

An essential role for the interferon-inducible, double-stranded RNA-activated protein kinase PKR in the tumor necrosis factor-induced apoptosis in U937 cells

(tumor necrosis factor α /cytokine)

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ABSTRACT Tumor necrosis factor α (TNF- α) is well-characterized for its necrotic action against tumor cells; however, it has been increasingly associated with an apoptosis-inducing potential on target cells. While the signaling events and the actual cytolytic mechanism(s) for both TNF- α -induced necrosis and apoptosis remain to be fully elucidated, we report here on (i) the ability of TNF- α to induce apoptosis in the promonocytic U937 cells, (ii) the discovery of a cross-talk between the TNF- α and the interferon signaling pathways, and (iii) the pivotal role of interferon-inducible, double-stranded RNA-activated protein kinase (PKR) in the induction of apoptosis by TNF- α . Our data from microscopy studies, trypan blue exclusion staining, and apoptotic DNA ladder electrophoresis revealed that a subclone derived from U937 and carrying a PKR antisense expression vector was resistant to TNF- α -induced apoptosis. Further, TNF- α initiated a generalized RNA degradation process in which the participation of PKR was required. Finally, the PKR gene is a candidate "death gene" since overexpression of this gene could bring about apoptosis in U937 cells.

Most cells in metazoan animals can activate a self-destruct process known as apoptosis or programmed cell death when they are no longer needed during development, homeostasis, and pathogenesis (1). Once committed, these cells undergo new rounds of protein synthesis and various morphological/physiological changes including cell shrinkage, chromatin condensation, membrane blebbing, and DNA degradation into a characteristic oligonucleosomal ladder composed of multiples of 200 bp, leading eventually to cell death.

Unlike the morphological transformation events, the genetics of apoptosis is less well-defined. Some of the more well-known "death genes" include *bcl-2*, *p53*, *ced-3*, *ced-4*, *ced-9*, *ice*, and *c-myc* (2). Recently, the growth-suppressor gene *PKR* was demonstrated to be associated with apoptosis in HeLa (3) but not in NIH 3T3 cells (4). PKR is a serine/threonine protein kinase inducible by interferon (IFN) and activated by double-stranded RNA following viral infection (5). By phosphorylating the translation initiation factor-2, PKR inhibits further protein synthesis and thus contributes to the host viral defense. Not surprisingly, overexpressing PKR inhibits cell growth, whereas mutated PKR results in tumorigenesis. The growth-inhibiting property of PKR may partly account for its apoptosis-inducing potential in some systems. Other functions associated with PKR include induction of IFN- α genes (6), and phosphorylation of I- κ B α leading to the release and activation of the nuclear transcription factor (NF)- κ B (7).

Tumor necrosis factor α (TNF- α) is a polypeptide cytokine produced primarily by activated macrophages during infection

and injury, as well as during pathogen and tumor invasion (8). Existing as membrane-bound and soluble forms, TNF- α is an important, pleiotropic cytokine that is responsible for signaling a plethora of cellular responses including antiviral activity, various immunoregulatory activities, and the transcriptional regulation of many genes including NF- κ B. Despite its various beneficial action, TNF- α is instrumental in a multitude of lethal effects including septic shock syndrome, tissue injury, inflammation, cachexia, suppression of adipocyte lipoprotein lipase, and reduction of myocyte resting potential. Historically, TNF- α is noted for its tumoricidal activity through its tissue necrosis-inducing action. However, TNF- α is now known also to induce apoptosis in select cell types and in the presence of metabolic inhibitors (9). Although the exact mechanism of this apoptotic action is unclear, TNF- α has been shown to bind to its cognate receptors and to turn on the sphingomyelin pathway that generates ceramide as the second messenger, which then mediates the cytotoxic effects of TNF- α (10). How this is related to the TNF- α -induced apoptosis remains to be elucidated. Based on the observations (5) that (i) TNF- α treatment results in the activation of several serine/threonine protein kinases, (ii) TNF- α and PKR mobilize NF- κ B, and (iii) PKR is a serine/threonine protein kinase and is growth-inhibiting, we investigated a possible involvement of PKR in the TNF- α signaling pathway and, in particular, TNF- α -induced apoptosis. Here we present evidence that PKR is critical in the apoptotic action of TNF- α on U937 cells.

