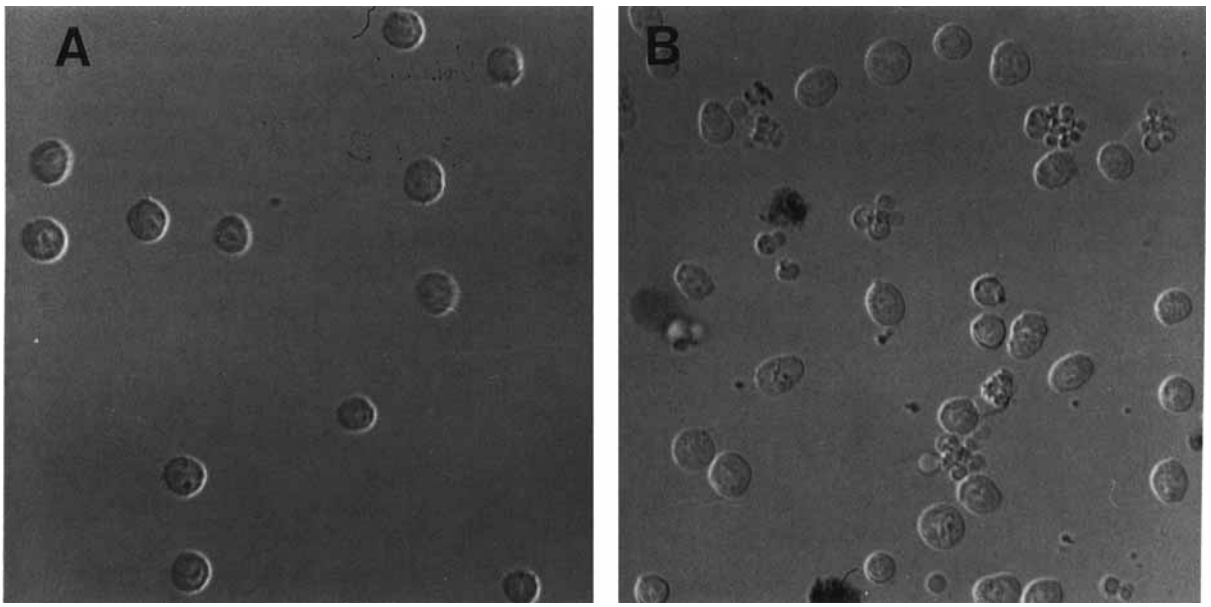

Fig. 1. Morphology of normal (**A**) and apoptotic (**B**) U937 cells. U937 cells were incubated in RPMI 1640 plus 2.5% FCS for 2 h at 37°C without (A) or with (B) TNF (10 ng/ml).

have undergone necrosis as opposed to apoptosis. However, the results do show that TNF induces apoptosis in a significant percentage of cells, and that it should be feasible to assay this response microscopically. In setting guidelines for a routine apoptosis assay, we identify any cell exhibiting 2 or more cytoplasmic blebs as apoptotic, whereas trypan blue uptake identifies dead cells.

Experiments were then performed to determine the dose response of TNF-induced apoptosis in U937 cells. As shown in Figure 2, the percentage of apoptotic cells was directly proportional to the TNF concentration ranging from 1–5 ng/ml. The TNF dose response was similar when one measures cell viability after 4 h of incubation (data not shown). At higher TNF concentrations, the frequency of apoptotic cells was seen to plateau at 20%.

Relative Kinetics of Apoptosis, DNA Fragmentation, and Cell Death

If TNF induces programmed cell death in U937 cells, it would be predicted that both apoptosis and DNA fragmentation should be detectable prior to cytolysis. This was tested directly. Parallel cultures were set up to measure apoptosis as well as DNA fragmentation in the presence and absence of 10 ng/ml TNF after different periods of incubation. As can be seen in Figure 3, the percentage of apoptotic cells peaked at 2 h and then declined. At 2 h there was also a significant increase in DNA fragmentation which continued to increase for up to 4 h. In contrast, there was no significant increase in the release of ^{51}Cr until after 3 h of incubation. Similar results were obtained when cytotoxicity was measured by trypan blue exclusion in the apoptosis assay (data not shown). We have performed similar kinetic experiments many times and have observed some day to day variation in the kinetics of onset and peak of apoptosis. Peak apoptotic responses have been observed to occur following 1.5–3.0 h of incubation. However, the relative sequence of events is always the same. The peak of apoptosis coincides with a significant increase in DNA fragmentation prior to any appearance of cell death. It is only when the percentage of apoptotic cells declines that a significant increase in dead cells is observed.

