Tumor Necrosis Factor-induced Apoptosis Stimulates p53 Accumulation and p21WAF1 Proteolysis in ME-180 Cells*

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Tumor necrosis factor (TNF)-mediated apoptotic signaling has been characterized by activation of specific protease or protein kinase cascades that regulate the onset of apoptosis. TNF has also been shown to induce oxidative or genotoxic stress in some cell types, and apoptotic potential may be determined by the cellular response to this stress. To determine the role of genotoxic stress in TNF-mediated apoptosis, we examined cellular accumulation of p53 in TNF-treated ME-180 cells selected for apoptotic sensitivity (ME-180S) or resistance (ME-180R) to TNF. Although TNF was able to activate receptor-mediated signaling in either cell line, p53 accumulation was measurable only in apoptotically sensitive ME-180S cells. TNF-induced changes in p53 levels were detected 1 h after treatment, and peak levels were measurable 4–8 h after TNF exposure. TNF was unable to induce p21WAF1 in either cell line but affected the stability of this protein in apoptotically responsive ME-180S cells. Evidence of p21WAF1 proteolysis was detected by monitoring the appearance of a 16-kDa immunoblotable p21WAF1 fragment, which became detectable 4 h after TNF addition and increased in content before the onset of DNA fragmentation (16–24 h). The kinetics of p21WAF1 proteolysis closely paralleled those of poly(ADP-ribose) polymerase, suggesting cleavage of p21WAF1 by activation of an apoptotic protease. Pretreatment of ME-180S cells with the apoptotic protease inhibitor YVAD blocked TNF-induced apoptosis and prevented both poly(ADP-ribose) polymerase and p21WAF1 degradation but did not affect p53 induction. These results provide evidence for the early onset of genotoxic stress in cells committed to TNF-mediated apoptosis and for divergence in propagation of this signal in non-responsive cells. In addition, TNF-induced p21WAF1 proteolysis may be mediated by an apoptotic protease and may contribute to the apoptotic process by disrupting p53 signaling, altering cell cycle inhibition, and limiting cellular recovery from genotoxic stress.

The process of cell death or apoptosis has gained wide recognition as an important mediator of organismal development and pathology. Inappropriate onset or defects in sensitivity to an apoptotic stimulus can give rise to a number of clinical conditions including autoimmunity, neurodegenerative disorders, and cancer (1–3). Therefore, research efforts have focused on understanding both the molecular mechanisms of apoptosis and the elements that constitute an apoptotic signal. While the terminal events in the apoptotic process are evolutionarily conserved and involve activation of proteases with specificity for aspartic acid residues in target proteins (termed caspases; Refs. 4 and 5), the elements that signal an apoptotic event and controls that regulate the apoptotic signal are varied and numerous. There have been reports of apoptotic cell death by activation of death cascades coupled to cytokine receptors (6, 7), withdrawal of growth factors (8), physical alterations in DNA through structural damage after cytotoxic drug exposure or radiation (9–11) and by expression of specific genes in tumor cells that alter cell cycle control (12, 13). Each of these conditions may increase the level of cellular stress and activate cellular signals that alleviate stress or promote elimination of damaged cells by apoptosis (14, 15). Evidence suggests that cellular or genotoxic stress above a threshold level, controlled by many elements and varied within cell types, will activate a cell death program (16). Understanding these elements and their coupling to the apoptotic signaling cascade will be of critical importance in understanding and treating diseases associated with aberrant apoptosis.

Several proteins have been described that may be involved in regulating cellular stress and stress-induced signaling, and functional defects or alterations in their expression have been associated with specific diseases (1–3, 8, 9, 15, 16). One of the most investigated proteins for which loss of function is associated with cancer is p53, which may function as a critical regulator of genotoxic stress and apoptosis (14–16). Studies of the wild-type protein have shown that DNA damage or oxidative stress can enhance the cellular accumulation of this protein by its increased stability in stressed cells (15–17). Induced changes in the level of p53 may directly facilitate DNA repair or through transactivation of other genes, inhibit cell cycle progression, or induce apoptosis (15, 16). Loss of p53 function may allow inappropriate survival of damaged cells and permit the accumulation of DNA damage, further promoting cellular transformation (17–20). Therefore, genotoxic stress surveillance and concomitant p53 accumulation are important primary responses in damaged cells.

