Enhanced Synthesis of Tumor Necrosis Factor-inducible Proteins, Plasminogen Activator Inhibitor-2, Manganese Superoxide Dismutase, and Protein 28/5.6, Is Selectively Triggered by the 55-kDa Tumor Necrosis Factor Receptor in Human Melanoma Cells*

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We have demonstrated that A375 melanoma cells express mRNA for both types of tumor necrosis factor (TNF) receptors and receptor proteins on their plasma membranes. Specific agonist and blocking antibodies to either 55-kDa (TNF-R1) or 75-kDa (TNF-R2) TNF receptors combined with two-dimensional gel analysis were employed to determine which receptor type is responsible for mediating the induction of individual melanoma proteins. Our results indicate that the enhanced synthesis of proteins 217/7 (M/pI), 28/5.6, and 41/5.7 is selectively induced through TNF-R1. TNF induces these proteins; antagonist antibody to TNF-R1 prevents their induction by TNF, and TNF-R1 agonist induces them in the absence of TNF. Identification of these proteins by immunoblot analysis proved that 217/7 is manganese superoxide dismutase, protein 28/5.6 is unrelated to 27/280-kDa heat shock protein, and protein 41/5.7 is plasminogen activator inhibitor-2. Furthermore, TNF cytotoxicity for A375 cells is also mediated by TNF-R1. These studies indicate that TNF-R1 is a critical signaling receptor for TNF action on A375 cells and demonstrate the potential use of TNF-R1 antibodies to selectively block or enhance specific effects of TNF on melanoma cells.

Tumor necrosis factor (TNF),1 originally defined for its ability to cause hemorrhagic necrosis of certain tumors (1), is a hormone-like protein that binds to specific receptors on a variety of different cell types and subsequently transduces a broad spectrum of immunological, inflammatory, and regulatory activities (reviewed in Refs. 2–5). These pleiotropic activities include growth inhibition of some tumor cells, activation of phagocytic and endothelial cells, induction of prostaglandins and alterations in lipid metabolism, bone resorption, and regulated expression of major histocompatibility complex antigens, genes, and transcription factors. The therapeutic application of TNF or agents directed against it is complicated by the fact that TNF has been implicated in the regulation of normal development and homeostasis as well as in the pathogenesis of certain diseases. The diversity of biological effects attributed to TNF suggests that the specificity of its action probably resides in the receptors to which it binds and/or the intracellular components that become activated as a result of TNF binding to its receptors. Therefore, it is essential to characterize not only the receptors themselves, but also the individual intracellular molecules that are induced by TNF in various cell types and to determine the pathways by which these molecules are regulated.

Two distinct high-affinity receptors for TNF have been identified and cloned (6–9). In most human cells examined, both 55 kDa (TNF-R1) and 75 kDa (TNF-R2) TNF receptors are coexpressed (10). Cross-linking studies have shown that the TNF trimer must complex with two or three receptor molecules to generate an intracellular signal (11, 12). Furthermore, immunoprecipitation studies with receptor type-specific antibodies revealed that the two types of TNF receptors segregate into two species of complexes, each containing only one type of receptor (13). Both TNF-R1 and TNF-R2 have the typical structure of a membrane-spanning protein with a single transmembrane region, and both are members of a new superfamily of cell surface proteins which include the p75 chain of nerve growth factor receptor, the B cell antigen CD40, the OX-40 T cell activation marker, the SFV-T2 open reading frame of Shope fibroma virus (6–9, 14), and the Fas antigen (15). Although TNF binds with high affinity to both receptors, the extracellular domains of the two TNF receptors show only 28% amino acid identity and are immunologically distinct. No significant similarities exist between their intracellular domains (6, 16), suggesting that the two receptors use different signal transduction pathways, therefore contributing to the diverse biological effects of TNF.

