Tumor Suppressor p53 as a Component of the Tumor Necrosis Factor-induced, Protein Kinase PKR-mediated Apoptotic Pathway in Human Promonocytic U937 Cells*

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Despite what is known about the early signaling events in tumor necrosis factor (TNF-α)-induced apoptosis, characterization of the downstream events remains largely undefined. It is now known that a cross-talk exists between the interferon and TNF-α pathways. This linkage allows recruitment of the cell proliferation suppressor PKR (dsRNA-dependent protein kinase) from the interferon pathway to play a pivotal role in TNF-α-induced apoptosis. In this study, we took advantage of the differential TNF-α susceptibilities of human promonocytic U937 sublines, deficient in or overexpressing PKR, to further characterize the role of PKR in apoptosis. By reverse transcription-polymerase chain reaction, we demonstrated that TNF-α transiently induces the tumor suppressor p53 in U937 cells. This p53 induction lags behind the TNF-α induction of PKR by 1 h. By cell viability determination, ultrastructural studies, apoptotic DNA laddering, and antisense techniques, it was shown that inhibition of p53 expression in PKR-overexpressing U937 cells abrogates the TNF-α-induced apoptosis in these cells. Conversely, overexpressing wild type p53 in PKR-deficient U937 cells confers the susceptibility of these cells to TNF-α-induced apoptosis. This latter result indicates that p53 induction is an event downstream of TNF-α-induced up-regulation of PKR, thereby further establishing the critical role of p53 in TNF-α-induced apoptosis in U937 cells. PKR-overexpressing U937 cells were found to possess a constitutively higher level of p53, which partly explains why these cells spontaneously undergo apoptosis even without TNF-α treatment. Finally, a model is presented on the interplay between PKR and p53 in effecting TNF-α-induced apoptosis in U937 cells.

Responding to injury, microbial infection, and tumor invasion, many cell types including activated macrophages, lymphocytes, polymorphonuclear cells, Kupffer cells, and astrocytes may produce tumor necrosis factor (TNF)1, α, a polypeptide cytokine (1).

In general, TNF-α is inducible by bacterial endotoxins, viruses, parasites, antibody/antigen complexes, complements, and other cytokines. On one hand, this pleiotropic cytokine is associated with a plethora of cellular defense responses and activation of many beneficial and cell-protecting genes, including NF-κB (2–4). On the other hand, TNF-α is instrumental in a myriad of lethal effects, including septic shock syndrome, tissue injury, inflammation, cachexia, suppression of adipocyte lipoprotein lipase, and reduction of myocyte resting potential. This bipolar beneficial and detrimental potential of TNF-α reflects the duality also evident in other aspects of TNF-α: TNF-α exists both as membrane-bounded 26-kDa and soluble 17-kDa forms; TNF-α can interact with either a 60-kDa or an 80-kDa receptor with vastly different consequences; TNF-α can render death in susceptible target tumor and non-tumor cells by both extracellular (necrosis) and intracellular (apoptosis) mechanisms (5, 6).

Historically known for its necrotic action, TNF-α is being increasingly appreciated for its ability to induce apoptosis in susceptible cells (7). Apoptosis, or programmed cell death, is a genetically determined program in which a committed cell undergoes a new round of protein synthesis and various morphological/physiological changes such as cell shrinkage, chromatin condensation, membrane blebbing, and ultimately DNA degradation into multiples of 198-base pair oligonucleosomal fragments (apoptotic DNA laddering). The last few years have seen much progress in characterizing the postreceptor binding signaling events in TNF-α-induced apoptosis. Factors such as TRADD, FADD/MORT, RIP, FLICE/MACH, and TRAFs are now known to associate with the p60 and p80 TNF-α receptors in initiating the TNF-α-induced signaling pathway (8). Other than the demonstration of the involvement of the sphingomyelin pathway, ceramide, and the transcription factor NF-κB, much work is needed to map the events connecting the initial receptor binding and the final apoptotic processes. Recently, it was demonstrated that there is a cross-talk between the TNF-α and the interferon signaling pathways and that the double-stranded RNA-activated protein kinase PKR, a key component in the interferon pathway, is recruited to mediate the downstream events in the TNF-α-induced apoptotic pathway (9). PKR is essential for the synthesis of interferon-α and contributes to antiviral activities by phosphorylating and inactivating the translation initiation factor eIF2α in the presence of double-stranded viral RNA or single-stranded viral RNA with double-stranded characteristics. PKR also possesses antiproliferative and apoptosis-promoting activities, including the induction of Fas and mediation of stress-induced apoptosis (10–12).

