Fas and Tumor Necrosis Factor Receptor-mediated Cell Death: Similarities and Distinctions

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Summary

Fas antigen and two tumor necrosis factor receptors (TNFR), p55 and p75, are implicated in the triggering of cell death upon stimulation by natural ligands and specific monoclonal antibodies. However, the relative efficiency of each receptor, the mechanisms that regulate their function and the signaling pathways they employ, remain to be elucidated. In this study, fusion proteins, composed of the extracellular domain of CD40 and the intracellular and transmembrane domains of Fas, TNFRp55 and TNFRp75, were stably expressed in a human melanoma cell line that is deficient in Fas and TNFR expression. Transfectants were stimulated by a soluble recombinant form of the CD40 ligand gp39, and the effect on cell viability determined. Engagement of all three fusion proteins by the gp39 ligand induced lethal signals, but the rate at which cell death occurred was distinct. Fas-derived signals were observed to have the most rapid effect, killing most cells within hours of stimulation, whereas TNFRp55- and TNFRp75-associated signals resulted in cell death within 2-3 days after engagement by ligand. It is interesting to note that optimal cell killing by all three fusion proteins was dependent on a critical, low to intermediate, cell surface expression level. High levels of fusion protein expression, on the other hand, were associated with inhibition of cell death. Our results provide a model to study Fas and TNFR-mediated cell death and suggest a novel mechanism for the regulation of death signals triggered by members of the TNFR family.

Mechanisms that regulate cell death are essential for normal development and maintenance of homeostasis. Cell death can be developmentally controlled, by the expression of novel genes that induce the death signal at a specific stage of differentiation in response to defined physiologic stimuli (1). The most common morphologic expression of such programmed cell death is apoptosis, characterized by cell shrinkage, vesiculation, or blebbing, of the plasma membrane, and nuclear collapse and fragmentation of the nuclear chromatin at internucleosomal sites due to activation of an endogenous nuclease (1-5). However, lethal cellular programs that lead to apoptosis may also be triggered by a variety of exogenous or environmental stimuli. Some of these are cell type specific, including glucocorticoid-dependent lymphocyte killing (6), antigen receptor-associated thymocyte death (7, 8) and TGF-β-induced hepatocyte death, whereas others are effective on a broad range of cells, including target cell lysis by cytotoxic T cells (9), radiation-induced DNA damage (10, 11) and engagement of specific cell surface receptors by natural ligands or antibodies (3, 4). Recently, three structurally related cell surface receptors implicated in transduction of death signals in a broad range of cell types have been identified and characterized. They include the Fas antigen (12, 13), and two distinct TNFR chains, p55 (14-16) and p75 (17, 18). All three receptors belong to a family of cell surface glycoproteins that includes nerve growth factor receptor (19), the T cell antigen CD27 (20), the B cell receptor CD40 (21), and the T cell-associated receptors CD30 (22), and 4-1BB (23).

TNFRs are broadly distributed in both normal and neoplastic cells and bind two related TNF molecules, TNF-α and TNF-β (24). TNF-α is one of the principal mediators of inflammation, and interaction between TNF and TNFRs produces a range of effects depending on the nature of the target cell (24). In certain tumor cells, engagement of TNFR by TNF induces death (24-28). However, tumor cells and cell lines display variable sensitivity to TNF, some cell lines being readily lysed by low concentrations of TNF, whereas others appear to be TNF resistant even at high TNF concentrations. Several TNF-resistant tumor cell lines are killed by TNF in the presence of inhibitors of protein synthesis. The mechanisms responsible for sensitivity or resistance to TNF are largely unknown. Furthermore, the respective role of the two TNFR chains in triggering cell death is disputed, some studies suggesting that TNFRp55 is the principal mediator (27, 29) whereas others contend that p75 may be equally effective (28, 30). Because several of the commonly used target cells constitutively express one or both TNFRs, it has been difficult to assess the effect of each receptor chain indepen-
dently, and the possibility of direct or indirect cooperation between the two polypeptides has not been excluded.