METHODS AND MATERIALS

Plasmids and Cultivation of Cells. Sense and antisense PKR expression vectors derived from the parental control expression vector pRC-CMV (Invitrogen) as well as methods to isolate and characterize clonal cell lines derived from American Type Culture Collection U937 cells and containing the three PKR vectors have been described (6). To demonstrate that results were reproducible in multiple isolates for each of the three U937 derivatives, new U937 clones were generated for this work; new clonal cells containing pRC-CMV control, sense, and antisense PKR expression vectors were designated U9K-C, U9K-S, and U9K-A, respectively, throughout this study. The expected PKR expression levels in these clones were confirmed by reverse transcription-PCR (RT-PCR) and Western blot analysis. Because old and new U937-derived clones exhibited similar responses to TNF- α treatment, only results from the new clones are reported here. All cells were main-

Abbreviations: IFN, interferon; TNF, tumor necrosis factor; PKR, double-stranded RNA-dependent protein kinase; NF- κ B, nuclear factor κ B; RT-PCR, reverse transcription-PCR.

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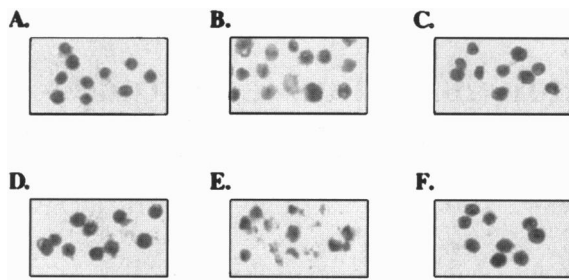


FIG. 1. Light microscopy studies of U937 subclones treated with TNF- α . U937 subclones were treated with 0.5 ng/ml TNF- α for 18 hr, and diluted 10-fold with 0.5% neutral red before examination by light microscopy. (A–C) Untreated controls U9K-C, U9K-S, and U9K-A cells, respectively. (D–F) TNF- α -treated (0.5 ng/ml; 18 hr) U9K-C, U9K-S, and U9K-A cells, respectively. ($\times 100$.)

tained in suspension culture in RPMI medium 1640, 5% fetal bovine serum, and 400 μ g/ml Geneticin (GIBCO/BRL).

Induction of Apoptosis and Cell Viability. To induce apoptosis, recombinant human TNF- α (R & D Systems) was added to the medium to a final concentration of 0.5–5 ng/ml. Alternately, polyriboinosinate-polyribocytidylate [poly(I-C); Pharmacia LKB] was used at a concentration of 0.1 μ g/ml. To determine cell viability, cells were mixed with 0.1 vol of 0.4% trypan blue and were counted with a hemocytometer. Percentage cell viability is defined as the percentage of the number of viable test cells over the number of viable control cells of the same cell line at the end of each experimental treatment.

Microscopy Studies. For light microscopy, cells were diluted with 0.5% neutral red. For transmission electron microscopy, samples were fixed in Karnovsky solution, followed by cacodylate buffer for ultrastructural examination. The cells were postfixed in 1% osmium tetroxide, and dehydrated for staining with uranyl acetate and lead citrate. Thin sections were examined in a JEOL 100 SX electron microscope.

Isolation of Apoptotic DNA Fragments. Apoptotic DNA fragments were isolated according to Herrmann *et al.* (11) and were analyzed in 1% agarose gels. The PKR-specific sense (control) and antisense oligodeoxynucleotides used in some experiments to inhibit apoptosis were 5'-GAAGAAATG-GCTGGTGATC-3' and 5'-GATCACCAGCCATTTCTTC-3', respectively.

RT-PCR. Total RNA extraction and RT-PCR for steady-state RNA levels have been described (12). PCR reaction was allowed to proceed for 30 cycles (95°C for 0.5 min, 55°C for 1 min, and 72°C for 1 min) before arresting the reaction in its logarithmic phase by rapid cooling to 4°C. PCR products were resolved on 0.7% agarose gel, followed by Southern blot analysis (13) with gene-specific probes. PCR primer sets used were as follows: (i) glyceraldehyde-3-phosphate dehydrogenase: upstream, 5'-CAAAAGGGTCATCATCTCTG-3' and downstream, 5'-CCTGCTTACCACCTTCTTG-3'; (ii) PKR: upstream, 5'-GGCACCCAGATTTGACCTTC and downstream, 5'-TCCTTGTTTCGCTTTCCATCA-3'; (iii) interleukin 1 β converting enzyme: upstream, 5'-AATGCTGCT-ACAAAATCTGG-3' and downstream, 5'-ATCATCCTCA-AACTTCTCTG-3'; and (iv) 18S ribosomal RNA: upstream, 5'-CGCAGCTAGGAATAATGGAA-3' and downstream, 5'-TTATGACCCGCACTTACTGG-3'.