The tumor suppressor gene p53 has long been linked to cell cycle G1 arrest and inhibition of cell proliferation (13, 14). Mutations of this gene are strongly associated with tumorgenesis. Despite the critical role of p53 in cell survival, protein...
levels of p53 are extremely low in nontransformed cells because of the rapid turnover of p53 (15). Still to be fully elucidated, p53 is believed to act through its transcriptionally transactivating activities (13). Considerable interest has also been shown in p53 for its role in apoptosis (13). Ectopic overexpression of wild type p53 promotes apoptotic death in many tumor cell lines (16, 17). Moreover, another apoptosis-promoting gene, bax, is upregulated by p53 (18, 19). Cytokines and growth factors can protect cells from p53-mediated apoptosis. Because TNF-α induces NF-κB (21), which can in turn up-regulate p53, and in view of the apoptosis-promoting potential of p53, we investigated in this study a possible role of p53 in the apoptotic pathway induced by TNF-α. In particular, we sought to gather evidence in support of the hypothesis that the molecular mechanism of the TNF-α-induced apoptosis in U937 cells involves the induction of PKR, which, already a potent growth suppressor by itself, further enhances the expression of another powerful growth suppressor, the anti-oncogene p53, leading eventually to apoptosis and cell death.

MATERIALS AND METHODS

Plasmids and Cultivation of Cells—Construction of expression vectors with sense and antisense PKR inserts driven by the CMV promoter in pRC-CMV (Invitrogen) and isolation and characterization of stable U937 derivatives harboring these plasmids have been described. End point dilution-subcloned U937-PKR stables with control, sense, and antisense PKR plasmids were designated U9K-C, U9K-S, and U9K-A, respectively (9, 22). Expression vectors for p53 were constructed with standard cloning techniques in pBK-CMV (Stratagene), with the CMV promoter driving full-length sense or antisense p53 cDNA inserts obtained from RT-PCR on mRNA isolated from apparently healthy volunteers’ peripheral blood mononuclear cells. The upper and lower p53 cloning primers were 5'-TGGGCTGTCAGATCATCGAGGAGGCCAG-3' and 5'-AACGTCTTACATGCTTACGTCAGGCCC-3', respectively; p53 inserts were confirmed by restriction mapping and sequencing. Sense and antisense p53 expression vectors were designated pBK-p53S and pBK-p53A, respectively. Derivatives doubly overexpressing sense PKR and either antisense p53 or parental pBK-CMV were isolated from transformation of U9K-S cells with either pBK-p53S or control pBK-CMV and were designated U9K-S53A and U9K-S53C, respectively. Because the selection markers on both pRC-CMV and pBK-CMV were both neomycin, the additional transfection of pBK-53A into U9K-S cells was performed in the presence of another plasmid, pSTK7, which were both neomycin, the additional transfection of pBK-53A into U9K-S cells was performed in the presence of another plasmid, pSTK7. Because the selection markers on both pRC-CMV and pBK-CMV were both neomycin, the additional transfection of pBK-53A into U9K-S cells was performed in the presence of another plasmid, pSTK7. Because the selection markers on both pRC-CMV and pBK-CMV were both neomycin, the additional transfection of pBK-53A into U9K-S cells was performed in the presence of another plasmid, pSTK7. Because the selection markers on both pRC-CMV and pBK-CMV were both neomycin, the additional transfection of pBK-53A into U9K-S cells was performed in the presence of another plasmid, pSTK7. Because the selection markers on both pRC-CMV and pBK-CMV were both neomycin, the additional transfection of pBK-53A into U9K-S cells was performed in the presence of another plasmid, pSTK7.

Induction of Apoptosis and Determination of Cell Viability—Induction of apoptosis in U937 cells and determination of cell viability have been described (23). Briefly, U937 cells and derivatives were treated with TNF-α at 0.5 ng/ml for 24 h before cells were stained with 0.1 volume of 0.4% trypan blue to quantify viable cells with a hemacytometer. 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. For some experiments, the control and anti-p53 oligodeoxynucleotides (phosphodiester; synthesized by Genosys Biotechnologies, Inc.) used were 5'-AAGCAGCTTAGAATGACCGG-3' and 5'-TCCGGGTCTAGACCATGGAGGAGGCCG-3', respectively. Percentage cell viability was determined by trypan blue exclusion.