To date, few studies have addressed at the molecular level whether the two TNF receptors activate distinct biochemical pathways in cells which result in the selective expression of one or more of the biological effects of TNF. In previous studies, we have employed 2D polyacrylamide gel electrophoresis and computer-based gel analysis to characterize proteins induced by TNF and/or IFN-γ in several cell types, including human fibroblasts (17), ME-180 cervical carcinoma (18), and A375 melanoma (19, 20). In the present study we employed this technology in combination with antibodies that act as specific agonists or antagonists to TNF-R1 and TNF-R2, to determine which type of TNF receptor is responsible for triggering the enhanced synthesis of individual melanoma proteins by TNF. We describe three TNF-inducible proteins that are selectively induced by...
TNF through TNF-R1 in a human melanoma cell line that expresses both types of TNF receptors. In view of the fact that we find TNF cytotoxicity for A375 cells is also induced through TNF-R1, and our 2D gel analysis shown no proteins to be significantly induced by TNF through TNF-R2, it is probable that TNF-R1 plays a major role in mediating TNF effects in A375 melanoma cells.

MATERIALS AND METHODS

Cytokines—Pure human recombinant TNF (specific activity, 4 x 10^7 units/mg), and pure human recombinant IFN-y (specific activity, 9 x 10^7 units/mg) from Genentech, Inc. (South San Francisco, CA). At the concentrations employed in our experiments, these cytokines are inactive for endotoxin as determined by the Limulus assay.

Antibodies—The following antibodies specific for either TNF-R1 or TNF-R2 were obtained from Genentech, Inc.: TNF monoclonal antibodies (mAb) 984 and 1947 (antibodies that bind to human TNF-R1 and TNF-R2, respectively, and act as TNF antagonists) and polyclonal antibodies 11706-34B (TNF-R1 agonist) and 12074-16B (TNF-R2 agonist). Polyclonal antibody 10373-33, a very low titered antiserum to nerve growth factor that was generated in a manner similar to the TNF receptor agonist antibodies, was also obtained from Genentech and used as a control serum. Lipopolysaccharide (LPS, 100 ng/ml) was used as a positive control antibody was obtained from Organon Teknika Corp., West Chester, PA and rat anti-mouse pE conjugated was obtained from Rector-Dickinson, Mountain View, CA. Polyclonal antibodies to human manganese superoxide dismutase (MnSOD) were obtained from Dr. Larry Oberley at the University of Iowa, Iowa City, and antibodies to plasminogen activator inhibitor-2 (PAI-2) were obtained for Dr. Robert Oberley at the University of California in San Francisco, mAb to human heat shock protein 27/28 (hsp 27) that recognizes all known forms of the protein was purchased from StressGen Biotechnologies Corp., Victoria, British Columbia, Canada.

Polymerase Chain Reaction (PCR) Primers and Positive Controls—PCR primers and annealing temperatures were selected with the help of Oligo 4.0 Primer Analysis software (National Biosciences Inc., Plymouth, MN). Forward (sense) and reverse (antisense) PCR primers for TNF-R1 were 5’CCTC~~ACCGTGCA~CTCT3~ and 5’GTGACT- eten Biotechnologies Carp., Victoria, British Columbia, Canada.

Immunoblot Analysis—The location of human MnSOD, hsp 27, and PAI-2 on 2D gels was determined by immunoblot analysis as follows. Proteins were separated on isoelectric point and relative mobility in polyacrylamide and SDS by the method of method as modified by Patton et al. (24) and by our laboratory (22) using the Inventorat 2-D Electrophoresis System (Millipore, Corp., Bedford, MA). Briefly, gels were prepared, scanned, and computer-analyzed. The PDQUEST system (17-20, 22, 23) was used to match gels and assign molecular weights (M) and isoelectric points (pI) within 15% error to individual gel proteins. Optical density was converted into dpm using 14C calibration standards, and films were normalized to account for small differences in gel loading. The relative abundance in parts per million (ppm) of TNF or mononuclear antibody-induced proteins was then determined using a Computing Densitometer and ImageQuant software (Molecular Dynamics, Mountain View, CA). Our criterion for a significant change is a 2-fold increase or decrease in protein expression over the control level in at least two experiments. In addition, significant changes in the synthesis of individual proteins were confirmed by visual examination of autoradiograms.