Tumor necrosis factor (TNF)1 has been shown to initiate several biological activities including apoptosis of some tumor cells (21). These activities are mediated through TNF receptor oligomerization, which activates several downstream signaling events including protein kinase and apoptotic protease cascades (22–24). Recent identification of proteins with distinct affinities for specific TNF receptor domains and the recruitment of other proteins to these signaling complexes have pro-

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1 The abbreviations used are: TNF, tumor necrosis factor; PARP, poly(ADP-ribose) polymerase; PCNA, proliferating cell nuclear antigen; PAGE, polyacrylamide gel electrophoresis.
vided insight into the process of TNF-mediated signaling and apoptosis (22, 25, 26). However, some of the signal cascades activated by TNF are also activable by a number of stress-inducing stimuli, suggesting common stress signals as mediators of TNF signal transduction and apoptosis (24, 27). There is also evidence for oxygen radical formation, lipid peroxidation, and other oxidative damage in TNF-treated cells and the signaling pathway that mediates these responses is unknown (21, 28–30). Importantly, these responses may be altered in tumor cells that are unable to undergo apoptosis after TNF incubation (21, 29). Therefore, the nature, onset, and requirement of these stress-activated signals in TNF-initiated apoptosis will need to be more fully defined.

In the present study, we examine the affects of TNF on p53 accumulation as a measure of the onset of genotoxic stress in ME-180 clonal cells previously selected for apoptotic sensitivity or resistance to TNF (31, 32). The results suggest that although TNF activated stress signaling cascades (JNK, NF-κB) in both cell types, early genotoxic stress, as measured by p53 accumulation, was detected only in apoptotically sensitive cells. These results suggest a possible defect in propagation of a genotoxic signal, with little change in TNF receptor-associated signaling in cells resisting TNF-induced apoptosis. Interestingly, p53 accumulation in response to TNF did not result in the induction of p21WAF1 but stimulated its proteolysis with characteristics similar to that of other apoptotic protease substrates (33).

EXPERIMENTAL PROCEDURES

Cell Lines and Their Apoptotic Sensitivity—ME-180S and ME-180R clonal response variants have been previously described and characterized (31, 32). Recent studies have shown similarities between cell lines in TNF-initiated signal transduction but defects in TNF-mediated apoptosis in non-responsive ME-180R cells. Cells were tested for quantitative TNF sensitivity by crystal violet assays and apoptotic potential by monitoring the appearance of DNA fragments as described previously (31, 35).

Antibodies and Reagents—Antibodies used in these studies were anti-p53 (Ab-6; Oncogene Sciences, Cambridge, MA), anti-p21WAF1, anti-PARP, (PharMingen, San Diego, CA), anti-bax (N-20), and anti-PCNA (Santa Cruz Biologicals, Santa Cruz, CA). YVAD-cmk was obtained from Bachem (King of Prussia, PA) and was dissolved in Me2SO as a stock solution of 40 mM. Peroxidase-conjugated anti-murine Ig was purchased from Bio-Rad. ECL reagent was obtained from Amersham Corp. and was used according to the procedure provided by the supplier. Recombinant human TNF was kindly provided by Genentech Corp. (South San Francisco, CA).

Protein Extraction and Analysis—Cells were grown to 80% confluence in 60-mm culture dishes (~2 × 10⁶ cells) and treated with TNF for the interval described. Dishes were placed on ice and rinsed three times with ice-cold phosphate-buffered saline, and cells were released with a cell scraper. Cells were pelleted by centrifugation (1 min, 4 °C, 15,000 × g), and the cell pellet was resuspended in lysis buffer (20 mM sodium phosphate, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). After centrifugation (15,000 × g, 15 min), equal protein aliquots from each supernatant were resolved by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting. ECL reagent was used for antigen detection in all experiments.