RESULTS

Induction Kinetics of p53 by TNF-α in U937 Cells—As an initial step to demonstrate the involvement of p53 in the TNF-α-induced apoptotic pathway in U937 cells, we first investigated if TNF-α induces p53 in the promonocytic U937 cells. Because of the high turnover rate of p53, it is commonly known that p53 protein levels in U937 cells are extremely low (25). Like others, we experienced considerable difficulties in measuring p53 protein in U937 cells by Western blotting or enzyme-linked immunosorbent assay, even when employing sensitive chemiluminescence detection techniques from various manufacturers (data not shown). Similarly, fluorescence-activated cell sorter analysis for p53 expression in U937 cells also did not render reliable data. Consequently, we employed the ultra-sensitive technique of RT-PCR to monitor p53 up-regulation by TNF-α. Fig. 1 shows that TNF-α at a low concentration of 0.5 ng/ml up-regulated the expression level of p53 in U937 cells with time, peaking at around 3 h (lane 3) post-TNF-α treatment.

Induction of p53 in U937 PKR Subclones—It has been demonstrated that PKR is pivotal in mediating TNF-α-induced apoptosis in U937 cells (9). As documented previously (9), PKR overexpressing U9K-S cells have 5-fold the amount of PKR proteins as in U9K-C control cells, whereas U9K-A cells harboring an antisense PKR-expressing vector produce negligible levels of PKR protein. We determined PKR protein levels in the p53 up-regulation in U937 cells by TNF-α. U937 PKR subclones were treated with 0.5 ng/ml TNF-α for 3 h to allow maximal p53 expression. It is apparent in Fig. 2 that even without TNF-α, constitutively PKR-expressing U9K-S cells had already twice the steady-state p53 mRNA levels (Fig. 2, lane 2) as that in the control U9K-C and PKR-deficient U9K-A cells (Fig. 2, lanes 1 and 3, respectively). Upon TNF-α treatment, the steady-state p53 mRNA level in U9K-S cells was 5-fold that...
in unstimulated U9K-C parental controls (compare lanes 5 and 1 in Fig. 2), whereas the steady-state p53 mRNA levels in U9K-C and U9K-A cells both increased by less than 2-fold after treatment with TNF-α (Fig. 2, compare lanes 1, 4, and 6).

**Inhibition of TNF-α-induced Apoptosis by Inhibiting p53 Expression**—To demonstrate if expression of p53 was crucial for induction of apoptosis by TNF-α in U937 cells, expression of p53 in U9K-S cells was inhibited by three antisense techniques before TNF-α treatment. U9K-S cells were chosen for these studies because it was demonstrated that, whereas U9K-C and U9K-A cells are resistant to TNF-α at 0.5 ng/ml, U9K-S cells are susceptible to this low concentration of TNF-α (9). To inhibit p53 expression, the first approach was to pre-incubate U9K-S cells with 5 nm of a p53 antisense oligodeoxynucleotide, 5′-CGGCTCCTCCATGGCAGT-3′, which has previously been shown to be effective in inhibiting p53 expression in a dose-dependent manner in many cell types, including human acute myeloblastic leukemia blasts, the human erythroleukemia cell line TF-1, and the human neuroblastoma SK-N-SH cells (26, 27). After 24 h of pre-incubation with this anti-p53 oligodeoxynucleotide, U9K-S cells were treated with 0.5 ng/ml TNF-α and in the presence of fresh anti-p53 oligodeoxynucleotides (5 nm) for another 18 h, before the cells were counted with trypan blue for cell viability. Fig. 3A, bar 1, shows that, consistent with previous results (9), U9K-S cells sustained a 25% cell death induced by TNF-α. In contrast, this TNF-α susceptibility was apparently eliminated by the anti-p53 oligodeoxynucleotide (Fig. 3A, bar 2).