Immunoblot Analysis—The location of human MnSOD, hsp 27, and PAI-2 on 2D gels was determined by immunoblot analysis as follows. Lysates were prepared from cells stimulated for 24 h with TNF (1000 units/ml) plus IFN-y (100 units/ml) for optimal expression of the three proteins. Protein lysates (200 µg), spiked with 9 x 10^5 dpm of 14C-labeled lysate, were electrophoresed as above and gels were blotted to polyvinylidene difluoride (Immobilon-P, Millipore Corp.) at 400 µA/cm² for 60 min in a modified Towbin transfer buffer (25). Immunoblotts were prepared by the method of Lopez et al. (26) using the following primary antibodies: antiseraum to MnSOD (1:1500), antiseraum to PAI-2 (1:500), or mAb to hsp 27 (1 µg/ml), and the appropriate immunoglobulin-conjugated anti-rabbit or anti-mouse secondary antibodies (AuroProbe BI- plus; Amersham Corp.). Positive spots on immunoblotts were then matched to autoradiograms and stained gels and quantitated with Imagequant software. In other experiments, 1D minigels were loaded with proteins from cell lysates for 24 h and/or IFN-R1 antibodies as described previously, run at 100 V, and blotted and probed with MnSOD or PAI-2 antibodies as indicated above.

TNF Cytotoxicity Assays—A375 cells were seeded at 10⁵ cells/well in 200 µl of medium in a 96-well microtiter tray and grown to confluence over 18-24 h at 37°C. The cells were then treated in the presence of 1 µg/ml cycloheximide with medium alone, TNF, and/or the type-specific TNF receptor antibodies at the concentrations indicated above. All controls and treatments were set up in replicates of six wells.
and the trays were incubated an additional 24 h. Under these conditions TNF induces significant cell death which is apparent by microscopic examination. Staining with crystal violet was used to quantitate cell death where absorbance at 600 nm is inversely correlated with cell death. The dye was eluted, quantitated spectrophotometrically, and dye binding was correlated with cell growth as described previously (17).

RESULTS

A375 Cells Express mRNA and Cell Surface Protein for Both Types of TNF Receptors—PCR-amplified reverse-transcribed TNF receptor mRNA studies demonstrated that A375 cells express message for both TNF-R1 and TNF-R2. Fig. 1 shows the ethidium bromide stained gels of the PCR products. In A, a band of 425 base pairs was found in lanes containing TNF-R1 cDNA (positive control) and reverse-transcribed cDNA from either U937 or A375 cells. This band was missing in the lane containing the appropriate primers but lacking RNA (negative control). In B, a band of 450 base pairs was found in the lanes containing TNF-R2 cDNA and reverse-transcribed cDNA from either U937 or A375 cells. Both bands are the size predicted by the respective primers of the two TNF receptor genes. The additional PCR fragment found in A375 (Fig. 1B) was occasionally observed and its identity is presently not known.

Indirect immunofluorescence analysis verified that mRNA for each type of TNF receptor was translated into protein and expressed on the plasma membranes of A375 cells. Fig. 2 shows immunofluorescent microscopic images of single cells stained with either monoclonal antibodies to TNF-R1, TNF-R2, or with IgG1 isotype control, followed by rat anti-mouse κ PE conjugate. Fluorescence intensity was quantitated in arbitrary units of 0–4095 and represented in the gray scale, where white represents the highest amount of fluorescence above background.
highest amount of fluorescence above background. The average total cellular fluorescence per cell above background (Fl/Cell) times $10^4$ for TNF-R1, TNF-R2 and IgG1 isotype stained cells was 125 ± 18, 85 ± 9.4, and 33 ± 5.7, respectively. The level of fluorescent staining for TNF-R1 and TNF-R2 was significantly greater than that of the isotype control at p < 0.002 (analysis of variance, Student-Neumann-Kuels multiple range test).

**TNF Induction of Individual Proteins in A375 Cells—**
Previously, we demonstrated that TNF inhibits the growth of A375 melanoma cells (20). To define some of the molecular mechanisms which may be involved in growth suppression or other functional manifestations of TNF activity in A375 cells, we examined the synthesis of individual cellular proteins during cytokine treatment using 2D gel electrophoresis and computerized gel analysis. Fig. 3 shows an autoradiogram of a representative 2D gel loaded with lysate from A375 cells treated with TNF and the location of three TNF-inducible proteins of interest, designated by MW/pl.