We also analyzed the pattern of DNA fragmentation induced by TNF at different time points. The results shown in Figure 4 indicate that marked DNA fragmentation is detectable after 1 h of incubation with TNF. As discussed in Materials and Methods, this procedure does not quantitatively reflect total cell DNA fragmentation. The method only reveals intracellular DNA frag-

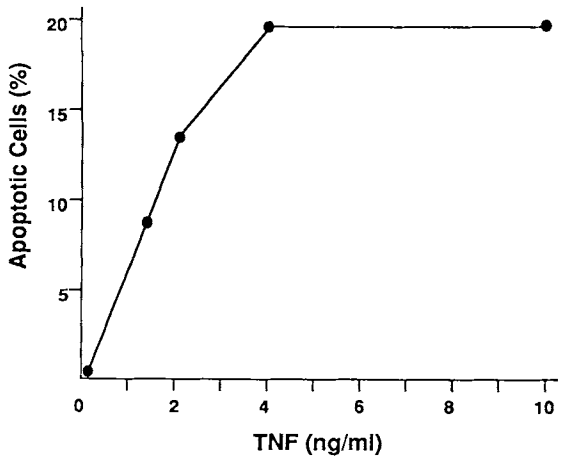

Fig. 2. Dose response of TNF-induced apoptosis. U937 cells were incubated with different concentrations of TNF for 2 h at 37°C and then the percentage apoptotic cells assessed microscopically in the presence of trypan blue. Control cells incubated without TNF always exhibited less than 2% apoptotic cells (data not shown).

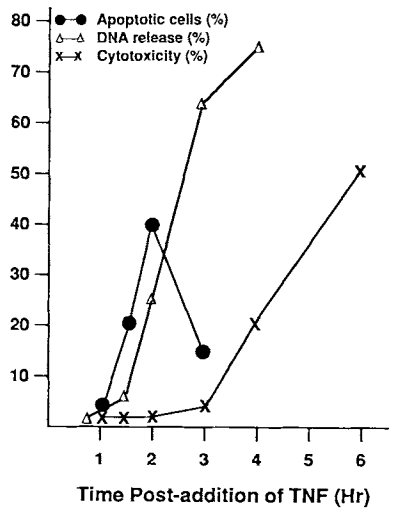

Fig. 3. Kinetics of apoptosis, DNA release, and cell death. Parallel cultures of U937 cells were set up in the presence of TNF (10 ng/ml). At different time points, cells were removed to determine apoptosis, DNA release, or cell death. DNA release was determined by measuring release of ^{3}H-thymidine labeled DNA as described in Materials and Methods. Cell death was determined by ^{51}Cr release. In control cultures lacking TNF there were less than 2% apoptotic cells and less than 5% dead cells as determined by trypan blue exclusion (data not shown).