As a second approach to inhibit p53 expression, 5 × 10⁶ U9K-S cells were transiently transfected by electroporation with 30 μg of the anti-p53 plasmid pBK-53A (or pBK-CMV control). After 24 h, TNF-α at a final concentration of 0.5 ng/ml was added to the cells, which were further incubated for 18 h before viable cell counting with trypan blue. Fig. 3B shows that, as in the case of using anti-p53 oligodeoxynucleotides, PKR-overexpressing U9K-S cells became much less susceptible to TNF-α when p53 expression was inhibited.

To further illustrate the involvement of p53, U9K-S cells stably transfected with pBK-53A (or pBK-CMV) were isolated and treated with 0.5 ng/ml TNF-α for 18 h before cell viability determination. Fig. 3C (bars 3 and 4) shows once again that inhibition of p53 expression led to resistance to TNF-α-induced apoptosis. As controls, bars 1 and 2 demonstrate the normal responses of PKR-overexpressing U9K-S and PKR-deficient U9K-A cells, respectively, to TNF-α.

**Electron Microscopy Studies of TNF-α Resistance in U9K-S53A**—To confirm resistance to TNF-α as described above, U9K-S53A cells treated with TNF-α at 0.5 ng/ml for 18 h were subjected to transmission electron microscopy studies. Consistent with published data (9), U9K-C cells showed early signs of apoptosis with chromatin condensation following TNF-α treatment (Fig. 4, panels A and D). As expected, U9K-S53C cells also exhibited spontaneous chromosomal condensation and retained their susceptibility to TNF-α, which was evident in the appearance of total chromosomal condensation, a hallmark of apoptosis, and in the appearance of vacuoles (Fig. 4, panels B and E). In contrast, U9K-S53A cells showed much reduced deleterious effects because of TNF-α. The chromosomal condensation spots were just making their appearance along the inner nuclear membrane (Fig. 4, panels C and F). The extent of this chromosomal condensation was similar to that of TNF-α-treated parental U9K-C control cells (Fig. 4, compare panels D and F).

**Nucleosomal DNA Laddering Studies of TNF-α Resistance in U9K-S53A**—To further demonstrate that p53 expression was
involved in resistance to TNF-α, U9K-S53C cells, which overexpressed PKR, and U9K-S53A cells, which overexpressed PKR and antisense p53 mRNA, were treated with 0.5 ng/ml TNF-α for 18 h before extraction of small molecular weight oligonucleosomal DNA. As reported previously (9), Fig. 5 shows (lanes 2) but not in PKR-deficient U9K-A cells (compare lanes 3 and 5). As expected, U9K-S53C cells retained the characteristics of parental U9K-S cells in terms of their low-level spontaneous apoptosis (Fig. 5, compare lanes 2 and 6) and sensitivity toward TNF-α (Fig. 5, compare lanes 4 and 8). In contrast, when p53 expression was inhibited, U9K-S53A cells exhibited reduced levels of spontaneous apoptosis (Fig. 5, compare lanes 2 and 7) and susceptibility toward TNF-α (Fig. 5, compare lanes 4 and 9), implicating again the role of p53 in TNF-α-induced apoptosis in U937 cells.

Overexpression of p53 Rendered U9K-A Cells Susceptible to TNF-α—As a complementary approach described above, 5 × 10⁶ TNF-α-resistant and PKR-deficient U9K-A cells were transiently transfected by electroporation with 30 μg of the wild type p53 expression plasmid pBK-53S (or pBK-CMV control). After 24 h, TNF-α at a final concentration of 0.5 ng/ml was added to the cells, which were further incubated for 18 h before viable cell counting with trypan blue. Fig. 6 shows that overexpression of p53 effectively conferred TNF-α susceptibility to U9K-A cells (bars 3–5), which now behaved similarly to the TNF-α-sensitive U9K-S cells (bars 1 and 2).

DISCUSSION

Most of the current research on TNF-α-induced apoptosis has focused on the early signaling events after TNF-α binds to its membrane receptors. One notable exception is the elucidation of a cross-talk between the TNF-α and interferon pathways, by which a key enzyme in the interferon pathway, PKR, is recruited to play a pivotal, albeit undefined, role in the TNF-α-induced apoptotic process (9). In this study, we took advantage of the unique TNF-α responses of U937 subclones overexpressing sense or antisense PKR mRNA and identified the tumor suppressor p53 as another crucial downstream participant in the TNF-α-induced apoptosis in U937 cells.

