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Role of Protein Phosphorylation in TNF-Induced Apoptosis: Phosphatase Inhibitors Synergize With TNF to Activate DNA Fragmentation in Normal as Well as TNF-Resistant U937 Variants

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Abstract This study examined the role of protein phosphorylation in TNF induction of apoptosis in several tumor cell lines by testing the effects of agents that either stimulate or inhibit protein phosphorylation. The serine-threonine phosphatase inhibitors, okadaic acid (OKA) and calyculin A (CLA), synergistically augmented TNF-induced apoptosis in several TNF-sensitive tumor cell lines including the U937 histiocytic lymphoma, the BT-20 mammary carcinoma, and the LNCap prostatic tumor cell line. Furthermore, the phosphatase inhibitors completely reversed the TNF resistance of a variant (U9-TR) derived from U937. CLA also inhibited phosphatase activity in cell-free extracts from both U937 and U9-TR at the same concentrations (0.4–2.0 nM) that it synergized with TNF. In contrast, TNF treatment of U937 cells did not result in inhibition of phosphatase activity mediated by protein phosphatase 1 (PP1) and PP2A in cell extracts. Since the phosphatase inhibitors are known to increase the overall levels of protein phosphorylation in cells, this suggested that TNF may act by stimulating protein kinase (PK) activity. This hypothesis was supported by the results of testing a panel of relatively specific protein kinase inhibitors. TNF activation of DNA fragmentation was blocked by a potent inhibitor of myosin light chain kinase (MLCK) but was unaffected by inhibitors of cAMP or cGMP-dependent PKs. We postulate that a defect in the activation of MLCK or possibly some other as yet unknown PK may be responsible for the TNF resistance of U9-TR. Furthermore, this resistance may be circumvented by promoting protein phosphorylation with the serine-threonine-dependent phosphatase inhibitors.

Key words: tumor necrosis factor, apoptosis, okadaic acid, calyculin A, protein kinase, phosphatase inhibitors

Despite the fact that TNF has been studied for many years, its mechanism of action is still not well understood [for review see Larrick and Wright, 1990; Vilcek and Lee, 1991]. Since this molecule can elicit widely divergent responses in various cell types including stimulation of proliferation, differentiation, and death, it may activate multiple signalling pathways in different cell types. Our studies have focussed on the mechanism of TNF-mediated cytotoxicity and factors determining sensitivity and resistance in tumor cell lines. The human histiocytic lymphoma, U937, has been widely studied as a TNF-responsive cell [Shalaby et al., 1990; Unglaub et al., 1987; Cassatella et al., 1989; Osborn et al., 1989; Hasegawa and Bonavida, 1989; Schutze et al., 1989], and most of our studies have employed this line as well as a TNF-resistant variant (U9-TR) to dissect the cytotoxic pathway.

To elucidate any cytolytic mechanism, it is important to first determine whether cell death occurs via necrosis as opposed to apoptosis, since these two mechanisms of cell death are quite different even though they may be initiated by

Abbreviations used: CLA, calyculin A; MLCK, myosin light chain kinase; OKA, okadaic acid; PK, protein kinase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; PP, protein phosphatase; TNF, tumor necrosis factor alpha; U9-TR, TNF resistant variant of U937.

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the same stimuli in diverse cells. Apoptosis is the normal physiological mechanism of cell death characterized by cytoplasmic membrane blebbing, cell shrinking, and DNA fragmentation into multiples of 180 base pairs prior to death [for review see Walker et al., 1988; Duvall and Wyllie, 1986]. Necrosis is seen in response to pathological trauma and is characterized by cell and organelle swelling, loss of membrane integrity, and digestion of DNA into fragments of a continuous spectrum of sizes after cell death. Our initial studies established that TNF activates apoptosis in U937 cells based on both biochemical and morphological criteria [Wright et al., 1992a]. The TNF-resistant variant (U9-TR) derived from U937 did not undergo either apoptosis or necrosis in response to TNF. Subsequent studies analyzed the mechanism of TNF activation of apoptosis and sought possible defects responsible for the resistant phenotype of the variant.

Many diverse stimuli activate apoptosis in different cell types, yet the mechanism is poorly understood. It is generally accepted that signalling pathways ultimately lead to activation of an endogenous endonuclease(s) that cuts DNA between the nucleosomes in the linker regions. Very little is known about the mechanism of endonuclease activation; however, there is evidence to indicate it involves elevation of intracellular calcium in some [Cohen and Duke, 1984; Kyprianou et al., 1988; McConkey et al., 1990] but not all [Alnemri and Litwak, 1990] systems. Likewise, apoptosis often [Cohen and Duke, 1984; Ucker et al., 1989; Sellins and Cohen, 1987] but not always [Duke et al., 1983; Martin et al., 1990] requires de novo protein synthesis. Studies have demonstrated that neither extracellular calcium [Hasegawa and Bonavida, 1989; our unpublished observations] nor protein synthesis is required for TNF-induced apoptosis of U937 cells [Wright et al., 1992a]. Furthermore, the mechanism of resistance of U9-TR did not depend on protein synthesis since cycloheximide did not render the cells TNF-sensitive. In addition, this variant still expresses functional TNF receptors since TNF activated the nuclear regulatory factor, NF-κB, in both U9-TR and parental U937 cells [Wright et al., 1992a]. U9-TR will undergo apoptosis in response to other stimuli, such as UV light [unpublished observations], indicating that it still expresses the endonuclease(s) and other molecules essential for the process of DNA fragmentation. Therefore we postulated that there was a block at some intermediate step distal to TNF-receptor interaction but prior to endonuclease activation that was responsible for the TNF resistance of U9-TR. In this investigation we tested the hypothesis that protein phosphorylation may function as a factor determining sensitivity or resistance to TNF-induced apoptosis. We found that inhibitors of protein phosphatases, PP1 and PP2A, synergize with TNF in normal U937 and completely reverse the resistance of U9-TR.