Western Blot Analysis. Total protein extracts (20–30 μ g) per sample in PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 45 μ g/ml aprotinin were separated by SDS/10% PAGE, electroblotted onto nitrocellulose membranes, and were probed with anti-PKR or anti-actin antibodies (Sigma) using the Enhanced Chemiluminescence system (Amersham) and following the manufacturer's protocol.

RESULTS

Cell Morphology Post-TNF- α Treatment. Despite the potency of TNF- α , not all tumor cell lines are susceptible to this cytokine. To first ascertain if TNF- α is cytotoxic to U937 cells, the three U937 subclones (see *Materials and Methods*) were first treated with 0.5 ng/ml TNF- α for 18 hr. By light microscopy, both TNF- α -treated U9K-C and U9K-A cells did not exhibit any significant sensitivity to TNF- α (Fig. 1 D and F). However, extended TNF- α treatment for 48 hr in U9K-C cells resulted in signs of cell injury including cell shrinkage, increased cellular granularity, and cell debris (data not shown). U9K-A cells were apparently resistant even to prolonged treatment of TNF- α (data not shown). In sharp contrast to U9K-C and U9K-A cells, the constitutively PKR-expressing U9K-S cells displayed a heightened susceptibility to TNF- α (Fig. 1E). After only an 18-hr incubation with TNF- α , these cells sustained considerable cell injury and changes in cell morphology similar to, albeit with a higher degree of severity,

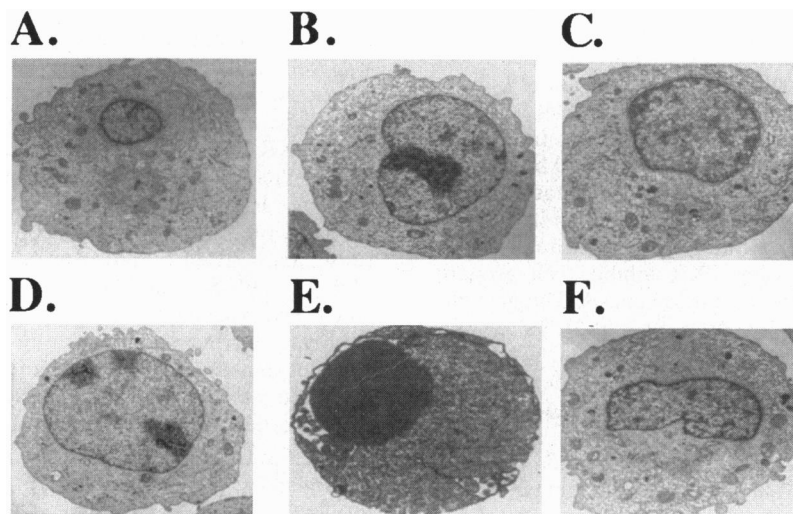


FIG. 2. Electron microscopy of U937 subclones treated with TNF- α . U937 subclones were treated with 0.5 ng/ml TNF- α for 18 hr and processed for electron microscopy as described. (A–C) Untreated controls U9K-C, U9K-S, and U9K-A cells, respectively. (D–F) TNF- α -treated (0.5 ng/ml; 18 hr) U9K-C, U9K-S, and U9K-A cells, respectively. (A–D and F, $\times 41,000$; E, $\times 82,000$.)