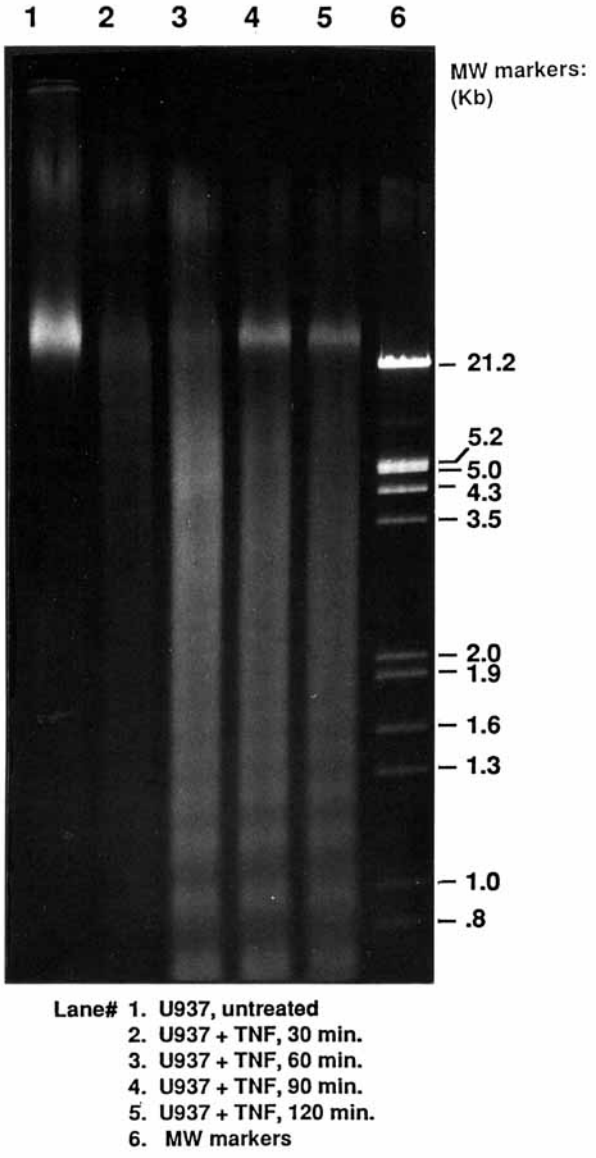

Fig. 4. DNA fragmentation induced by TNF. U937 cells were incubated in the presence of TNF (10 ng/ml) for different lengths of time and then DNA was extracted and analyzed by gel electrophoresis as described in Materials and Methods.

ments and not those released into the cell supernatant. However, it can readily be seen that the DNA is fragmented into multiples of ~ 180 base pairs, which is the typical pattern seen in programmed cell death and not in necrosis. This result, along with the kinetics experiments, demonstrates that TNF activates apoptosis in at least a subpopulation of U937 tumor cells.

Cycloheximide Augments TNF-Induced Apoptosis

It has been shown that some types of PCD, such as that induced by glucocorticoid treatment of thymocytes [5], can be blocked by inhibitors of protein synthesis. However, other examples of apoptosis, such as that mediated by cytotoxic T lymphocytes [7] are not affected by such inhibitors. Therefore, it was of interest to determine if cycloheximide has any effect on TNF-induced apoptosis in U937 cells. Parallel cultures were set up in which U937 was incubated with cycloheximide (1.0 μg/ml) alone, TNF (1.0 ng/ml) alone, or both combined, and cells were examined microscopically at different time points. The results shown in Figure 5A indicate that cycloheximide alone does not induce apoptosis. However, when combined with TNF, it causes a twofold increase in the percentage of apoptotic cells over that seen with TNF alone. The peak of apoptosis was observed at 2 h of incubation and declined at 3 h. Consistent with

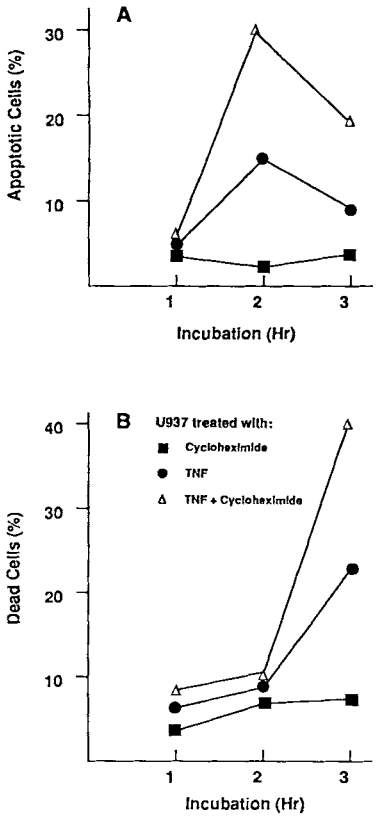

Fig. 5. Cycloheximide augments TNF-induced apoptosis. Cultures of U937 were set up with cycloheximide (1.0 μg/ml), TNF (1.0 ng/ml), or a combination of both and incubated at 37°C. At different time points cells were removed and assessed microscopically for the percent apoptotic cells (**A**) and the percent dead cells by trypan blue exclusion (**B**). Cells cultured in the absence of TNF or cycloheximide exhibited less than 2% apoptotic cells and less than 5% dead cells at any time point (data not shown). This experiment was repeated on two other occasions with similar results.

previous observations, at 2 h very few dead cells were observed in these cultures (Fig. 5B). However, by 3 h there was a substantial increase in the percentage of dead cells and cycloheximide also caused a twofold increase in cell death over that seen with TNF alone. Our preliminary studies have shown that under these conditions of treatment with cycloheximide the uptake of ^{3}H-amino acids is inhibited by 95–99%. Therefore, these results suggest that protein synthesis is not required for TNF to activate apoptosis in U937 cells. Furthermore, since cycloheximide appears to increase target cell sensitivity to TNF, these two agents were used in combination in subsequent experiments to study the activation of apoptosis.