MATERIALS AND METHODS

Cell Lines

The human histiocytic lymphoma, U937, the human breast carinoma, BT-20, and the human prostate cancer cell line, LNCap, were all obtained from the ATCC. All cell lines were maintained in RPMI-1640 supplemented with 10% FCS, penicillin-streptomycin, and l-glutamine (2 mM). The selection and characterization of the TNF-resistant U937 variant clone (U9-TR) has been described in detail previously [Wright et al., 1992b]. This line is highly resistant to TNF, and in fact is routinely cultured in the presence of TNF at 50 ng/ml while maintaining a viability $>95\%$. The variant is cultured in the absence of TNF at least 5 days prior to use in an experiment, and its resistant phenotype is stable in the absence of TNF for at least 1 month. 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 R&D Systems (Minneapolis, MN). The phosphatase inhibitors, OKA and CLA, were obtained from Wako Chemicals (Richmond, VA). The protein kinase inhibitors, KT5720, KT5823, and KT5926, were purchased from Kamiya Biomedical (Thousand Oaks, CA).

Quantitative Assay of DNA Fragmentation

The assay we routinely employ to quantitate DNA fragmentation is a modification of a previously described assay [Sellins and Cohen, 1987] which was adapted to the 96-well microtiter format. The major advantage of our modified assay is that it facilitates processing of large numbers of samples. This assay was described in detail previously and found to produce results

similar to other standard DNA fragmentation assays [Wright et al., 1992a]. 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 0.1 ml. Target cells were suspended at 1×10^6/ml and 0.05 ml was added to each well. Wells for total counts received an additional 0.05 ml of medium, whereas experimental wells received 0.05 ml of the appropriate concentrations of TNF. Plates were usually incubated for 20 h at 37°C but in some experiments the incubation time was shortened as indicated. The assay was terminated by addition of 150 μl 10 mM EDTA, and 0.3% Triton X-100 to each well. Plates were harvested using a Packard harvester and samples were counted using a Packard Matrix 96 beta 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 percentage spontaneous DNA release varied from 0–5% over a 5 h incubation period and did not exceed 10% release in a 20 h incubation.

DNA Fragmentation Assay Using Adherent BT-20 and LNCap Target Cells

Cells were trypsinized and seeded at 1×10^4 cells/well in the presence of ^{3}H-Tdr in 96-well microtiter plates 24 h prior to the assay. Just before the assay, monolayers were washed free of unincorporated ^{3}H-Tdr. The rest of the protocol was the same as for U937 except that the assay was incubated for 24 h.

Visualization of DNA Fragmentation by Gel Electrophoresis

The second method to analyze DNA fragmentation employed agarose gel electrophoresis. Target cells were treated with TNF or the various agents and the DNA was harvested as described previously [Schmid et al., 1986] 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. 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.

Protein Phosphatase Assay

U937 cells were suspended at 10^6/ml in RPMI-1640 plus 2.5% FCS and incubated with TNF or CLA at 37°C for the indicated length of time. Cytosolic and nuclear extracts were isolated as described by Kuret et al. [1986]. Briefly, cells were lysed by adding ice-cold extraction buffer containing 50 mM tris/HCl pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 1 mM PMSF, 0.5 μg/ml pepstatin, 0.5 μg/ml leupeptin, 1 mM benzamidine, 0.1% 2-mercaptoethanol, 250 mM sucrose, and 0.1% triton X-100 (buffer A). The lysate was centrifuged for 5 min at 1,500 rpm and the supernatant used as the cytosolic extract. The pelleted nuclei were resuspended in buffer A + 10 mM EDTA, then 0.33 volumes of 50 mM tris/HCl, pH 7.5; 0.1% 2-ME and 2 M NaCl were added and the samples placed on ice for 30 min. The nuclear lysates were then centrifuged for 1 h at 13,000 rpm. Phosphatase assay kits were purchased from GIBCO/BRL. Protein phosphatase was measured using ^{32}P-labeled phosphorylase-a as a substrate, which will detect PP1 and PP2A. The phosphatase activity was determined by measuring the TCA-soluble counts released after 10 min of incubation of the ^{32}P-labeled substrate in the extract.

RESULTS

Phosphatase Inhibitors Augment TNF-Induced Apoptosis and Reverse TNF Resistance

Phosphatase inhibitors known to increase overall levels of protein phosphorylation [Chartier et al., 1991; Haystead et al., 1989] have been used to evaluate the role of protein phosphorylation in regulating various intracellular processes. The specificity of two inhibitors is shown in Table I. Calyculin A (CLA) potently inhibits both PP1 and PP2A at subnanomolar concentrations [Ishihara et al., 1989]. Okadaic acid (OKA) is an equally potent inhibitor of PP2A: however, approximately 100-fold higher concentrations are required to inhibit PP1 [Bialojan and Takai, 1988]. Both OKA and CLA are known to penetrate cell membranes, although the efficiency may vary with different cell types. Since the IC_{50} values were determined in cell-free enzyme assays, one cannot necessarily expect the same concentrations to be effective in cell assays.