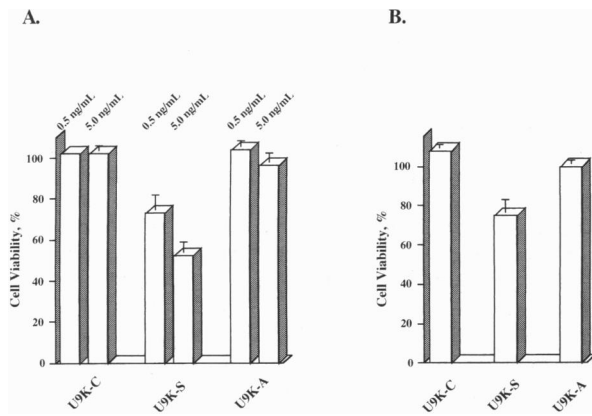


FIG. 3. Cell viability after TNF- α and poly(I-C) treatments. Cells were treated with TNF- α or poly(I-C) for 18 hr and were stained with 0.4% trypan blue in PBS for viable cell counts. Results were derived from three experiments. (A) U9K-C, U9K-S, and U9K-A cells were treated with 0.5 or 5 ng TNF- α (B) U9K-C, U9K-S, and U9K-A cells were treated with 0.1 μ g/ml poly(I-C).

those seen in U9K-C cells treated with TNF- α for 48 hr as described above. By the more sensitive and defining transmission electron microscopy, morphological changes were evident in the nuclei of both U9K-C and U9K-S cells after an 18-hr treatment with TNF- α (Fig. 2 D and E); these cytopathic effects were particularly pronounced for U9K-S cells. Suggestive of apoptosis, the condensation of chromatin into crescents juxtaposing the inner nuclear membrane was clearly seen in these cells. While these chromatin crescents were induced in U9K-C cells only after TNF- α treatment, they were formed spontaneously at a low level in U9K-S cells in the absence of TNF- α (Fig. 2B). Consistent with the light microscopy results described above, this chromatin condensation was drastically aggravated by TNF- α treatment in U9K-S cells (Fig. 2E). Significantly, no appreciable nuclear condensation was apparent in the U9K-A cells in the presence of TNF- α (Fig. 2F), thus establishing a crucial role of PKR in TNF- α -induced cytotoxicity in U937 cells. Interestingly, with continuous routine subculturing and even in the absence of TNF- α , U9K-S cells replicated at a slower rate than the other two U937 subclones and tended to undergo spontaneous cell lysis (apoptosis, see below). Thus, a given culture of U9K-S cells would eventually

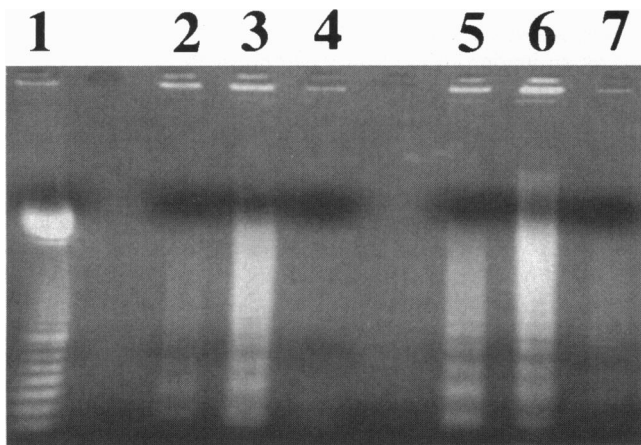


FIG. 4. Oligonucleosomal DNA ladder analysis of apoptotic U937 cells post-TNF- α treatment. After the U937 subclones were treated with 0.5 ng/ml TNF- α for 18 hr, small molecular weight DNA was extracted and was fractionated by 1% agarose gel electrophoresis. Lane 1, 100 base pair DNA marker; lanes 2-4, untreated U9K-C, U9K-S, and U9K-A cells, respectively; lanes 5-7, TNF- α -treated U9K-C, U9K-S, and U9K-A cells, respectively.