The Fas antigen is a 43-kD cell surface glycoprotein that is constitutively expressed in a variety of normal tissues and tumor cell lines while being inducible in others (12, 13). Administration of anti-Fas mAb in vitro (12) and in vivo (31), triggers apoptosis of Fas-expressing cells, suggesting that Fas may play a critical role in the regulation of cell death in a broad range of tissues. Mutations that inactivate Fas have been shown to be associated with the lymphoproliferative disorder of lpr/lpr mice (32), and Fas has recently been implicated in cytotoxic T lymphocyte–mediated target cell death (33).

Although Fas and TNFRs are currently grouped together as the principal cell death receptors, it is unclear whether they trigger the death signal along the same or distinct pathways, whether the potency of each receptor in any given cell is comparable, and whether their function is subject to similar or distinct regulatory circuits. To study the mechanisms that regulate Fas and TNFR-mediated cell death, we have developed fusion proteins composed of the extracellular domain of the B cell–associated antigen CD40 and the transmembrane and intracellular domain of Fas, TNFRp55, and TNFRp75. CD40 was chosen to provide the extracellular domain of the fusion proteins because it is structurally related to TNFR and Fas but has no known cytotoxic function. On the contrary, stimulation of CD40 by a natural ligand, gp39, induces B cell activation and Ig class switching (34–37). CD40-Fas and -TNFR fusion proteins were stably expressed in a human melanoma cell line that is deficient in Fas and TNFRs, and the transfectants tested for responses to stimulation by the CD40 ligand, gp39. Our results show that all three fusion proteins induce cell death upon engagement by the gp39 ligand. However, cellular response to the lethal signal cascade associated with each receptor is distinct, raising the possibility that Fas, TNFRp55, and TNFRp75 may operate via different pathways. Furthermore, the efficiency of lethal signal delivery was observed to be dependent upon a critical level of cell surface receptor expression which may constitute a novel regulatory mechanism of Fas and TNFR function.

Materials and Methods

Development of CD40, Fas, TNFRp55, and TNFRp75 Fusion Proteins. A cDNA encoding Fas was amplified from a human PHA blast cDNA library by PCR using synthetic oligonucleotide primers based on the published sequence (12). Primers complementary to the 5' and 3' extremities of the coding sequence were designed to contain an XhoI and a PstI endonuclease restriction site respectively, to facilitate insertion into the CDM8 vector. Fas cDNA was amplified by 30 cycles of PCR (94°C for 1 min; 60°C for 2 min; 72°C for 3 min) using AmpliTaq polymerase (Perkin Elmer, Norwalk, CT) and buffer conditions recommended by the vendor. The amplified sequence was subjected to XhoI and PstI endonuclease digestion and ligated into XhoI/PstI-cut CDM8 vector. The oligonucleotide primers were: Forward: 5'GGG CTG CAG CTA GAC CAA GCT TTG GAT TTC ATT 3'; and Reverse: 5'CGC GGG CTC GAG ATG CGT CTG TGG ACC CTA GAC CAA GCT TTG GAT TTC ATT 3'.

Development of the CD40-Fas chimera, was facilitated by the presence of the BamHI site in the membrane-proximal region of Fas (12). To exploit this site, the BamHI site in CDM8 was removed by cutting the vector with BamHI, filling in with dNTPs, and religating. Fas was excised from CDM8 with XhoI/PstI and inserted into XhoI/PstI-cut CDM8Bam. All subsequent constructs were made in this vector. The extracellular domain of CD40 was amplified from the CD40 cDNA (21) using synthetic 5' and 3' oligonucleotide primers containing XhoI and BamHI sites, respectively. The BamHI site was designed to allow an infame ligation to the Fas stalk. The oligonucleotide primers were: Forward 5': CAC GGG CTC GAG ATG ATG CGT CTG TGG ACC CTA GAC CAA GCT TTG GAT TTC ATT 3'; and Reverse 5': CAC GGG GGA TTC ATC CTG GGG ACC ACA GAC AAC ATC AGT; BamHI restriction sites to allow inframe ligation with Fas transmembrane and intracellular sequences. Oligonucleotide primers were: Forward: 5' CGC GGG GGA TCC ACA GTG ACC AAC ATC AGT. Amplified CD40 sequences were inserted into XhoI/PstI-cut CDM8Bam and religated. Fas was excised from CDM8 with XhoI/PstI and replaced by ligation of corresponding XhoI/BamHI-cut PCR-amplified TNFRp55 sequences, creating a TNFRp55-Fas chimera.