Figure 1A shows the TNF dose response for induction of DNA fragmentation in U937 cells. Figure 1B depicts levels of DNA fragmentation

induced by different concentrations of OKA tested with and without the suboptimal concentration of TNF (2.5 ng/ml). OKA alone at 25 nM did not cause significant DNA fragmentation, although it synergistically augmented TNF-induced apoptosis. At higher concentrations OKA alone was toxic (data not shown). These results suggest that augmentation of the state of protein phosphorylation by inhibition of protein phosphatases promotes TNF-induced apoptosis.

TABLE I. Specificity of Phosphatase Inhibitors Used in This Investigation

Phosphatase	IC_{50} values OKA	CLA
PP1	10–15 nM	0.3 nM
PP2A	0.1 nM	0.1 nM
PP2B (calcineurin)	5 uM	
PP2C	> 10 μM	
Endogenous phosphatase of smooth muscle myosin	15–70 nM	3.7 nM
Acid phosphatase	NI[a]	NI
Alkaline phosphatase	NI	NI
Phosphotyrosine phosphatase	NI	NI

[a]NI = not inhibited.

Similar studies were performed using the TNF-selected U9-TR variant. Compared to parental U937, U9-TR is relatively resistant to TNF-induced DNA fragmentation (Fig. 1C). The level of resistance varies somewhat from day to day and in some experiments approached complete resistance. OKA synergizes with TNF to induce DNA fragmentation in U9-TR (Fig. 1D). Resistance was completely reversed since U9-TR was just as sensitive to the combined effect of OKA and TNF as the parental U937. This finding suggests that protein phosphorylation is one element regulating sensitivity to TNF-induced apoptosis.

These results were verified by demonstrating similar effects of a second structurally unrelated

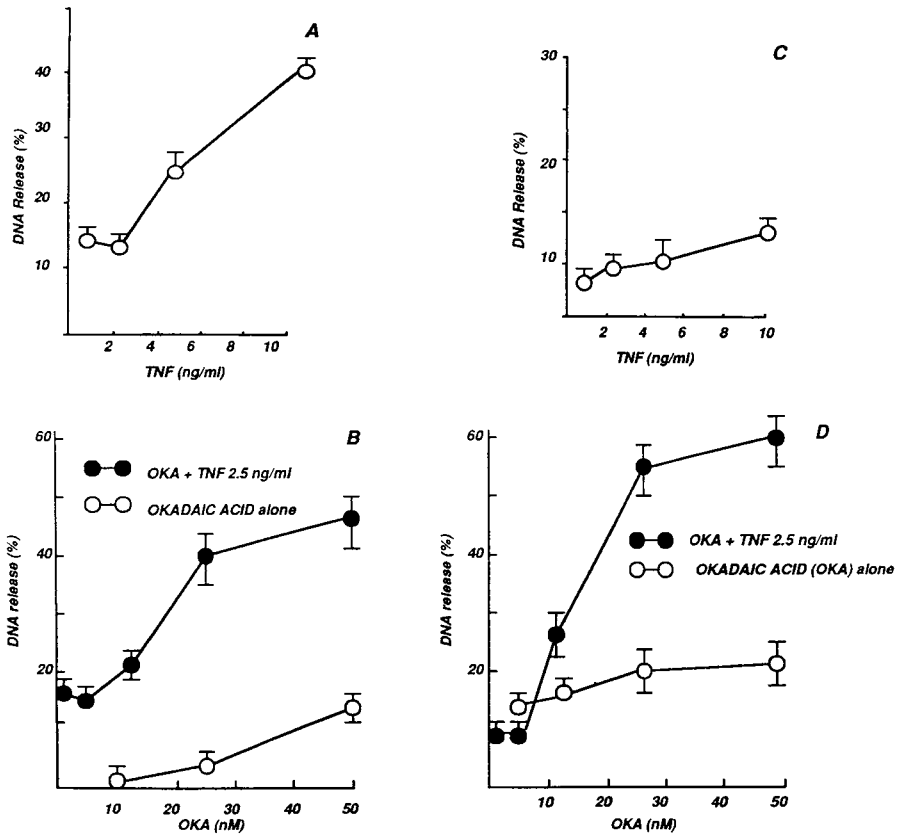

Fig. 1. OKA augments TNF-induced DNA fragmentation in U937 (**A,B**) and U9-TR (**C,D**) cells. Fig. 1A,C shows the dose response for TNF alone. In Fig. 1B,D, target cells were pretreated with OKA for 30 min prior to adding TNF and continuing the assay for 20 h. DNA fragmentation was assessed by measuring release of ^{3}H-labelled DNA fragments as described in Methods. These are the results of one representative experiment out of three total. Each point is the mean ± standard deviation of triplicates.

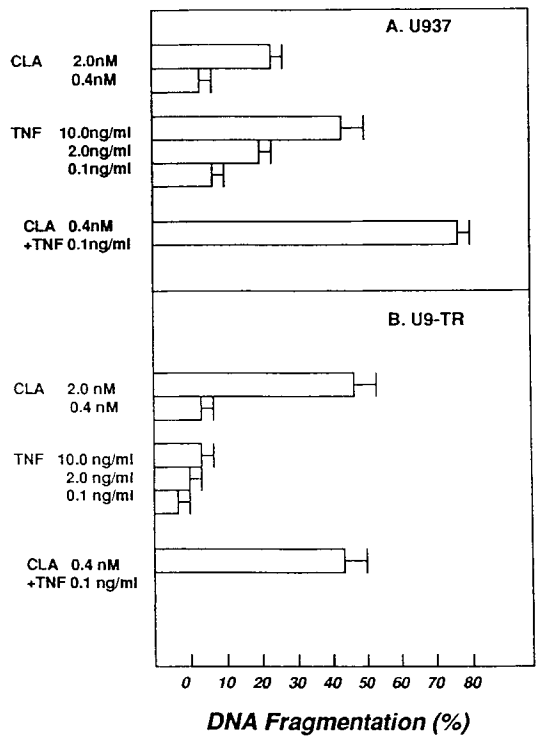

Fig. 2. CLA augments TNF-induced DNA fragmentation in U937 and U9-TR. Target cells were pretreated with CLA for 30 min prior to adding TNF and the assay continued for 20 h. DNA fragmentation was assessed by measuring release of ^{3}H-labelled DNA fragments as described in Methods. These findings have been reproduced on three other occasions. Each point is the mean ± standard deviation of triplicates.