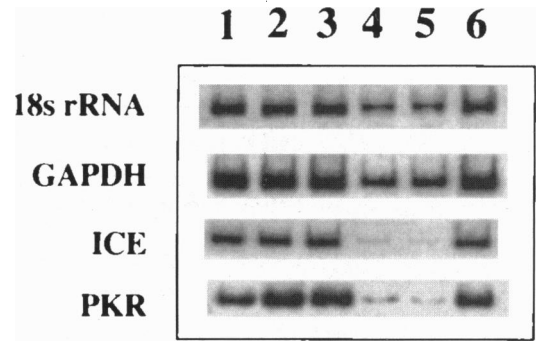


FIG. 5. TNF- α induces RNA degradation in U937 cells. After the U937 subclones were treated with 0.5 ng/ml TNF- α for 18 hr, total RNA was extracted and was subjected to RT-PCR analysis for steady-state levels of various RNA species. Lanes 1-3, untreated U9K-C, U9K-S, and U9K-A cells, respectively; lanes 4-6, TNF- α -treated U9K-C, U9K-S, and U9K-A cells, respectively.

die out after \approx 4-5 months of continuous subculturing (data not shown). The cytopathic effects on U937 cells as documented in this section was TNF- α -specific since anti-TNF- α antibodies apparently abrogated the TNF- α -induced morphological changes (data not shown).

Effect of TNF- α on Cell Viability. To further demonstrate the differential susceptibility of the three U937 subclones to TNF- α -induced cytotoxicity, cell viability was determined by trypan blue exclusion staining after the cells were treated with TNF- α for 18 hr. Trypan blue stains only dead cells; apoptosing cells, if still alive, are not stained. As expected, the viability of both U9K-C and U9K-A cells was not affected by TNF- α (Fig. 3A). However, overexpression of PKR seemed to predispose U9K-S cells to TNF- α cytotoxicity. Up to 25% and 50% of these cells were killed in a dose-dependent manner by an 18-hr treatment with TNF- α at final concentrations of 0.5 and 5 ng/ml, respectively. That PKR is a potential gatekeeper to cell death in U937 cells was underscored by the observation that poly(I-C), which activates PKR (14), killed U9K-S cells overexpressing PKR. Up to 25% of these cells were killed after an 18-hr incubation with poly(I-C) at a final concentration of 0.1 μ g/ml (Fig. 3B). Again, both U9K-C and U9K-A cells were not significantly affected by poly(I-C).

TNF- α -Induced Apoptosis Requires PKR. To further confirm the role of PKR in TNF- α -induced apoptosis, low M_r DNA was isolated from the three U937 subclones after an 18-hr treatment with TNF- α (0.5 ng/ml), using the ultra-sensitive low M_r DNA extraction method of Herrmann *et al.* (11). Because DNA ladder of 200 bp multiples is an unmistakable hallmark of apoptosis (15), TNF- α clearly effected a low level of apoptosis in U9K-C cells (Fig. 4, lane 5). With the sensitivity of this low M_r DNA extraction method, an otherwise insignificant level of spontaneous cell death common in a given culture of many cell lines was also registered for untreated U9K-C cells (Fig. 4, lane 2). Importantly, a deficiency in PKR rendered the U9K-A cells relatively protected from the apoptosis-inducing action of TNF- α (Fig. 4, compare lanes 4 and

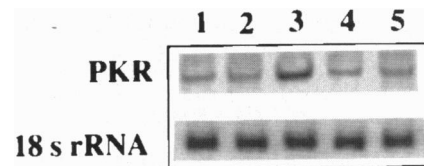


FIG. 6. TNF- α up-regulates PKR RNA expression. U9K-C cells were treated with 0.5 ng/ml TNF- α for indicated times. Total RNA was extracted and subjected to RT-PCR analysis for steady-state PKR and 18S RNA levels. Lanes 1-5 correspond to 0-4 hr post addition of TNF- α , respectively.

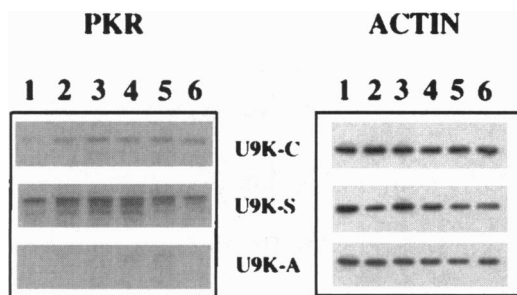


FIG. 7. TNF- α up-regulates PKR protein synthesis. Total protein extracts from U937 cells treated with 0.5 ng/ml TNF- α were immunoblotted with anti-PKR and anti-actin antibodies using the ECL system. Lanes 1–6 in both panels correspond to 0–4 and 6 hr post-TNF- α treatment, respectively.