**Melanoma Proteins 21/>7, 28/5.6, and 41/5.7 Are Induced through TNF-R1—**
In the next experiments we determined which specific type of TNF receptor is responsible for mediating the enhanced synthesis of these three TNF-inducible proteins. Cells were treated with specific antibodies that act as agonists and mimic TNF binding to one or the other type of TNF receptor. Cells were also treated with an antagonist antibody to TNF-R1 in the presence of TNF, TNF alone, or with the control antiserum. Results for proteins 21/>7, 28/5.6, and 41/5.7 are shown in A-C of Fig. 4, respectively. The autoradiograms depicted are matched sections from gels loaded with lysates from cells treated with medium alone, TNF, or the designated antibodies. In the medium control these proteins are expressed at relatively low levels or, in the case of protein 41/5.7, at nearly undetectable levels. TNF induces these proteins as does TNF-R1 agonist. In contrast, none of these three proteins are induced to a significant extent by TNF-R2 agonist. Furthermore, neutralizing, TNF-R1 antagonist antibodies prevent their induction by TNF. Our control antiserum gave results similar to that of the medium control. Table I shows the densitometric analysis of proteins 21/>7, 28/5.6, and 41/5.7 from the respective autoradiogram sections depicted in Fig. 4. The relative abundances of these three proteins is significantly greater in lysates of cells treated with either TNF or TNF-R1 agonist. It is interesting that protein 28/5.6 is somewhat suppressed by the TNF-R2 agonist. Presently, we do not know if this suppression is biologically significant.

**Identification of Melanoma Proteins by 2D Immunoblot Analysis—**
The location of protein 21/>7 on 2D gels is similar to that of a protein in fibroblasts that we identified previously by partial amino acid sequencing as MnSOD (27). Furthermore, we found that MnSOD mRNA is induced by TNF and IFN-γ in A375 melanoma cells (27, data not shown). In this study, we demonstrate that an antiserum to human MnSOD binds specifically to melanoma protein 21/>7 (Fig. 5A). Autoradiograms

![Fig. 3. Two-dimensional polyacrylamide gel electrophoresis of proteins induced by TNF in A375 melanoma cells. Autoradiogram of a representative 2D gel illustrating three individual proteins induced at least 2-fold by TNF. Cells were incubated with 1000 units/ml TNF in the presence of $^{35}$C-labeled amino acids for 24 h prior to preparation of whole cell lysates. Proteins, designated by M,pi, were separated first by their isoelectric points over a pH gradient of 3–10 and then by their relative mobility in 10% polyacrylamide with SDS.](image1)

![Fig. 4. Enhanced synthesis of melanoma proteins 21/>7, 28/5.6, and 41/5.7 is induced through TNF-R1. Lysates were prepared from cells treated with medium, TNF, and/or type-specific TNF receptor antibodies and subjected to 2D electrophoresis as indicated under "Materials and Methods." The locations of proteins 21/>7, 28/5.6, and 41/5.7 on the autoradiograms of matched gel sections are designated with an arrow in A–C, respectively.](image2)
55-kDa TNF Receptors Selectively Trigger Melanoma Proteins

Table 1

<table>
<thead>
<tr>
<th>Protein (M/μl)</th>
<th>Medium</th>
<th>TNF</th>
<th>TNF-R1 agonist</th>
<th>TNF-R2 agonist</th>
<th>TNF-R1 antagonist + TNF</th>
<th>Control serum</th>
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<tr>
<td>21/&gt;7</td>
<td>417&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1090</td>
<td>823</td>
<td>354</td>
<td>377</td>
<td>432</td>
</tr>
<tr>
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<td>1171</td>
<td>1141</td>
<td>22</td>
<td>232</td>
<td>144</td>
</tr>
<tr>
<td>41/5.7</td>
<td>14</td>
<td>122</td>
<td>291</td>
<td>30</td>
<td>45</td>
<td>19</td>
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</tbody>
</table>

<sup>a</sup> Relative abundance of proteins given in ppm. Film background levels are 10–50 ppm.

![Protein 21/>7](image1)

![Protein 28/5.6](image2)

![Protein 41/5.7](image3)

Fig. 5. Identification of melanoma proteins by 2D immunoblot analysis. Left panels, autoradiogram of representative blots of 2D gels loaded with lysates from cells treated with TNF + IFN-γ for optimal protein induction. Right panels, immunoblot of the same 2D Western blots probed with the appropriate primary antibodies and stained with the corresponding secondary anti-IgG conjugated to immunogold. A, melanoma protein 21/>7 migrates to the same location on 2D gels as MnSOD. B, protein 28/5.6 and hsp 27 proteins have different locations on 2D gels. C, melanoma protein 41/5.7 migrates to the same location on 2D gels as PAI-2.