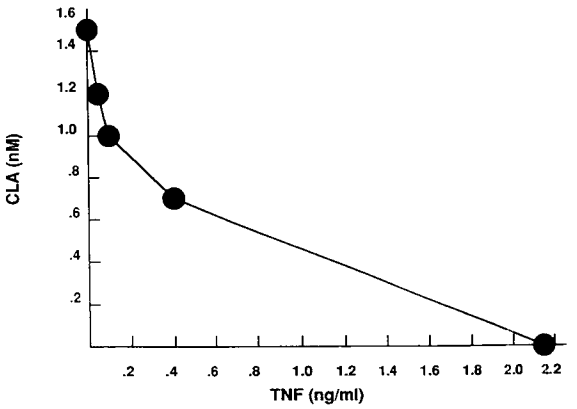

Fig. 3. CLA synergizes with TNF to induce DNA fragmentation in U937 cells. The sensitivity of U937 cells to apoptosis induced by CLA and TNF alone and combined at different concentrations was measured as described in Fig. 2. The graph shows the concentrations of each agent alone and in various combinations necessary to achieve 50% DNA fragmentation.

phosphatase inhibitor, CLA. Figure 2 shows that subnanomolar concentrations of CLA synergize with suboptimal concentrations of TNF to induce DNA fragmentation in both U937 and U9-TR. Furthermore, since CLA is effective at concentrations approximately fiftyfold lower than OKA, this suggests that the relevant phosphatase is PP1 based on the known IC_{50} values (Table I).

To verify that the interaction between TNF and CLA was in fact synergistic and not additive, DNA fragmentation mediated by different concentrations of TNF plus CLA was evaluated by an isobologram analysis. The concentrations of TNF and CLA alone and in different combinations required to achieve 50% DNA fragmentation are plotted in Figure 3. The resulting graph shows a concave curve indicating synergism, as opposed to a straight line which signifies an additive effect [Berenbaum, 1989].

DNA fragmentation induced by TNF and OKA was also evaluated by agarose gels. Treatment of U937 with TNF alone caused the DNA to fragment into multiples of 180 base pairs as expected (Fig. 4, lanes 2, 3). OKA alone at high concentration (100 nM) induced low levels of DNA fragmentation as shown by the faint banding patterns (Fig. 4, lane 4). However, the combination of both TNF and OKA resulted in the most intensely staining DNA "ladders" (lanes 5, 6) in the pattern typical of apoptosis. Although this is not a quantitative assay, the data agree with previous results (Fig. 1) showing OKA augments TNF-induced release of DNA fragments. When observed by light microscopy, U937 cells treated with TNF plus the phosphatase inhibitors undergo the morphological changes typical of apoptosis (unpublished observations) as previously documented for the effects of TNF alone [Wright et al., 1992a]. These findings confirm that the phosphatase inhibitors promote the process of apoptosis as opposed to nonspecific DNA degradation or necrosis.

Phosphatase Inhibitors Augment TNF-Induced Apoptosis in Other Sensitive Cells

To examine the spectrum of the effects of the phosphatase inhibitors, we tested two other lines which are not as highly susceptible to TNF-induced apoptosis as U937, but exhibit intermediate levels of TNF sensitivity. Our results demonstrate that CLA augments TNF-induced DNA fragmentation in the BT-20 mammary carcinoma (Fig. 5A) as well as the LNCap prostatic cancer cell line (Fig. 5B). Therefore, this phenomenon is not limited to U937 cells.