7). Consistent with the results described in the previous sections, PKR-overproducing U9K-S cells underwent spontaneous apoptosis (Fig. 4, lane 3), which was further aggravated by TNF- α treatment (Fig. 4, lane 6). The absolute requirement of PKR for the TNF- α -induced apoptosis in U937 cells was corroborated by experiments in which the sensitivity of U9K-C cells to TNF- α was much ablated in the presence of a PKR-specific antisense oligodeoxynucleotide but not a control oligodeoxynucleotide (see *Materials and Methods*; data not shown).

TNF- α Depressed Steady-State RNA Levels. To further elucidate the impact of TNF- α on U937 cells at the molecular level, steady-state levels of select RNA species were studied by RT-PCR. For the essential, housekeeping genes of 18S ribosomal RNA and glyceraldehyde-3-phosphate dehydrogenase, the steady-state RNA levels were depressed in both U9K-C and U9K-S cells by about 50% after an 18-hr TNF- α treatment (0.5 ng/ml) (Fig. 5, compare lanes 1 and 4, and lanes 2 and 5). For PKR and the interleukin 1 β converting enzyme, which is known to participate in select apoptotic pathways (16), the steady-state mRNA levels were severely depressed by TNF- α in these cells. Similar findings were obtained for other genes including IFN- α and p53 (data not shown). In contrast, the steady-state levels of the various RNA species tested were not appreciably affected by TNF- α in all cases in U9K-A cells (Fig. 5, compare lanes 3 and 6).

Induction of PKR by TNF- α . To investigate if TNF- α induces PKR as part of its apoptosis-promoting repertoire, steady-state PKR mRNA levels in U9K-C cells were monitored by RT-PCR during TNF- α treatment. Upon addition of TNF- α (0.5 ng/ml) to U9K-C cells, there was an early induction of PKR (Fig. 6); this induction peaked at 2 hr postaddition of TNF- α but was down-regulated thereafter (Fig. 6). This study was further extended by Western blot analysis. As expected, U9K-S cells had the highest endogenous level of PKR proteins (Fig. 7 *Left*, lane 1). Upon TNF- α treatment (0.5 ng/ml), PKR protein synthesis was up-regulated in both U9K-C and U9K-S cells. Consistent with the RT-PCR results in Fig. 6, this increased protein translation peaked at 2 hr post-TNF- α treatment (Fig. 7 *Left*, lane 3). In contrast, U9K-A cells exhibited negligible response to TNF- α treatment (Fig. 7 *Left*).

DISCUSSION

Despite the known functions of TNF- α , the exact mechanism(s) of cytotoxicity induced by this pleiotropic cytokine remains to be completely elucidated. In this paper, we presented evidence to demonstrate for the promonocytic cell line U937 that (i) overexpression of PKR alone was enough to induce apoptosis, (ii) TNF- α -induced cytotoxicity may be

effected via apoptosis, (iii) TNF- α induced PKR, and (iv) PKR was pivotal in the TNF- α -induced apoptotic pathway.

The chromatin condensation and oligonucleosomal DNA ladder demonstrated in the TNF- α -treated U9K-C and U9K-S cells in this study were hallmarks of apoptosis (15). That TNF- α exerted its cytotoxicity on U937 cells primarily through apoptosis rather than necrosis is consistent with previous data on the ability of TNF- α to induce either necrosis or apoptosis in tumor cells (17). The finding that TNF- α can induce apoptosis depending on the cell type and the presence of metabolic inhibitors (9, 17) suggests that some cell types may be susceptible to one but not the other cytotoxic action of TNF- α . It is remarkable that TNF- α has evolved such sophisticated cell killing mechanisms to eliminate unwanted cells in an organism, be it for development or immune surveillance.

In this study, we showed that an overexpression of PKR was enough to induce apoptosis in U937 cells. This induction of apoptosis was a slow process but could be hastened by poly(I-C), an activator of PKR. These results are a direct proof of the apoptosis-inducing potential of PKR in U937 cells. These data are corroborated by a previous indirect demonstration of apoptosis in HeLa cells using a vaccinia virus carrying a mutant PKR gene (3). It must be pointed out that the apoptosis-inducing potential of PKR may not be realized in all cell types, e.g., in NIH 3T3 cells (4). For future studies, it would be illuminating to examine a number of tumor cell lines for their susceptibility to PKR-induced apoptosis and to monitor the PKR status in cells resistant to TNF- α -induced apoptosis.