Susceptibility of Other Cell Lines to TNF-Induced Apoptosis

The development of TNF-resistant variants derived from the parental U937 has been a very useful tool to analyze the TNF lytic mechanism in this model system. Since the U9-TR variant still expresses TNF receptors that undergo TNF-induced internalization (unpublished observations), the TNF lytic pathway must be blocked at some subsequent step. Since our results indicate apoptosis is an intermediate step following TNF-receptor interaction and occurs prior to cell death, we wanted to determine if U9-TR will undergo apoptosis in response to TNF. The results shown in Figure 6 demonstrate that U9-TR treated with TNF plus cycloheximide does not undergo apoptosis or cell death over a 4 h incubation period, in contrast to parental U937. These findings suggest that U9-TR is TNF resistant because there is a block in the pathway leading to activation of apoptosis.

Although TNF receptors on U9-TR can undergo TNF-induced internalization, this does not necessarily mean they can effectively transduce a signal. Since TNF has been shown to activate the nuclear regulatory factor, NF-kB, in U937 [20] and other cells [21], we next tested this function in the U9-TR variant. This was analyzed using an electrophoretic mobility shift assay with U937 and U9-TR extracts from cells incubated for 2 h with and without TNF 1 ng/ml (Fig. 7). Migration of the NFkB probe was retarded by formation of TNF-induced complexes in extracts from both U937 (lane 3) and U9-TR (lane 5). Complex formation was specifically inhibited by unlabeled excess NFkB probe (lane 6) but not the unrelated OCTA probe (lane 7). The

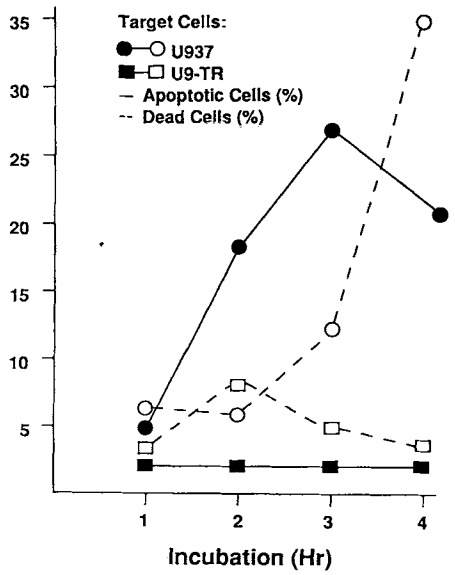

Fig. 6. U9-TR does not undergo apoptosis in response to TNF. U937 and the variant, U9-TR, were cultured with TNF (1.0 ng/ml) and cycloheximide (0.5 μg/ml) at 37°C. At different time points, cells were removed and analyzed for the percent apoptotic cells and the percent dead cells by trypan blue exclusion. Cells cultured in the absence of TNF and cycloheximide exhibited less than 2% apoptotic cells and less than 5% dead cells (data not shown). This experiment was repeated on two other occasions with similar results.

same cell extracts were also analyzed for complex formation with labeled probe recognized by the constitutively expressed OCTA nuclear regulatory factor to verify that approximately equal amounts of material are loaded in each lane (lanes 8–11). These results show that although very low levels of NFkB are found in the nuclei of untreated cells (lanes 2 + 4), TNF strongly activated NFkB in both U937 and U9TR (lanes 3 + 5). We have also compared the TNF dose response and kinetics of NFkB activation after treatments ranging from 5 min to 3 h and found no significant differences between U937 and U9-TR (data not shown). These findings demonstrate that the TNF receptor expressed on U9-TR can effectively transduce a signal leading to NFkB activation and translocation from the cytoplasm to the nucleus. Therefore, either NFkB is not involved in TNF-induced apoptosis, or else there is a block downstream from NFkB activation in the lytic pathway in U9-TR.