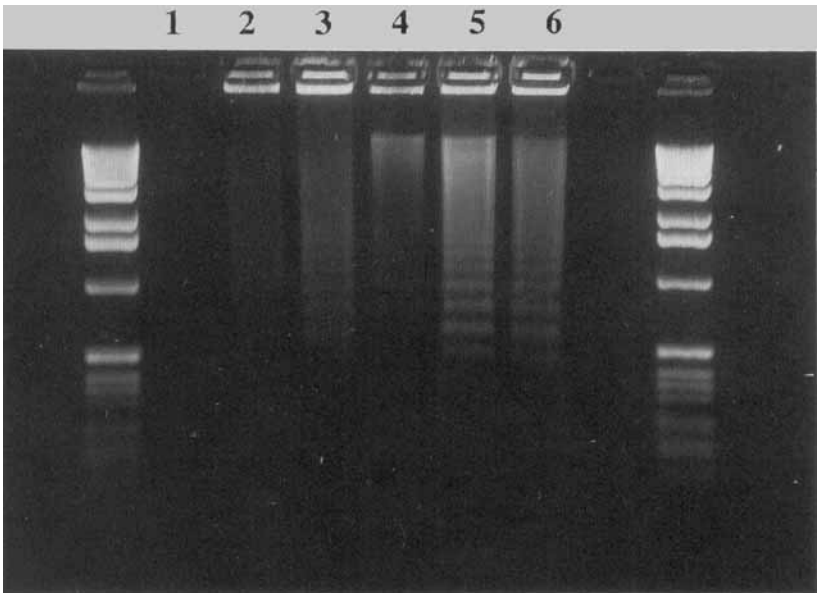

Fig. 4. OKA augments TNF-induced endonuclease activation. U937 cells were incubated for 20 h in the presence of the various agents and the DNA was extracted and analyzed by agarose gel electrophoresis as described in Methods. **Lane 1:** Untreated U937. **Lane 2:** U937 + TNF 1.0 ng/ml. **Lane 3:** U937 + TNF 5.0 ng/ml. **Lane 4:** U937 + OKA 100 nM. **Lane 5:** U937 + TNF 5.0 ng/ml + OKA 100 nM. **Lane 6:** U937 + TNF 1.0 ng/ml + OKA 100 nM.

Since the phosphatase inhibitors could reverse the resistance of the U9-TR variant, it was of interest to determine if they could also sensitize naturally resistant tumor cells to the cytotoxic action of TNF. Therefore we tested the effects of CLA and OKA on several cell lines that are resistant to the cytostatic or cytotoxic effects of TNF. We found that the murine B16 melanoma, the human prostatic cancer line PC3, and the human carcinoma KB-3-1 were still resistant to TNF-mediated growth inhibition or cytotoxicity (through either apoptosis or necrosis) even in the presence of the phosphatase inhibitors (data not shown). This is evidence that there may be multiple mechanisms of TNF resistance operating in various tumor cell types that differ from the mechanism of resistance in the U9-TR variant.

CLA, but not TNF, Inhibits Intracellular Phosphatase Activity

Since phosphatase inhibitors promote apoptosis, it is possible that the TNF mechanism of action involves inhibition of phosphatase activity. This was tested directly by measuring phosphatase activity in extracts from U937 cells treated with different concentrations of TNF or with CLA as a positive control. In preliminary experiments, we examined the kinetics of CLA inhibition of phosphatase activity and found maximum inhibition occurred as early as 10 min after treatment and persisted for up to 2 h (data not shown). TNF at concentrations which induce DNA fragmentation had no significant effect on phosphatase activity in nuclear extracts, whereas CLA lowered the activity as expected (Fig. 6A). We obtained similar results testing cytoplasmic extracts (Fig. 6B). Extracts from cells treated with TNF for 5 min up to 2 h demonstrated no inhibition of phosphatase activity (data not shown). Longer incubation times were not tested due to the accumulation of dead cells in the culture. These results suggest that TNF does not activate apoptosis by inhibiting PP1 and PP2A. However, we cannot rule out the possibility that TNF specifically inhibits a minor subpopulation of phosphatases that cannot be detected in this assay which measures the ubiquitous enzymes, PP1 and PP2A.

Further experiments were performed to determine if CLA also inhibits phosphatase activity in the U9-TR variant. The results show that CLA was just as effective at suppressing nuclear phosphatase activity in U9-TR (Fig. 6D) as in the parental U937 (Fig. 6C). Similar results were obtained on testing cytoplasmic extracts (data not shown). These data also show that the concentrations of CLA required to suppress phos-

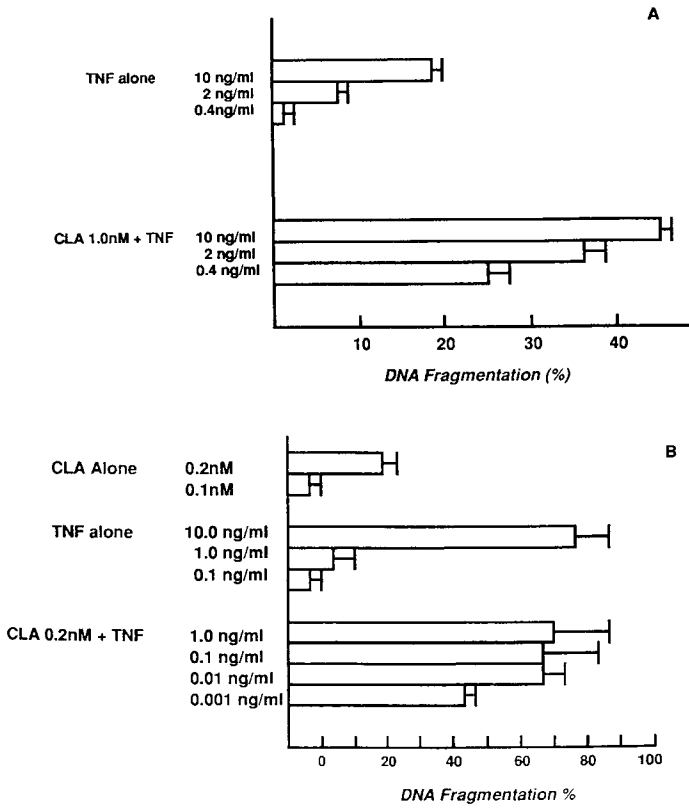

Fig. 5. CLA augments TNF-induced apoptosis of other TNF-sensitive cell lines. BT-20 cells (**A**) or LNCap cells (**B**) were tested for sensitivity to apoptosis induced by TNF with and without CLA. CLA 1.0 nM alone was nontoxic to BT-20 cells in this assay (data not shown). Each point is the mean ± standard deviation of triplicates.

phatase activity (0.4–2.0 nM) coincide with those that synergize with TNF to induce DNA fragmentation in both U937 and U9-TR. This supports the hypothesis that inhibition of phosphatase activity promotes apoptosis and may be involved in the mechanism of TNF resistance.