RESULTS

Previously, we reported the isolation and characterization of ME-180 clonal populations, which expressed complete sensitivity or resistance to TNF-induced apoptosis (31, 32). These studies, and more recent analysis, suggested that resistance to TNF was not associated with TNF receptor-associated signaling defects but was restricted to the loss of TNF-induced apoptosis. As shown in Fig. 1, DNA fragmentation was detectable in ME-180S cells 16–24 h after TNF addition but TNF had no effect on DNA integrity in ME-180R cells. Therefore, distinc-

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![Fig. 1. TNF-mediated DNA fragmentation in ME-180 cells. ME-180 Sen or Res cells were treated with TNF (1 nM) for the interval indicated, cells were collected, and DNA resolved by agarose gel electrophoresis (as described above). The etidium bromide-stained gel was photographed under UV light and is shown.](image1)

![Fig. 2. Induction of p53 by TNF in ME-180 cells. ME-180 Sen (left) or Res (right) cells were treated with TNF (1 nM) for the interval indicated and cell lysates were prepared and analyzed for protein concentration. Equal protein aliquots (40 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for p53. Total protein was stained with Coomassie Blue and is shown at the bottom (Total Protein).](image2)
The influence of TNF-induced p53 accumulation on p21WAF1 expression was also examined in both apoptotically responsive and non-responsive cells. As shown in Fig. 4, TNF failed to induce p21WAF1 in either cell line. However, the stability of p21WAF1 was affected by TNF in ME-180S cells, as determined by the appearance of a 16-kDa p21WAF1 fragment, which correlated with a reduction in the level of the full-length p21WAF1 protein. This cross-reactive protein was detectable 4 h after the addition of TNF and increased in intensity with increasing time after TNF addition. Forty-four hours after TNF addition and after the onset of DNA fragmentation, p21WAF1 protein was not detectable by immunoblotting apoptotic cell lysates. Other proteins, such as PCNA (as shown in Fig. 4), were unaffected by TNF incubation, providing evidence for specific proteolysis of p21WAF1. Together, the results provide evidence for the induction of p53 and proteolytic process-
inhibited PARP cleavage, ME-180S and ME-180R cells were pretreated with YVAD (40 \mu M) for 60 min before the addition of 1 nM TNF for 9 h. Cell lysates were prepared and analyzed for PARP cleavage by immunoblotting. As shown in Fig. 8, TNF induced PARP proteolysis in ME-180S cells but had no effect on PARP expressed in ME-180R cells. The tetrapeptide analogue YVAD-cmk was an effective inhibitor of TNF-mediated PARP proteolysis (and apoptosis) in ME-180S cells.

The effect of YVAD on TNF-initiated p21WAF1 proteolysis was also examined in TNF-sensitive and -resistant ME-180 cells. As shown in Fig. 9, when p21WAF1 was examined 8 h after the addition of TNF, the 16-kDa fragment was again detectable and its appearance correlated with a concomitant decrease in the level of full-length p21WAF1 in apoptotically sensitive ME-180S cells. The 16-kDa fragment was not detected in ME-180R cells under similar conditions (longer film exposures also failed to provide evidence for a 16-kDa fragment in ME-180R cells). YVAD treatment alone (9 h; 40 \mu M) failed to alter p21WAF1 levels in either cell line. Pretreatment with YVAD (1 h) effectively blocked proteolysis of p21WAF1 when analyzed 8 h after TNF addition. Similar results were obtained with the CPP32 caspase inhibitor (43, 44), DEVD (data not shown). Together, these results provide evidence for the possible involvement of apoptotic proteases as mediators of p21WAF1 cleavage in ME-180S and their deficiency in ME-180R cells.