This study has revealed a cross-talk between the TNF- α and the IFN signaling pathways in U937 cells. This cross-talk may have both beneficial and deleterious consequences. First, consider a host fighting off a viral infection. It is known that TNF- α is inducible by virus infection (18). We have recently shown that PKR is absolutely required for the induction of IFN- α in U937 cells (6). Thus, the induction of PKR by TNF- α may be advantageous to the host because a downstream effect of this is the priming of the cells by PKR, followed by induction of IFN- α upon additional stimulatory signals. Because the production of IFN- α is an important virus-combating weapon, TNF- α can participate and augment the IFN production pathways by up-regulating the PKR gene. On the other hand, it is possible that an aberrant up-regulation of TNF- α , for instance in a disease state, may ultimately lead to a prolonged expression of PKR, resulting in disjointed immune responses as well as activation of the apoptotic pathway. Thus, it is reasonable to postulate that the apoptotic destruction of T cells as documented in some AIDS patients can be partly accounted for by an inappropriate up-regulation of PKR caused by the activation of the TNF- α system commonly seen in HIV infection (19).

It remains to be defined as to how TNF- α up-regulates PKR as described in this report. TNF- α is known to activate phospholipases A₂, C, and D, serine/threonine phosphatases, 2'-5'-oligoadenylate synthetase to synthesize 2'-5'-oligoadenylate, (which in turn activates the RNase L that degrades viral mRNA), the PKC pathway that leads to phosphorylation of serine/threonine kinases, and the sphingomyelin pathway generating the second messenger ceramide, which in turn leads to activation of NF- κ B (8). TNF- α has also been shown to up-regulate IRF-1, a transactivator of many genes involved in the IFN signaling pathway and a direct up-regulator of the PKR gene itself (4). Thus, the apoptosis induction potential of IRF-1 as reported recently (20) may actually turn out to be a consequence of induction of PKR and its pathway of death by IRF-1.

An immediate challenge is to decipher the workings in the PKR-induced apoptotic pathway. An obvious mechanism is the inhibition of protein synthesis through the phosphorylation of translation initiation factor 2 by PKR, so that a prolonged

and uncontrolled activation of PKR will adversely affect normal cell functions leading eventually to cell death. This may partly explain why it took a long time for a culture of U9K-S cells to die out spontaneously by apoptosis, since without an activating agent such as double-stranded RNA, PKR may not be fully active even though there may be substantial amounts of PKR proteins inside the cell. This also leads to the question as to how TNF- α activates PKR activity once it up-regulates expression of the *PKR* gene. Does TNF- α induce some molecules with double-stranded RNA characteristics that will fully activate PKR? Other possibilities to account for the mechanism(s) of PKR-induced apoptosis include induction of some proteases, RNases or endonucleases by PKR either directly, or by some downstream effects such as the PKR-induced mobilization of the transactivation factor NF- κ B, which may then up-regulate the genes for these proteases and nucleases.

Data in this paper did show a relationship between TNF- α and steady-state levels of various RNA species, so that TNF- α treatment of U9K-C and U9K-S cells resulted in a pronounced repression of the steady-state levels of RNA in these cells (Fig. 5). This repression was probably not at the transcriptional level but rather at the level of RNA degradation, because treatment of U937 cells with TNF- α in the presence of actinomycin D revealed that TNF- α activated an RNA degradation pathway independent of the usual virus/double-stranded RNA-induced 2'-5'-oligoadenylate synthetase and RNase L route of RNA degradation (unpublished data).

Finally, the recruitment of PKR by TNF- α in the induction of apoptosis seems to be a recurrent theme of synergism in the repertoire of TNF- α . TNF- α has been shown to synergize with other molecules in executing its various functions. For instance, TNF- α synergizes with IFN- γ in its antiviral activities (21), and transforming growth factor- β in induction of cell differentiation (22). Perhaps the recruitment of PKR by TNF- α is another example of nature's way to economize, to evolve new functions, and to enhance existing functions through the synergism of specific molecules. As it happens, the interaction this time between the two potentially cytotoxic molecules of TNF- α and PKR is fatal.

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