Inhibition of TNF-Induced DNA Fragmentation by a Protein Kinase Inhibitor

The synergistic effects of TNF and phosphatase inhibitors may be explained if TNF signal transduction involves activation of a protein kinase. Although numerous studies have shown TNF stimulates protein phosphorylation, there is little information available concerning the effects of PK inhibitors on TNF cytotoxicity. Therefore we tested the effects of a panel of protein kinase inhibitors on TNF-induced DNA fragmentation. The specificity of these commercially available reagents is presented in Table II. The results of testing the KT series of PK inhibitors at their highest nontoxic concentrations demonstrate that only KT5926 could block TNF-induced DNA fragmentation (Fig. 7). The specificity of these compounds (Table II) indicates the most likely targets are cGMP-dependent PK and myosin light chain kinase. PKG is probably not involved since KT5823 (an inhibitor of PKG) did not inhibit and also because we have been unable to detect any increases in cGMP levels in TNF-treated cells (unpublished observations). Therefore, we conclude that myosin light chain kinase or perhaps some other as yet unidentified kinase that is inhibited by KT5926 may be involved in TNF activation of DNA fragmentation.

DISCUSSION

This investigation examined the role of protein phosphorylation in TNF-induced apoptosis in several tumor cell lines. In general, overall levels of protein phosphorylation are controlled by two classes of enzymes, protein kinases and protein phosphatases. This study employed relatively specific inhibitors of these enzymes to examine their effects on TNF-induced DNA fragmentation in sensitive as well as resistant tumor cell variants. The new finding that emerges from

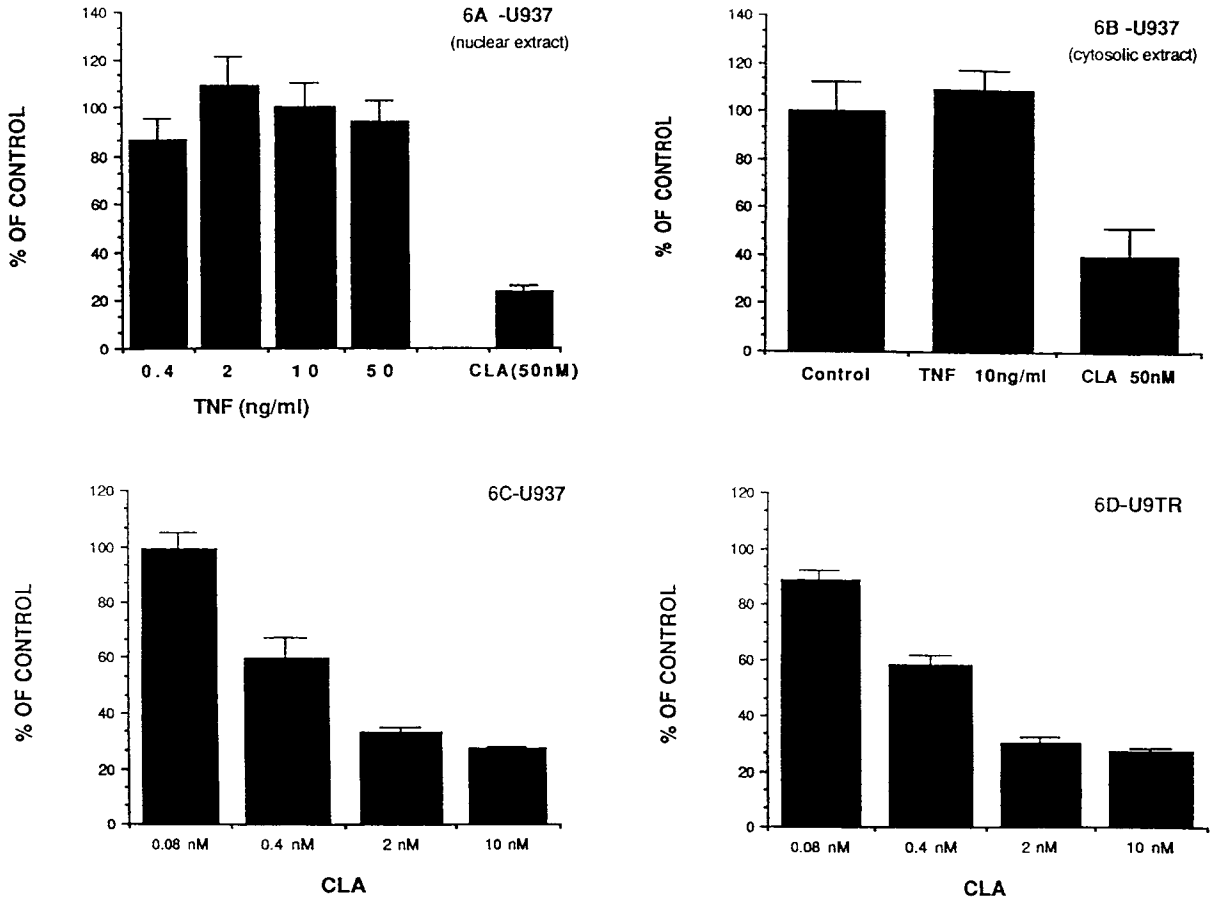

Fig. 6. Inhibition of phosphatase activity in cell extracts by CLA but not by TNF. U937 cells (**A–C**) or U9-TR cells (**D**) were pretreated with TNF or CLA for 10 min. Phosphatase activity was measured using ^{32}P-labelled phosphorylase as a substrate as described in Materials and Methods. All data are expressed as the mean percent of control ± SEM. Control phosphatase activity in U937 nuclear extracts (A) was 1.7 nM/min/10^6 cells and in cytoplasmic extracts (B) was 0.97 nM/min/10^6 cells.