CD44 extracellular domain sequences were amplified using the following oligonucleotide primers: Forward: 5' CAC GGG CTC GAG ATG AGC ACA AGG AAA TTG TGG AGG AAC GCA CAC CTA ATC ATC AGG TAT TAT 3'; and Reverse: 5' CAC GGG AAG TCT TCT TCG TGG AAT TGG TGG TGT CTT TAT 3'. Amplified CD44 extracellular domain sequences were digested with XhoI and BglII and ligated to XhoI/BamHI-cut CD40-Fas vector, from which CD40-specific sequences had been excised.

Development of Stable Transfectants. The human melanoma cell line MC has been described previously (38). MC cells were detached from tissue culture plates with PBS containing 0.5 mM EDTA and resuspended in RPMI (Irvine Scientific, Santa Ana, CA) at a concentration of 10^7 cells/ml. CD40, CD40-Fas, CD40-TNFRp55, CD40-TNFRp75, and TNFRp55-Fas expression plasmids were added to 0.4 ml of cell suspension each along with the pSV2Neo selection vector at a ratio of 15:1 µg. Plasmids were introduced into the cells by electroporation, using a Gene Pulser (Bio-Rad Laboratories, Richmond, CA), at 625 V/cm, 960 µF. Cells were
seeded onto culture plates in DMEM/20% fetal bovine serum (FBS)1 (Irvine Scientific), and 48 h later, the medium was replaced by fresh DMEM/10% FBS supplemented with 1 mg/ml G418 (GIBCO BRL, Gaithenbury, MD). Resistant clones were detached and tested for CD40 and TNFR expression by indirect immunofluorescence.

Production of Soluble Recombinant CD40 Ligand. The development of a CD8-gp39 soluble fusion protein has been described previously (35). The CD8-gp39 expression plasmid kindly provided by Sandro Aruffo (Bristol-Myers Squibb, Seattle, WA) was transfected into COS cells using DEAE-dextran/chloroquine. 12 h after transfection, the culture medium was removed and replaced by fresh serum-free DMEM. Cells were maintained in serum-free medium for 6-8 d, whereupon the medium was harvested. The concentration of CD8-gp39 was estimated at 0.5-1 μg/ml on Coomassie-stained SDS gels.

mAbs and Immunofluorescence. Parental MC cells and transfectants were detached from culture plates with EDTA and incubated with 1-5 μg/ml anti-CD40 mAb S2C6 (39), anti-Fas mAb (Upstate Biotechnology, Inc., Lake Placid, NY), anti-TNFRp55 mAb htr-9 (18) or anti-TNFRp75 mAb utr-1 (18) for 45 min at 4°C in PBS, washed, and incubated with fluorescein-conjugated affinity-purified goat anti-mouse antibody (Cappel, Malvern, PA) for 30 min. at 4°C. Cells were washed, resuspended in PBS, and analyzed by flow cytometry on a FACS® (Becton Dickinson & Co., Mountain View, CA). To determine gp39-CD40 interaction, transfected clones were incubated with CD8-gp39 supernatants for 45 min at 4°C, washed, incubated with fluorescein-conjugated anti-CD8 mAb (Becton Dickinson & Co.), for 30 min, washed, and observed under an epifluorescence microscope.