By RT-PCR, it was demonstrated that TNF-α could induce p53 in U937 cells. This p53 induction was transitory and tightly regulated: the p53 induction peaked at 3 h and then quickly subsided. Incidentally, the PKR induction by TNF-α is also transitory and peaks at 2 h (9). Thus, the p53 induction peak lagged that of PKR by 1 h (see also below). This tight regulation of PKR and p53 expression reflects both the potency of these two cell growth inhibitors and the potential adverse effects of having abundant amounts of these two proteins. This may partially explain the difficulty in detecting p53 in U937 cells at the protein level. In addition, some undefined characteristics of the cellular milieu of U937 cells may reduce the already short half-life of p53. Despite the common findings of mutations in p53 in many cell lines, solid documentation of mutation in either the transcriptional regulatory sequences or the actual gene coding sequences of p53 in U937 cells has not been reported in the literature. In the event that these p53 sequences are mutated, the possibility exists that TNF-α is able to induce and activate wild type p53 while suppressing the activity of mutant p53 in U937 cells. This unusual property of TNF-α inducing wild type p53 has been reported in the rat glioma C6 cells, which normally express mutant p53 (28).
any case, these questions are beyond the scope of this paper and will be addressed elsewhere. What is important is that our results on p53 in U937 cells as discussed below provide strong evidence for the crucial role of p53 in the TNF-α-induced apoptosis in U937 cells.

Using three different antisense techniques to inhibit p53 expression in U937 cells, we demonstrated by cell viability, ultrastructural studies, and apoptotic DNA laddering that p53 is essential for the TNF-α-induced apoptotic process. This obligatory need for p53 is reflected also by data in the present study that the uninduced steady-state p53 mRNA level in PKR-overexpressing U9K-S cells was already higher than that of either the control U9K-C or the PKR-deficient U9K-A cells (Fig. 2). This increased p53 expression level may partially account for the spontaneous apoptosing nature of U9K-S cells and the heightened susceptibility of these cells to TNF-α. The finding that induction and overexpression of wild type p53 rendered the previously TNF-α-resistant U9K-A cells susceptible to TNF-α (Fig. 6) is the most telling evidence of the role of p53 in TNF-α-induced apoptosis in U937 cells. This conclusion is independent of the mutation status of p53 in U937 cells. Lastly, this data also establishes that p53 acted downstream of PKR in the TNF-α-induced apoptosis in U937 cells; as mentioned above, this finding is further supported by the observation that the induction of p53 by TNF-α lagged that of PKR by 1 h.

The mechanism of p53 up-regulation remains undefined. Because p53 functions downstream of PKR, there is a possibility that PKR may up-regulate NF-κB, which in turn up-regulates p53. It has been demonstrated that PKR phosphorylates IκB, leading to the release and activation of NF-κB (29). A NF-κB recognition sequence has been detected 5′ to the p53 gene, and NF-κB by itself can up-regulate p53 (30). To resolve the issue that NF-κB has actually been found to protect cells from apoptosis in certain circumstances, it is proposed that initial mobilization of NF-κB may activate some protective pathways. However, once a NF-κB-induced threshold level of p53 is reached as a result of continual cell stress, the cells become committed to apoptosis, and self-destruction ensues. Moreover, continued exposure to stress may actually suppress further NF-κB activation, as reported for normal peripheral blood T cells exposed to oxidative stress for 2 days (31). Another question remains as to the identity of events downstream of p53. One obvious candidate is p21 (WAF), a cell growth suppressor, that has often been found to be coupled to up-regulated p53 activities in many systems (32, 33). However, our preliminary results did not support a significant linkage of p21 and p53 activities in U937 cells in response to TNF-α treatment (data not shown). This may not be surprising because it is now known that p21 can operate independently of p53 (7, 34).

In summary, evidence was provided to support a significant role of p53 in TNF-α-induced apoptosis in U937 cells. Up-regulation of p53 expression appears to be a downstream event following TNF-α-induced activation of PKR. Increase in p53 expression may explain the heightened sensitivity to TNF-α in PKR-overexpressing U937 cell lines. A model was proposed linking the contribution of PKR, NF-κB, and p53 to TNF-α-induced apoptosis in U937 cells. Support of this model and identification of events downstream of p53 activation remain to be clarified.

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