To determine whether TNF-induced p53 accumulation required activation of apoptotic proteases as cellular stress mediators, p53 levels were measured in control and YVAD-pretreated ME-180S after TNF incubation. As shown in Fig. 10, TNF induced a 3-fold increase in cellular p53 levels in the presence or absence of YVAD, suggesting that p53 accumulation in TNF-treated ME-180S cells does not require early activation of a YVAD-sensitive protease.

**DISCUSSION**

In this report, the distinctions in specific genotoxic stress signals were investigated in ME-180 cells selected for sensitivity or resistance to TNF-induced apoptosis. We had shown previously that TNF signaling was not defective in resistant cells since both JNK and NF-\kappa B activities mediated through TRADD/TRAF2 recruitment to the TNF receptor complex (27), were activated by TNF in either cell line.2 Importantly, in this study, we demonstrate that p53 accumulation occurred rapidly after TNF exposure in apoptotically sensitive cells and preceded the onset of PARP cleavage and DNA fragmentation. Despite evidence for activation of stress signaling by TNF in resistant cells, there was no evidence for alterations in p53 levels in resistant cells. Mutations in p53 and general defects in apoptosis in ME-180R cells do not appear to account for the loss of sensitivity to TNF since other apoptotic stimuli (cis-platinum, adriamycin, radiation) were able to induce equivalent or greater levels of apoptosis in ME-180R and ME-180S cells (45). In addition, both p53 and p21WAF1 levels could be coordinately elevated in response to DNA damage in either cell line (UV exposure; Fig. 3). Other TNF-sensitive cell lines with wild-type p53 function (MCF-7) have also been shown to accumulate p53 in response to TNF (46), and our studies of MCF-7 cells have shown similar rapid accumulation of p53 only in apoptotically responsive MCF-7 cells but not in a TNF-resistant MCF-7.
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proteins may alter its function and nuclear distribution (50). State suggests that association of this inhibitor with other factors may regulate the growth factor-stimulated proliferation (36). In addition, p53 induction may not contribute to TNF-mediated apoptosis in all tumor types but may participate in stress signaling and destabilization of cell survival in some populations. The cellular mediator of TNF-promoted p53 accumulation and genotoxic stress is unknown but is currently being investigated. Importantly, this activity appears to be upstream of cellular activation of apoptotic proteases and does not appear to be correlated with activation of other stress signaling cascades (such as JNK; Refs. 14 and 27) or require the early activation of an apoptotic protease cascade. Tumor cell sensitivity to TNF-induced apoptosis may be determined by the combined but independent activation of stress and stress response signaling events (such as those mediating p53 accumulation) as well as those controlling activation of caspase cascades.

Examination of the downstream effectors of p53 in TNF-treated ME-180S cells provided evidence for the proteolytic processing of p21WAF1 but not other p53-regulated proteins (e.g. bax; Fig. 5). The onset of p21WAF1 proteolysis was similar to that of PARP and was inhibited by protease inhibitors active in protecting cells from apoptosis. Neither PARP or p21WAF1 proteolysis was detected in ME-180R cells after TNF incubation, providing evidence for defects in TNF-mediated protease activation in these cells that correlate with specific loss of TNF-directed apoptosis. Therefore, this study provides first evidence for p21WAF1 proteolysis in TNF-mediated apoptosis and is one of the first reports of tumor cell resistance to TNF through specific loss of a TNF-activated protease cascade (25–27). The specific defects in this cascade have not as yet been determined, but initial evidence suggests defects in TNF receptor-complexing caspases but not downstream apoptotic proteases. Importantly, TNF-stimulated degradation of p21WAF1 may be mediated by specific proteases expressed in ME-180 cells since p21WAF1 degradation has not been described in other cell lines that induce p53 in response to TNF (46).