Cytotoxicity Assays. To determine the cytolytic effect of CD8-gp39 on MC transfectants, MC-C, MC40, MC40-Fas, MC40-p55, and MC40p75 were seeded in 96-well microtiter plates (Falcon Labware, Oxnard, CA) at 2 × 104 cells/well and cultured for 24 h in DMEM/10% FBS. Medium was then aspirated and replaced with CD8-gp39 COS cell supernatant or supernatant from mock-transfected cells. Cells were incubated with COS cell supernatants for various times, after which the supernatants were aspirated and cells incubated with 0.75% crystal violet (Sigma Chemical Co., St. Louis, MO) in a 50% ethanol, 0.25% NaCl, and 1.75% formaldehyde solution. Cells were then washed with water, air-dried, and dye eluted with PBS/1% SDS. Cell viability was reflected by dye absorbance determined by optical density measurement at 595 nm on an automated ELISA reader.

Results

Development of CD40-Fas,-TNFRp55, and -TNFRp75 Fusion Proteins. A cDNA insert containing the coding sequence of Fas (12) was amplified by PCR from a human PHA blast cDNA library and inserted into the CDM8 expression vector. Sequences encoding the secretory signal peptide and extracellular domain of human CD40 (21) were amplified by PCR using synthetic oligonucleotide primers designed to contain endonuclease restriction sites that facilitate inframe ligation to the Fas expression vector. The nucleotide sequence encoding the membrane proximal region of the extracellular domain of human Fas contains a BamHI restriction site (12) that can be conveniently exploited for domain substitution. The CD40-Fas construct served as a cassette for subsequent switching of sequences encoding the transmembrane and intracellular regions. PCR-amplified transmembrane and intracellular sequences of p55 and p75 TNFRs could thus be readily substituted for their FAS counterparts. The chimeric constructs are illustrated in Fig. 1.

Expression of CD40 and CD40-Fas, CD40-TNFRp55, and CD40-TNFRp75 Fusion Proteins in a Human Melanoma. Wild-type CD40 cDNA and each of the chimeric constructs were introduced into COS cells by the DEAE-Dextran method and the cells tested for expression, 48 h later, of cell surface CD40 by indirect immunofluorescence, using the anti-CD40 mAb S2C6 (39). Cells expressing all of the constructs showed appropriate reactivity with anti-CD40 mAb (data not shown). The human melanoma MC (38) was chosen for establishing stable transfectants because it shows no reactivity with anti-Fas, anti-TNFR, or anti-CD40 mAb (Fig. 2), and is insensitive to anti-Fas mAb and TNF-α (data not shown). cDNA clones encoding wild-type CD40 and CD40 fusion proteins were cotransfected with the pSV2neo selection plasmid, at a ratio of 15:1, into MC cells by electroporation and the cells selected for neomycin resistance. Single neomycin-resistant clones were picked and tested for reactivity with anti-CD40 mAb. Several independent clones of each transfectant, expressing variable levels of the corresponding fusion protein (Fig. 3) were obtained, and selected for further study.

Figure 1. Development of TNFR family fusion proteins. Extracellular and transmembrane/intracellular components of each of the fusion proteins used are indicated. Amino acid sequences corresponding to the extracellular domain (plain typeface) and the transmembrane/intracellular domain (italics) at each junction are shown. The vertical line represents the cell membrane.

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1 Abbreviations used in this paper: FBS, fetal bovine serum; MFI, mean fluorescence intensity.
The parental MC melanoma cell line is deficient in CD40, Fas, and TNF receptor expression. The parental MC cell line was tested for the expression of Fas (A), CD40 (B), TNFRp55 (C), and TNFRp75 (D) by flow cytometry. Cells were stained with anti-Fas mAb, anti-CD40 mAb 2C6, anti-TNFp55 mAb htr-9, and anti-TNFRp75 mAb utr-1, followed by a fluorescein-conjugated, affinity-purified goat anti-mouse antibody, and analyzed by FACScan®. The relative cell number is plotted against log fluorescence intensity.