Although this study is focused on analyzing the TNF lytic mechanism in the U937 model system, we wanted to determine if TNF could activate PCD in a different tumor cell line. For this purpose we tested the TNF-sensitive murine WEHI-164 fibroblast to determine if TNF

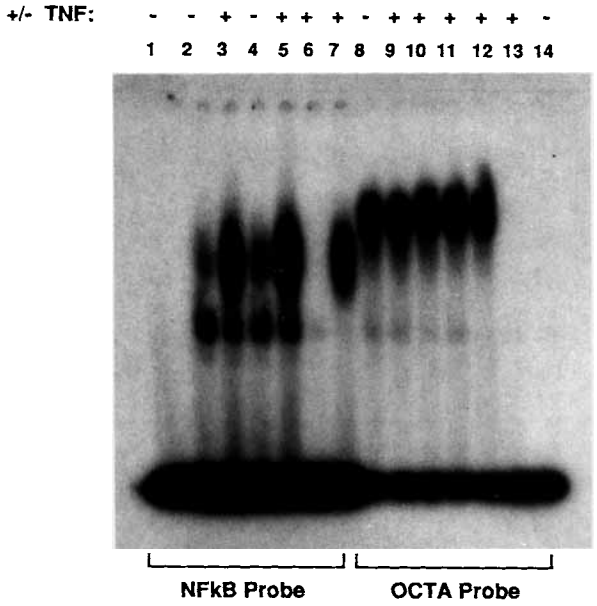

Fig. 7. TNF-induced activation of NFkB in U937 and U9-TR. Nuclear extracts from untreated and TNF (1 ng/ml)-treated U937 and U9-TR were analyzed using the electrophoretic mobility shift assay as described in Materials and Methods. Oligonucleotide probes alone are in lanes 1 and 14. Nuclear extracts from U937 cells are in lanes 2,3,6,7,10–13. Nuclear extracts from U9-TR cells are shown in lanes 4,5,8,9. Specificity of DNA-protein complex formation was demonstrated by competition with excess unlabeled NFkB probe (lane 6,12) and unlabeled OCTA probe (lane 7,13).

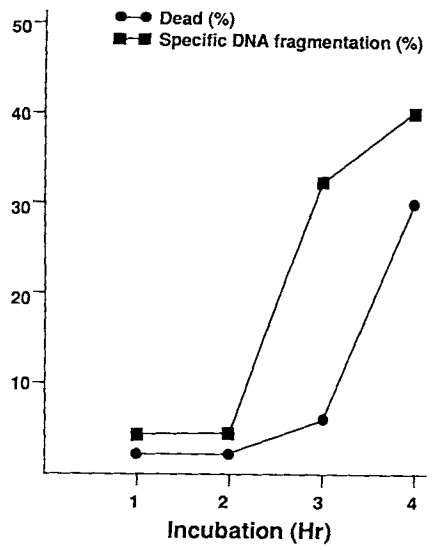

Fig. 8. Kinetics of TNF-induced DNA fragmentation and cell death in WEHI-164 fibroblasts. ^{3}H-labeled WEHI-164 were detached from the flask by scraping and cultured in polypropylene Eppendorf tubes with and without TNF (1.0 ng/ml) and cycloheximide (0.5 μg/ml). At different time points, cells were removed to determine viability by trypan blue exclusion and other tubes were processed to measure percent specific DNA fragmentation as described in Materials and Methods. This experiment was repeated on three other occasions with similar results.

could induce DNA fragmentation. The results shown in Figure 8 demonstrate that not only does TNF induce DNA fragmentation, but also that this occurs 1 h before there are significant numbers of dead cells observed in these cultures. We have also observed the characteristic pattern of DNA fragmentation into multiples of 180 base pairs in TNF-treated WEHI-164 analyzed by DNA gels (data not shown). These results confirm that U937 is not unique in its ability to undergo DNA fragmentation in response to TNF. Furthermore, the sequence of events is similar in both cell lines.