![Protein 21/>7](image4)

![Protein 28/5.6](image5)

![Protein 41/5.7](image6)

Fig. 6. Immunoblot analysis confirms that TNF selectively induces MnSOD through TNF-R1 in A375 cells. A, immunoblot of a representative 1D gel loaded with MnSOD protein standard and lysates from A375 cells treated with TNF and/or type-specific TNF receptor antibodies. Electrophoresis, blotting, and detection of MnSOD were as indicated under “Materials and Methods.” B, densitometric analysis of the 21-kDa band detected by MnSOD antisera in lanes containing A375 lysates (lanes a–h) shown above.

of the 2D Western blots are shown on the left and the corresponding immunoblots on the right. Lysates were prepared from cells treated with a combination of TNF and IFN-γ for optimum protein expression and MnSOD was detected using a polyclonal antibody to human MnSOD and goat-anti rabbit IgG conjugated to immunogold. Two other proteins of identical molecular mass but lesser abundance also reacted with the MnSOD polyclonal antibody and may represent another form of MnSOD. Naturally occurring polymorphic variants (i.e. Ile-58 to Thr) of human MnSOD (28, 29) have been described with differences in charge and activity which could account for these additional forms.

Since melanoma protein 28/5.6 migrates to a similar location in the gel as hsp 27, a protein phosphorylated by TNF and associated with TNF signaling in some cells (3, 30, 31), we probed Western blots of 2D gels with a monoclonal antibody to hsp 27. Fig. 5B demonstrates that melanoma protein 28/5.6 is probably not hsp 27, as the location of 28/5.6, as shown in the autoradiogram of the blot on the left, is not the same as the proteins detected by immunoblot analysis (shown on the right).

Melanoma protein 41/5.7 was identified as PAI-2. As shown in Fig. 5C, an antiserum to human PAI-2 binds specifically to protein 41/5.7. A second spot of identical molecular mass with a greater PI was also detected by the antiserum. Autoradiograms derived from gels loaded with lysates from cytokine-treated cells show that this additional protein is induced by TNF as well (data not shown).

Immunoblot Analysis Confirms the Selective Induction of MnSOD and PAI-2 by TNF through TNF-R1 in A375 Cells—To confirm that MnSOD and PAI-2 are induced by TNF only when the 55-kDa TNF receptor is triggered, we probed Western blots...
of gels loaded with lysates from cells treated with TNF or the TNF receptor antibodies with specific antiserum to either MnSOD or PAI-2. Fig. 6A depicts the immunoblot probed with the MnSOD antiserum. A specific band of approximately 41-kDa is visible in the lanes loaded with lysates from cells treated with medium alone (lane a), TNF-R2 agonist (lane d), the control rabbit serum (lane e), or with TNF-R1 agonist + TNF (lane g) or murine IgG1 + TNF (lane h). In contrast, this band appears much reduced in lanes loaded with lysates from cells treated with medium alone (lane a), TNF-R2 agonist (lane d), the control rabbit serum (lane e), or with TNF-R1 agonist + TNF (lane f). The densitometric analysis of the immunoblot is illustrated in Fig. 6B and shows a 5- and 3-fold induction of MnSOD protein by TNF (lane b) and the TNF-R1 agonist (lane c), respectively, over medium control levels (lane a).

Fig. 7A depicts the immunoblot probed with PAI-2 antiserum. A band of approximately 41 kDa is visible in the lanes loaded with lysate from cells treated with TNF (lane b), TNF-R1 agonist (lane c), or with either TNF-R2 antagonist + TNF (lane g) or murine IgG1 + TNF (lane h). In contrast, this band appears nearly absent in all other lanes (lanes a and d–f). The densitometric analysis of the immunoblot is shown in Fig. 7B. PAI-2 protein is induced approximately 3- and 2-fold by TNF (lane b) and the TNF-R1 agonist (lane c), respectively, over medium control levels (lane a).