Transfectants expressing wild-type CD40 (MC40) and selection vector only (MC-C) were used as controls. The panel of transfectants used throughout the study are listed in Table 1.

*Engagement of CD40 Fusion Proteins by CD8-gp39 Induces Lethal Signals in MC Transfectants.* To assess the functional properties of the fusion proteins, we used a soluble recombinant form of a cell surface CD40 ligand (35). CD40 has recently been shown to interact with gp39, a type II integral membrane protein expressed on activated T cells (34, 35).

A recombinant soluble form of gp39, fused to the extracellular domain of CD8 has been developed and shown to retain its CD40-binding and stimulatory properties (35). The CD8-gp39 fusion protein was observed to bind all transfectants expressing wild-type CD40 and CD40 fusion proteins (data not shown), and thus provided a suitable means to determine whether ligand-dependent engagement of CD40 might trigger activity of the intracellular Fas and TNFR domains.

Representative MC40-Fas, MC40-p55, and MC40-p75 clones were incubated in microtiter wells with serum-free COS cell supernatants containing CD8-gp39, and observed by light microscopy for signs of cell death at multiple intervals over a 48-h period. MC40-Fas cells showed marked morphologic changes within 2 h of incubation, characterized by cell rounding, formation of surface blebs and chromatin condensation, corresponding to those associated with apoptosis (Fig. 4 and data not shown). MC40-p55 and MC40-p75 cells displayed similar morphologic changes but at a later

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**Table 1. Fusion Protein Expression Level in MC Transformants**

| MC transformants | Fusion protein expression level |
|------------------|--------------------------------|
| MC40-Fas         | 19 +                            |
|                  | 13 ++                           |
|                  | 5bis ++                         |
|                  | 5 + +                           |
|                  | 10 + +                          |
|                  | 6 ++                            |
|                  | 14 +++                          |
|                  | 4 +++                           |
| MC40-p55         | 15 + +                          |
|                  | 31 + +                          |
|                  | 2 +                             |
| MC40-p75         | 15 + +                          |
|                  | 17bis + +                       |
|                  | 20 +                            |
| MCC              | -                              |
| MC40             | 9 + + +                         |
| MCTNFp55-Fas     | 3 + +                           |
|                  | 5 + +                           |
|                  | 12 + +                          |
|                  | 13 + +                          |
|                  | 15 +                            |

MFI is graded as follows: (+) MFI < 20, low expression; (++) 20 > MFI < 150, intermediate expression; (+++) MFI > 150, high expression.

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time point, typically between 24 and 48 h after the start of incubation with CD8-gp39 supernatant. Apoptosis of MC40-Fas cells could be observed after pulsing the cells with CD8-gp39 for 15 min and replacing the supernatants with fresh, serum-free media, suggesting a high degree of sensitivity to Fas-associated death signals. MC40 transfectants, expressing wild-type CD40, were unaffected by CD8-gp39 supernatants as were MC-C and parental cells (data not shown), indicating that cell death was not due to the potential toxicity of the supernatants themselves. It is interesting to note that none of the transfectants were susceptible to anti-CD40 2C6 mAb, which recognizes a CD40 epitope distinct from that bound by gp39 (Clement, M.-V., and I. Stamenkovic, unpublished results), even at high mAb concentrations (data not shown), nor did cross-linking of S2C6 by secondary antibodies have any effect.