TABLE 2. Specificity of Protein Kinase (PK) Inhibitors

	Inhibition constant (μM)[a]		
Enzyme	KT5720	KT5823	KT5926
PKC	> 2.0	4.0	0.723
cAMP-dependent PK	0.056	> 10.0	11.2
cGMP-dependent PK	> 2.0	0.234	0.158
Myosin light chain Kinase	> 2.0	> 10.0	0.018

[a]Values for inhibition constants were supplied by the manufacturer (Kamiya Biochemicals).

these studies is that inhibitors of PP1 and PP2A such as OKA and CLA synergize with TNF to promote apoptosis in U937 cells as well as completely reverse the resistance of the U9-TR variant.

The major intracellular serine-threonine protein phosphatases PP1, PP2A, PP2B, and PP2C are important regulators of cellular metabolism and mitosis [for review see Cohen, 1989]. The recent availability of inhibitors of these enzymes has provided a valuable tool for analyzing the regulatory role of protein phosphorylation in a variety of intracellular events. Treatment of cells with OKA [Haystead et al., 1989] or CLA [Chartier et al., 1991] increases the level of phosphorylation of many proteins presumably by allowing the unopposed activity of constitutive protein kinases. Interestingly, OKA has been shown to increase the level of phosphorylation of many of the same substrates that are phosphorylated in response to TNF stimulation in normal fibroblasts [Guy et al., 1991]. We confirmed that OKA increases incorporation of ^{32}P into many different proteins in U937 cells as revealed by one dimensional SDS-PAGE (unpub-

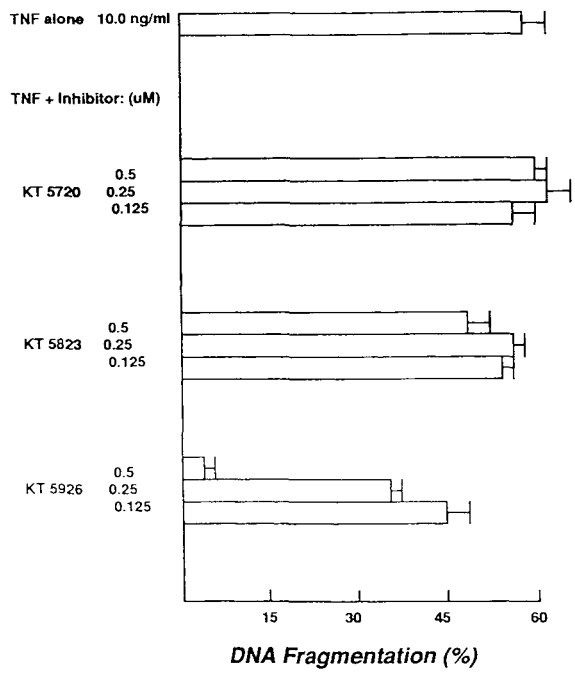

Fig. 7. Inhibition of TNF-induced apoptosis by a PK inhibitor. U937 cells were pretreated for 30 min with the inhibitors prior to adding TNF and continuing the assay for 20 h. Apoptosis was measured by release of ^{3}H-labelled DNA fragments as described in Methods. All inhibitors were tested at their highest nontoxic concentration. Similar results were obtained on two other occasions. Each point is the mean ± standard deviation of triplicates.

lished observations). Furthermore, the present studies also show that CLA suppresses protein phosphatase activity in U937 cell extracts at concentrations similar to those that synergize with TNF. Therefore, inhibition of protein phosphatases, as opposed to some nonspecific effect, is the most likely mechanism of OKA and CLA promotion of TNF-induced DNA fragmentation.

Other studies reported that OKA alone at high concentrations (1 μM) could induce apoptosis in various cell types [Song et al., 1992; Boe et al., 1991]. Since data was not reported testing OKA at concentrations similar to our study (10–50 nM), it is difficult to reconcile their results with ours. The fact that the IC_{50} values for OKA are 10–15 nM for PP1 and 0.1 nM for PP2A (Table I) raises the possibility that results obtained using 1 μM OKA may be due to some nonspecific effect unrelated to PP1 or PP2A.

It has also been reported that high concentrations of OKA (500 nM) caused down-regulation of U937 TNF receptors [Higuchi and Aggarwal, 1993]. The same authors stated that OKA at 100 nM did not decrease the number of TNF receptors, and therefore we do not expect this phenomenon to play any role in the present findings.

Sung et al. [1992] reported that high concentrations of CLA (10–30 nM) induced TNF production in human monocytes. Since the activity of PP1 and PP2A in CLA-treated cells was not measured, one wonders whether using CLA at levels closer it its IC_{50} values (0.1–0.3 nM) would also stimulate TNF production. In contrast, we found that CLA at concentrations ranging from 0.4–2.0 nM could synergize with TNF to activate DNA fragmentation. Furthermore we documented that these concentrations correlated with those that suppressed PP1 and PP2A activity in U937 cells. Although we have not tested for TNF production in our system, we consider it an unlikely explanation of our findings in view of the very high concentrations of CLA used by Sung et al. [1992].