Preliminary mapping of the proteolized p21WAF1 protein suggests that TNF-induced truncation of its C terminus produced the p16 fragment detectable by immunoblotting. If caspases are involved in the proteolysis of p21WAF1, aspartic acid residue cleavage site specificity of these proteases restricts the number of possible target sites on the p21WAF1 protein. Only four potential candidate cleavage sites exist in the C terminus of this protein, and only two of these would yield fragments of the appropriate size as those detected by immunoblotting (37, 38). These include Asp-109 and Asp-112, and the DHVD sequence bracketed by these aspartic acid residues bears similarity to the cleavage site on PARP (DEVD) recognized by CPP32 (33, 42, 43). Cleavage of this protein in vitro with recombinant protease will provide additional support for direct p21WAF1 proteolysis by apoptotic proteases.

p21WAF1 has been shown to suppress cell cycle progression by inhibition of cyclin-dependent kinases (37, 38). However, recent studies have shown that the expression of this protein may be regulated by growth stimulatory cascades, suggesting a more complex role for p21WAF1 in regulating cell cycle transition and evidence of a role for this protein in feedback regulation of growth factor-stimulated proliferation (36). In addition, the existence of p21WAF1 in both an active and inactive state suggests that association of this inhibitor with other proteins may alter its function and nuclear distribution (50), PCNA may play an important role in this sequestering by its affinity for the C terminus of p21WAF1 and its proposed role in DNA replication and excisional repair of DNA lesions (39, 40, 51). TNF-induced p21WAF1 proteolysis may alter the association of cdk with PCNA and disrupt the nature of the replication or repair complex. In addition, studies have shown that enhancement or disruption of cdk enzymatic activity alone can stimulate or repress TNF-induced apoptosis, respectively (12, 13). Interestingly, others have shown that expression of a C-terminally truncated form of p21WAF1, such that PCNA binding is disrupted, results in the slow onset of apoptosis (52). Thus, evidence suggests that modulation of cdk function and PCNA association with p21WAF1 may influence the apoptotic sensitivity of cells. TNF-induced proteolysis of p21WAF1 may alter both functions by first reducing PCNA/p21WAF1/cdk association and subsequently affecting cdk activity by loss of its inhibitory effects through its accelerated degradation in apoptotic cells. This may contribute to the changes in cell cycle progression seen in TNF-treated cells (53–55).

In this report, evidence for TNF-induced genotoxic stress and p53 accumulation in ME-180 cells committed to undergo apoptosis is presented. The accumulation of p53 as genotoxic damage is surveyed may induce the recruitment of repair proteins, which are known targets of apoptotic proteases (56). Both PARP and PCNA have been shown to play a role in DNA repair and may assemble at sites of DNA damage (39, 40, 44, 48, 51, 53). Complex formation between the C terminus of p21WAF1 and PCNA may direct p21WAF1 to the repair site and in proximity to activated caspases with affinity for other repair protein substrates (PARP). Proteins with sensitive aspartic acid sequences (e.g. DHVD in p21WAF1) may also present as specific targets of these proteases. Importantly, PCNA was not cleaved in TNF-treated cells, demonstrating target protein specificity. The identity of the protease active in cleaving p21WAF1 is unknown and additional analysis of the role of p21WAF1 proteolysis in apoptosis is required. However, although the mechanism is unknown, studies by other investigators have already provided evidence of a role for a truncated p21WAF1 in apoptosis (52).

We have reported that early activation of the apoptotic signal in ME-180 cells results in p53 accumulation and evidence of genotoxic stress that were not detectable in cells unable to commit to TNF-mediated apoptosis. Stress-induced p53 accumulation and TNF-mediated caspase activation may lead to the proteolysis of p21WAF1 and commit cells to apoptosis by altering p35 effectors, cell cycle controls, and recovery from genotoxic stress. Since p21WAF1 expression is involved in protection against a variety of apoptotic stimuli (34, 57), its proteolytic degradation may be an appropriate target of some apoptotic proteases.

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