Cytotoxicity for A375 Cells Is Induced by TNF through TNF-R1—In addition to exploring the impact of TNF-receptor interactions on the synthesis of intracellular proteins, we wish to examine the impact of these interactions on TNF-mediated functional changes in cellular activity. Therefore, we determined which TNF receptor is responsible for inducing TNF cytotoxicity in A375 cells. In these experiments, cells were incubated in the presence of cycloheximide with either medium alone, TNF, TNF-R1, or TNF-R2 agonists, or with TNF-R1 antagonist antibodies plus TNF, and cytotoxicity was measured after 24 h. Sixty-six percent of the cells were killed by TNF, and 74% were killed by TNF-R1 agonist (Fig. 8). TNF-R1 antagonist antibody prevented TNF killing completely. Less than 4% killing occurred with the TNF-R2 agonist and the control antiserum, indicating that TNF cytotoxicity for A375 cells is induced selectively through TNF-R1.

DISCUSSION

The presence of two distinct TNF receptors may explain, in part, some of the pleiotropic cellular responses to this cytokine and possibly permit regulation of specific responses by agents that trigger or block one or the other type of TNF receptor. In this study we detected the presence of mRNA for both TNF-R1 and TNF-R2 in A375 melanoma cells (Fig. 1) and have demonstrated the presence of TNF receptor proteins capable of binding specific antibodies to either TNF-R1 or TNF-R2 (Fig. 2). Since very few TNF receptors are required to transduce a signal (3), it initially appeared that TNF might be capable of inducing molecular and/or biological effects through either type of TNF receptor. However, our present studies employing specific antibodies to the two different TNF receptors indicate that TNF-R1 is a critical signaling receptor for TNF in A375 cells.

We examined the synthesis of cellular proteins as many of the functional effects of cytokines, including TNF, are regulated by proteins that are induced or suppressed following cytokine stimulation. We have shown by 2D gel analysis that TNF enhances the synthesis of three proteins of interest in A375 cells (Fig. 3), one of which we have identified by 2D immunoblot analysis as MnSOD (Fig. 5A), and another as PAI-2 (Fig. 5B). TNF induces these proteins; antagonist antibody to TNF-R1 prevents their induction by TNF; and TNF-R1 agonist induces them in the absence of TNF (Figs. 4, 6, and 7). The synthesis of
these three proteins could not be enhanced with a TNF-R2 antiserum that is a potent agonist in human thymocyte proliferation assays (32). Furthermore, a monoclonal antibody that blocks the binding of TNF to TNF-R2 did not inhibit TNF induction of MnSOD (Fig. 6) or PAI-2 (Fig. 7). Taken together, these data demonstrate the selective utilization of TNF-R1 for the enhanced synthesis of MnSOD, PAI-2, and melanoma protein 28/5.6 by TNF in A375 cells.

Most TNF-inducible events that have been studied are reported to be transduced through TNF-R1. Among these activities are cytotoxicity for some tumor cells and fibroblast proliferation (33, 34), resistance to the cytotoxicity of the surrounding tissue (47–51). Studies have shown that this process is regulated in part by a balance between tissue and urokinase-type plasminogen activators, matrix metalloproteinases, and their corresponding inhibitory proteins in melanoma (48, 49) and other tumor types (50, 51). In addition, some tumor lines showing an imbalance in favor of increased PAI-2 expression display a lower metastatic phenotype when compared to lines expressing little or no PAI-2 (50, 51). Furthermore, our present study and those of others (52, 53) demonstrate that TNF induces PAI-2 in more than one melanoma cell line. Taken together, these data and our observation that PAI-2 is induced in A375 cells by an agonist that specifically triggers TNF-R1, but not TNF-R2 (Figs. 4 and 7), suggest the possibility that TNF-R1 agonists may have potential as a therapeutic modality in the treatment of malignant melanoma. In fact, TNF-R1 agonists may possibly have an advantage over TNF itself, as TNF-R1 agonists capable of inducing PAI-2 may subsequently lower metastases while having reduced systemic toxicities exacerbated by TNF-R2 binding.