The phenomenon we observed is not limited to U937, because CLA also augments TNF-induced DNA fragmentation in BT-20 cells, which previously have been reported to undergo apoptosis in response to TNF [Bellomo et al., 1992] as well as in LNCap cells. Most tumor cells are resistant to the cytotoxic effects of TNF. Of those that are sensitive, some undergo apoptosis [Wright et al., 1992a; Schmid et al., 1987; Dealtry et al., 1987], whereas others die by necrosis [Laster et al., 1988]. We have not analyzed the effects of the phosphatase inhibitors on TNF-induced necrosis; however, other studies suggest the effects may be opposite since OKA was found to inhibit TNF lysis of L929 cells [Totpal et al., 1992]. Although this study did not specify whether the targets died by necrosis or apoptosis, another report indicated that some L929 sublines are killed by TNF through necrosis [Laster et al., 1988], whereas other L929 lines show a mixture of necrosis and apoptosis [Russell et al., 1972]. Further studies are required to determine if the effects of the phosphatase inhibitors depend on whether or not a cell undergoes apoptosis as opposed to necrosis in response to TNF.

The finding that phosphatase inhibitors promote apoptosis raised the possibility that TNF itself may directly or indirectly inhibit protein phosphatases to initiate DNA fragmentation. However, unlike CLA, TNF treatment of U937 did not result in reduced activity of phosphatases PP1 or PP2A in cell extracts. It is possible that TNF specifically inhibits only a subpopula-

tion of protein phosphatases that would not be detected in this assay which selectively measures PP1 and PP2A (such as the recently described novel serine-threonine phosphatase, PP3 [Honkanen et al., 1991]). Another study reported that TNF or Il-1 decreased the activity of an unidentified protein phosphatase in fibroblasts [Guy et al., 1993]. However, the fact that fibroblasts are growth stimulated by both these cytokines, whereas U937 cells undergo apoptosis in response to TNF but not IL-1, raises the possibility that completely different signalling pathways may be involved. Undoubtedly there exist many as yet unknown protein phosphatases, and their possible role in TNF-induced apoptosis will be the subject of future studies.

An alternative interpretation of our data is that TNF functions by activating a protein kinase. This hypothesis was tested using several of the newer commercially available PK inhibitors that exhibit much greater selectivity than many agents used in previous studies. By testing a panel of inhibitors with different potencies for PKC, PKA, PKG, and MLCK, we were able to determine that the most likely target for inhibition in this list is MLCK. Although KT5926 has been reported to be selective for MLCK [Nakanishi et al., 1990], we cannot rule out the possibility that it also inhibits some other, possibly unknown, PK. Although our data provide evidence for a cause and effect relationship between TNF-stimulated PK activity and induction of apoptosis, the substrate of the relevant kinase is not yet known.

Many studies have shown that proteins are phosphorylated subsequent to TNF stimulation of a variety of cell types [Schutze et al., 1989; Kaur and Saklatvala, 1986; Zhang et al., 1988; Bird and Saklatvala, 1989; Guy et al., 1991; Hepburn et al., 1988; Robaye et al., 1989; Marino et al., 1989, 1991; Donato et al., 1989; Arrigo, 1990]. However, none of the these studies was able to establish a causal linkage between TNF-induced protein phosphorylation and a variety of cellular responses including mitogenesis, differentiation, cytostasis, and cytotoxicity. A direct demonstration that TNF stimulates protein phosphorylation in cells responding by undergoing apoptosis has not yet been reported.

Although the above studies present abundant evidence that TNF stimulates protein phosphorylation, it is not clear whether this is an epiphenomenon or that this is an essential step in signal transduction. Studies of the effects of agents that either inhibit or promote protein phosphorylation are a different approach to elucidate the pathway of TNF-mediated cytotoxicity. Curiously, there are few reports in the literature evaluating the effects of protein kinase inhibitors on TNF cytolysis. However, two studies reported that the potent, although nonspecific, PKC inhibitor, staurosporine, did not inhibit TNF-mediated lysis of L929 cells [Kull and Besterman, 1989] or U937 [Hamamoto et al., 1990], although it was not stated whether the cells died by necrosis or apoptosis. Our unpublished results agree with these reports that activation of PKC is not required for TNF-mediated cytotoxicity.

Based on the findings of the present study, we propose that TNF activation of a protein kinase (other than PKC) is an essential step to induce apoptosis. The phosphatase inhibitors may synergize with TNF by increasing the amounts and prolonging the life of phosphorylated substrates through inhibition of endogenous phosphatases. The finding that OKA and CLA can reverse the resistance of U9-TR suggests the defect in TNF signalling may be due to an inability to activate a protein kinase. We postulate that the putative kinase is constitutively active at a basal level in U937 and U9-TR cells; however, the TNF signal to stimulate this kinase is disconnected in the variant. The activity of endogenous phosphatases maintains the enzyme's substrate primarily in the dephosphorylated state, thereby blocking apoptosis in the variant. According to this model, the phosphatase inhibitors would allow the accumulation of the phosphorylated substrate due to the basal activity of the kinase to levels sufficient to transmit the apoptotic signal. An alternative interpretation is that the phosphatase inhibitors stimulate a different signalling pathway that bypasses the unresponsive kinase to activate apoptosis.

The present study demonstrates that protein phosphorylation is an important factor determining tumor sensitivity to TNF-induced apoptosis. These studies provide the groundwork for future studies to isolate and identify the enzymes and substrates involved in this pathway. Understanding the biochemical events regulating apoptosis may lead to new concepts in cancer therapy based on promotion of apoptosis, that may be especially valuable in treating drug or cytokine resistant tumor cells.

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