Hypotonic Lysis Does Not Activate Apoptosis in U937 Cells

Although it has been shown for other cell types that apoptosis is not activated by toxic insults such as hypotonic conditions [22], we wanted to confirm that this is also true for U937. In preliminary experiments, we determined the appropriate mixture of water and RPMI 1640 which would lyse U937 with kinetics similar to TNF (1 ng/ml) plus cycloheximide (0.5 μg/ml). Cultures were then set up in parallel in the presence of TNF and cycloheximide or 90% H_2O and examined for DNA fragmentation or cytotoxicity by ^{51}Cr release. The results in Figure 9A show that ^{3}H-DNA is released 1–2 h prior to ^{51}Cr release in TNF-treated cells. In contrast, there is no significant amount of ^{3}H-DNA released from cells cultured in hypotonic conditions for up to 4 h of incubation (Fig. 9B). We obtained similar results when apoptosis was assessed microscopically and cytotoxicty measured by trypan blue exclusion. These results demonstrate that activation of nucleases and the resulting DNA fragmentation is not the inevitable result of *any* mechanism of U937 cell lysis, at least within the time frame studied here. Taken altogether, these data support the hypothesis that TNF activation of DNA fragmentation is the cause and not the result of TNF-mediated cytolysis in the U937 model system.

Inhibition of Apoptosis by Zn and 3-Aminobenzamide

If our hypothesis is correct, it is logical to postulate that TNF activates an endogenous enzyme which then cleaves U937 DNA resulting in cell death. Studies from other laboratories

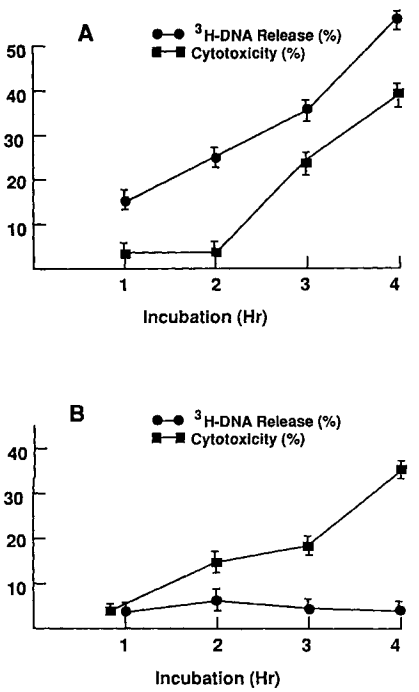

Fig. 9. Hypotonic conditions do not induce DNA release in U937 cells. ^{51}Cr-labeled and ^{3}H-labeled U937 were cultured with TNF and cycloheximide (**A**) or 90% H_2O and 10% RPMI 1640 (**B**). At different time points, assays were harvested to determine % DNA release and % cytotoxicity by ^{51}Cr release. This experiment was repeated on two other occasions with similar results.

have described various agents capable of inhibiting TNF-mediated cytotoxicity. We tested two of these compounds for their effects on TNF-induced apoptosis in U937 cells. It was found that both $ZnSO_4$ and 3-aminobenzamide could inhibit apoptosis (Table I) and cell death at concentrations similar to those reported to inhibit TNF-mediated cytotoxicity [23,24]. These compounds were of special interest since they have been reported to inhibit the activity or activation of endogenous endonucleases in other systems. Thus, it was shown that $ZnSO_4$ inhibits its activation of thymocyte endonucleases by glucocorticoids [5]. Inhibition of nuclear ADP-ribosylation reactions by 3-aminobenzamide could prevent calcium induced activation of endonucleases in rat liver nuclei [25]. The results of this experiment also suggest that the apoptosis assay may be a useful tool to analyze the effects of TNF inhibitors at this specific step in the TNF lytic pathway.

DISCUSSION

This study demonstrates that TNF activates apoptosis in U937 cells based on both morphological and biochemical criteria. These cells rapidly undergo the morphological changes typical of apoptosis within 3 h of exposure to TNF. This change coincides with the onset of DNA fragmentation as measured by release of ^{3}H-labeled DNA fragments. Furthermore, gel electrophoresis revealed that the DNA fragments into multiples of ~ 180 base pairs, typical of apoptosis and unlike necrosis in which DNA fragments into a continuous spectrum of sizes [4]. Apoptosis in this system does not require protein synthesis since the process is not inhibited by cycloheximide. In fact, addition of this inhibitor augments both apoptosis and DNA fragmentation, in accord with the known fact that metabolic inhibitors increase TNF sensitivity in various tumor cells [26]. These findings indicate that U937 already contains all the molecules/enzymes necessary to undergo apoptosis. This is similar to apoptosis induced by cytotoxic T lymphocytes which is also unaffected by protein synthesis inhibitors [7]. In contrast, the classical form of PCD, such as that seen in glucocorticoid induced thymic involution [5], IL-2 withdrawal from dependent cell lines [6], and activation induced T cell death [27,28], requires a period of protein synthesis before the onset of DNA fragmentation.