Studies investigating the function of MnSOD in normal and malignant cells have shown that production of MnSOD is the cell’s primary defense against mitochondrial damage and that malignant cells generally express much lower levels than their corresponding normal phenotypes (54). In addition MnSOD has been found to play a critical role in promoting cell differentiation and controlled cell growth (54, 55). Since the latter two activities are also attributed to TNF, we believe that in the absence of protein inhibitors, TNF functions primarily as a homeostatic agent driving some types of tumor cells toward a more normal phenotype. This may be achieved by the enhanced synthesis and/or activity of cellular proteins, for example, MnSOD, that subsequently regulate cellular function and growth. Although further studies are required to substantiate this hypothesis, additional support is provided by the recent work of Church et al. (56) who transfected extra copies of the MnSOD gene into several malignant cell lines including melanoma. They observed decreased cell proliferation, fewer numbers of cells entering the cell cycle and decreased ability to form colonies in soft agar when compared with a cells transfected with a control gene. Furthermore, additional studies (57) examining the expression of MnSOD in murine B16 melanoma reported that a B16 variant possessing a lower metastatic phenotype expressed a greater abundance of MnSOD protein and SOD activity than variants with higher metastatic potential. In the present study we have demonstrated that TNF is a potent inducer of MnSOD in human A375 melanoma cells and that the enhanced synthesis of MnSOD is mediated by TNF-R1 (Figs. 4 and 6). There are two additional mechanisms which may be associated with the synthesis of the hormone's receptor-induced MnSOD in melanoma cells. One possibility that TNF-R1 agonists may have potential as a therapeutic modality in the treatment of malignant melanoma. In fact, TNF-R1 agonists may possibly have an advantage over TNF itself, as TNF-R1 agonists capable of inducing PAI-2 may subsequently lower metastases while having reduced systemic toxicities exacerbated by TNF-R2 binding. Studies investigating the function of MnSOD in normal and malignant cells have shown that production of MnSOD is the cell’s primary defense against mitochondrial damage and that malignant cells generally express much lower levels than their corresponding normal phenotypes (54). In addition MnSOD has been found to play a critical role in promoting cell differentiation and controlled cell growth (54, 55). Since the latter two activities are also attributed to TNF, we believe that in the absence of protein inhibitors, TNF functions primarily as a homeostatic agent driving some types of tumor cells toward a more normal phenotype. This may be achieved by the enhanced synthesis and/or activity of cellular proteins, for example, MnSOD, that subsequently regulate cellular function and growth. Although further studies are required to substantiate this hypothesis, additional support is provided by the recent work of Church et al. (56) who transfected extra copies of the MnSOD gene into several malignant cell lines including melanoma. They observed decreased cell proliferation, fewer numbers of cells entering the cell cycle and decreased ability to form colonies in soft agar when compared with a hairs transfected with a control gene. Furthermore, additional studies (57) examining the expression of MnSOD in murine B16 melanoma reported that a B16 variant possessing a lower metastatic phenotype expressed a greater abundance of MnSOD protein and SOD activity than variants with higher metastatic potential. In the present study we have demonstrated that TNF is a potent inducer of MnSOD in human A375 melanoma cells and that the enhanced synthesis of MnSOD is mediated by TNF-R1 (Figs. 4 and 6). These data, along with our ability to induce PAI-2 through TNF-R1, provide a rationale for studies in which the in vivo administration of these or similar TNF-R1 targeted agents are evaluated for their ability to lower the incidence of metastases in melanoma.

Presently we do not know the identity of protein 28/5.6. However, immunoblot analysis indicates that this protein is not related to the small 27/28-kDa hsp that is phosphorylated in response to TNF treatment (3, 30, 31). We are attempting to sequence this protein, utilizing mass spectrometry and/or autoradiography (58), as the knowledge of its sequence and identity, or partial homology with known proteins, may indicate how enhanced levels of this protein can influence A375 melanoma cell growth. In conclusion, our studies indicate that although A375 melanoma cells express both mRNA and cell surface protein for both
types of TNF receptors, 55-kDa TNF receptors alone are capable of triggering TNF enhanced synthesis of PAI-2, MnSOD, and melanoma protein 28. In view of the fact that TNF cytotoxicity is also mediated by TNF-R1, and that these events are not triggered through TNF-R2, our studies indicate that TNF-R1 receptors are important for signaling-specific TNF-inducible biochemical and functional events in A375 melanoma. Furthermore, these studies show the potential use of type-specific TNF receptor agonist or antagonist antibodies to selectively enhance or block specific intracellular events regulated by TNF.

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