**The Efficiency of Fas-mediated Cytotoxicity Depends on a Critical Level of Receptor Expression.** To address the possible correlation between the level of fusion protein expression and the rapidity of cell death after triggering of CD40-Fas, eight independent MC40-Fas transfectant clones were tested for their response to CD8-gp39. Clones were designated as low, intermediate, and high expressors according to their mean fluorescence intensity (MFI) as determined by flow cytometry using the anti-CD40 S2C6 mAb: clones with MFI <20 were designated as low, 20-150 as intermediate, and >150 as high expressors (Table 1). Cell death was assessed at several time points by measuring the incorporation of crystal violet as an indicator of cell viability. Four of the clones, MC40-Fas 5, 5bis, 10, and 13 were killed rapidly, with a half maximal response of <2 h. Maximal cell death occurred at 6 h after stimulation, with only 20-30% of cells remaining viable (Fig. 5). Viability of these calls at 24 h remained at 15-25% (data not shown). The remaining four clones, MC40-Fas 4, 6, 14, and 19, displayed partial or near complete resistance to CD8-gp39 stimulation, with 60-95% viability at 6 h. At 24 h, however, viability of these clones fell to 40-70% (data not shown), consistent with reduced effectiveness of Fas-mediated cytotoxicity. Remarkably, three of the four resistant clones, MC40-Fas 4, 6, and 14 were high expressors of CD40-Fas (Fig. 5, and Table 1), whereas the fourth, MC40-Fas 19, expressed the fusion protein weakly. The four most
Figure 5. Time course of gp39-triggered MC40-Fas transfectant cell death. MC40-Fas transfectants were incubated with serum-free CD8-gp39 COS cell supernatants at 37°C and stained with crystal violet at 2, 4, and 6 h. The percentage of viable cells was determined as described in Materials and Methods. MC parental cells transfected with pSV2neo alone as well as the MC40 transfectants were unaffected by CD8-gp39 COS cell supernatants (data not shown). Mean values of five experiments are shown. SD were generally <5%.

Figure 6. Sensitivity of MC40-Fas transfectants to Gp39 cytotoxicity. Three MC40-Fas (CD40-FAS 5bis, CD40-FAS 19, and CD40-FAS 14) transfectants representing different fusion protein expression levels were incubated with serially diluted serum-free CD8-gp39 COS cell supernatants at 37°C for 24 h and stained with crystal violet. The percentage of viable cells was determined as described in Materials and Methods. The data shown are the means of four independent experiments. SD <5%.

Figure 7. Time course of gp39-mediated killing of MC40-p55 and MC40-p75 transfectants. MC40-p55 (A) and MC40-p75 (B) transfectants, and MC cells transfected with pSV2neo alone (Neo) were incubated with CD8-gp39 COS cell supernatants at 37°C for 3 d and stained with crystal violet at various time points to determine the percent viability. Data shown are representative of four independent experiments.

Sensitive clones expressed an intermediate level of the CD40-Fas fusion protein (Fig. 5, and Table 1). The difference in susceptibility to cell death among the clones could be more clearly demonstrated after a 24-h incubation with serial dilutions of CD8-gp39 (Fig. 6). The lowest expressor, MC40-Fas.19, was resistant to the lethal effect of CD8-gp39 at a 1:4 dilution of the supernatant. The intermediate expressor MC40-Fas.5bis, on the other hand, displayed high susceptibility...
MC40-p55 and MC40-p75 TNFRs Show Distinct Responses to CD8-gp39. Three independent MC40-p55 and MC40-p75 clones were obtained (Table 1). Both sets of transfectants responded more slowly to CD8-gp39 than MC40-Fas cells, 85–95% of the cells displaying viability at 24 h. Maximal cell death was observed at 72 h, when the most sensitive MC40-p55 and MC40p-75 clones displayed 30 and 50% viability, respectively (Fig. 7). Within each group, low expressors were the most sensitive to CD8-gp39 whereas intermediate expressors exhibited viability at 24 h, with 85–90% viability at 72 h. High expressors were consistently less sensitive than clones displaying intermediate level expression.

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expressors (according to the designation in Table 1) responded the least (Fig. 7, and Table 1).