To establish a role for apoptosis in TNF-

TABLE I. Inhibition of Apoptosis by Zinc Sulfate and 3-Aminobenzamide (ABA)

Inhibitor[a]	% Inhibition of apoptosis (± SEM)[b]	% Inhibition of DNA release[c]	% Inhibition of ^{51}Cr-release[d]
$ZnSO_4$ 100 μM	85 ± 6.1	69	81
$ZnSO_4$ 10 μM	46 ± 5.2	44	70
$ZnSO_4$ 1 μM	10 ± 6.0	17	40
3-ABA 100 μM	81 ± 4.6	N.D.	89
3-ABA 10 μM	66 ± 12.6	N.D.	77
3-ABA 1 μM	32 ± 14.9	N.D.	N.D.

[a]Each inhibitor was tested in at least 3 separate experiments. Inhibitors were added at the indicated final concentrations to U937 cells followed immediately by TNF (1.0 ng/nl) and cycloheximide (0.5 μg/ml).
[b]The percentage of apoptotic cells was enumerated after incubation for 2 h at 37°C. The average percentage of apoptotic cells in the absence of inhibitors for these experiments was 38 ± 3.1.
[c]DNA release was assessed by measuring release of H-Tdr labeled DNA fragments in a 2 h assay as described in Materials and Methods.
[d]The effect of inhibitors on cytotoxicity mediated by TNF (1.0 ng/nl) and cycloheximide (0.5 μg/ml) was measured in a 6 h. ^{51}Cr-release assay.

mediated cytotoxicity, it is necessary to show that DNA fragmentation is the cause and not the result of cell death. Thus, it may be argued that TNF kills a cell by some other mechanism resulting in the release of lysosomal enzymes including DNases which then solubilize the DNA. However, our kinetic studies clearly demonstrate that both apoptosis and DNA fragmentation are prelytic events. Both processes occur 30–90 minutes before there is significant cell death as assessed by either trypan blue exclusion or ^{51}Cr release. These studies show that DNA fragmentation is not a consequence of TNF-mediated cytolysis, but do not prove it is the cause of death. It is possible that TNF initiates some other cytotoxic mechanism and DNA fragmentation is an unrelated event that occurs prior to cytolysis. It is clear, however, that DNA fragmentation is not a nonspecific event that occurs prior to any form of cell death in the U937 cell line. Under hypotonic conditions, U937 cells do not undergo apoptosis or DNA fragmentation prior to or just after cell death.

Further evidence in support of a role for apoptosis in TNF-mediated cytotoxicity is derived from studies of our TNF-resistant variant, U9-TR. This cell line does not undergo apoptosis, DNA fragmentation, or cytolysis in response to TNF, even in the presence of cycloheximide. Thus, this variant differs from other TNF-resistant cells that have been reported to become TNF sensitive in the presence of metabolic inhibitors [29,30]. Therefore, U9-TR is not TNF resistant due to the activity of the postulated protein synthesis-dependent protective mechanism. U9-TR expresses TNF receptors that can transduce a cytoplasmic signal since TNF activates the DNA binding protein, NFkB, in both U9-TR as well as in parental U937. This suggests that NFkB activation is not involved in TNF-induced cytolysis or else that there is a block at some later step in the TNF lytic pathway in U9-TR.

We also found evidence that TNF activates apoptosis in the WEHI-164 tumor cell which undergoes DNA fragmentation prior to cell death. However, it is clear that not all sensitive lines undergo apoptosis since it has been shown that TNF induces necrosis in the L-M clone of the L929 fibroblast line [14]. The same study reported that F17 cells exposed to TNF exhibited the apoptotic morphology; however, release of fragmented DNA was not observed prior to cell death. A third cell line, C3HA, underwent TNF-induced apoptosis, but did not release DNA fragments at any time. It was suggested that degeneration of stress fibers may cause cytoplasmic apoptosis in the C3HA line in the absence of nuclear disintegration [31]. Other studies have shown that prolonged (24–48 h) treatment with TNF can cause DNA fragmentation in L929 cells [32], and MCF-7 breast cancer cells [33]. However, these studies did not determine whether the DNA fragmented before or after cell death. Furthermore, the failure of a different group to detect even single strand DNA breaks in L929 cells treated with TNF as high as 1 μg/ml for 24 h [34] indicates the role of apoptosis in TNF cytotoxicity is a controversial issue. The reported heterogeneity in the patterns of cell death induced by TNF make it important to analyze this phenotype when using any particular cell line to study the TNF lytic mechanism.

U937 differs from many other types of TNF-sensitive tumor cells with respect to its rapid response to TNF. We have consistantly observed a peak in the percentage of apoptotic cells, correlating with the release of DNA fragments, after only 1–2.5 h of exposure to TNF. Subsequently, the percentage of apoptotic cells declines. This suggests that at a given time, only a subpopulation of U937 cells are susceptable to TNF-induced apoptosis. The entire process of apoptosis has been reported to take place within 3 h [35], which is consistant with our findings. The susceptible subpopulation of U937 cells undergoes apoptosis and then presumably dies, with the remaining cells exhibiting a non-apoptotic morphology. Whether these cells may undergo apoptosis following a more prolonged exposure to TNF than was examined in this study is not known. Also, we cannot rule out the possibility that these cells may undergo the necrotic form of cell death following prolonged exposure to TNF. Alternatively, it is possible that these cells have become at least temporarily resistant to TNF, since it has been shown in other cell lines that pretreatment with TNF can induce TNF resistance [36]. Currently, studies are underway to separate the cells that rapidly undergo TNF-induced apoptosis, so the responsiveness of the remaining viable cells can be studied.

If apoptosis is an essential and irreversible step in the TNF lytic pathway, then it would be predicted that agents known to inhibit cytolysis

would also inhibit apoptosis in U937. On testing this hypothesis, we found that both 3-aminobenzamide and zinc could inhibit TNF-induced apoptosis at concentrations comparable to those reported to inhibit tumor cell lysis by TNF [23,24]. Zinc has been shown to inhibit the endonuclease that is activated in other systems of PCD [5]. The inhibitor of ADP-ribosylation, 3-amino-benzamide, has been shown to inhibit the calcium-dependent endonuclease found in rat liver nuclei [25]. Therefore, these compounds may be inhibiting the endonuclease activated by TNF in U937 cells undergoing apoptosis. Since we actually measured the morphological manifestation of PCD (i.e., apoptosis) it is also possible that these agents may be acting at some cytoplasmic location to inhibit these changes. Since apoptosis involves disruption of the microfilaments [37], it is possible that these inhibitors are interacting with the cytoskeleton to stabilize it. Further experiments are underway to test these possibilities.

A key event in the process of apoptosis is the activation of an endonuclease. Although numerous laboratories have studied apoptosis, the putative enzyme has not yet been purified and characterized. In protein synthesis dependent systems of apoptosis (e.g., developmental cell death), it is possible that the endonuclease must be synthesized de novo before the cell can undergo destruction. We believe that the putative endonuclease is constitutively expressed in U937 cells since protein synthesis is not required for TNF-induced apoptosis. This is supported by our preliminary findings that endonuclease activity can be isolated from lysates of U937 cells. Investigations under way in our laboratory to characterize this enzyme and examine its role in apoptosis will be the subject of a future communication.

This study has clearly demonstrated that TNF activates apoptosis in U937 cells. Although it was shown that DNA fragmentation precedes cell death, it is still not certain that this was the direct cause of death as defined by release of ^{51}Cr and inability to exclude trypan blue. It is possible that TNF activates other processes that more directly lead to cytolysis, such as generation of membrane damaging free radicals [38–40]. We speculate that it is actually the combination of both processes that leads to rapid cytolysis in U937 cells. Thus, cells may be able to repair free radical damage and continue to survive if their DNA remained intact.

This study has provided the foundation for further dissection of the TNF lytic mechanism in the U937 model system. Since the morphological changes of apoptosis and DNA fragmentation are easily measured in this cell line, it also provides an excellent model to study the process of PCD. Further analysis of the biochemical events underlying TNF-induced apoptosis may provide insights into how this process is regulated in other systems as well.

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