**Inhibition of Protein Synthesis Enhances Ligand-mediated Cytotoxicity in MC40-Fas and MC40-p55 but not in MC40-p75 Transfectants.** A clear distinction between MC40-p55 and MC40-p75 cell response to gp39 stimulation was observed in the presence of cycloheximide (Fig. 8). Whereas susceptibility of MC40-p55 cells to gp39 was markedly increased by cycloheximide, resulting in maximal cell death within 24 h, MC40-p75 sensitivity was not altered (Fig. 8). Similar to MC40-p55 cells, susceptibility of MC40-Fas transfectants to CD8-gp39 was enhanced by cycloheximide (Fig. 9). The rate at which the most sensitive MC40-Fas clones were killed after triggering by CD8-gp39 (maximal cell death was observed at 6 h) was not significantly altered by addition of cycloheximide (data not shown), but the fraction of cells susceptible to Fas-induced cytotoxicity was increased (Fig. 9). The potentiating effect of cycloheximide could be best demonstrated when MC40-Fas clones of different sensitivity were incubated with serial dilutions of CD8-gp39 supernatants in the presence or absence of the drug (Fig. 9). Cycloheximide significantly reduced the threshold of sensitivity to ligand-mediated Fas cytotoxicity in all transfectants tested (Fig. 9, and data not shown). Furthermore, maximal cell death, even of partially resistant clones, was observed within 6 h in the presence of cycloheximide (data not shown).

**TNFR-Fas and CD40-Fas Fusion Proteins Transduce Similar Cytotoxic Signals Whereas CD44-Fas Fails to Induce Cell Death.** To determine whether intracellular and transmembrane Fas segments retain cytotoxic signaling properties when fused to the extracellular domain of other receptors, a TNFRp55-Fas chimera was designed and expressed in MC cells. Five stable transfectants were obtained. One of the transfectants expressed TNFRp55-Fas in the low range (MFI <20) and was resistant to TNF (Fig. 10), reminiscent of low CD40-Fas expressor resistance to CD40 ligand (Fig. 5). Each of the other four transfectants displayed inhomogeneous expression of the fusion protein covering low and intermediate levels with an MFI in the intermediate range of 42-61. All four isolates were sensitive to TNF at a concentration of 6 ng/ml, with maximal cell death occurring within 6 h (Fig. 10), similar to corresponding CD40-Fas transfectants exposed to CD8-gp39. Augmenting TNF concentration to 25 ng/ml failed to increase its cytotoxic effect (Fig. 10), but addition of cycloheximide augmented transfectant sensitivity to TNF, rendering as little as 0.6 ng/ml lethal (data not shown). However, only about 50% of cells from each of the transfectants were killed by TNF (Fig. 10). This observation most likely reflects the broad range of TNFRp55-Fas fusion protein expression in each isolate. Roughly 50% of cells expressed a low level of TNFRp55-Fas which, in the case of CD40-Fas transfectants and one TNFRp55-Fas-expressing clone, was associated with resistance to ligand-induced, Fas-mediated cytotoxicity (Figs. 5 and 10). To determine the phenotype of surviving cells after exposure to TNF, a representative low to intermediate level TNFRp55-Fas expressor was treated with 100 ng/ml TNF for 6 h, and the supernatant removed and replaced with fresh culture medium. After 24 h of culture, surviving cells were incubated with anti-TNFp55 mAb htr-9 and subjected to FACS® analysis. The majority of the cells expressed low TNFRp55-Fas levels whereas most of the intermediate expressor population was depleted (Fig. 11). Thus, intermediate and low TNFRp55-Fas and CD40-Fas expressors displayed similar susceptibility to ligand-induced cytotoxicity.

It is interesting to note that cells expressing a CD44-Fas fusion protein were resistant to anti-CD44 mAb triggering, as well as to stimulation by soluble and surface-bound hyaluronate, the principal CD44 ligand (40, 41) (data not shown). Resistance was not due to high or low fusion protein expression, as transfectants expressing a broad range of CD44-Fas levels were obtained and tested.
Discussion

In the present work, we have developed an approach to facilitate the study and comparison of Fas and TNFR-mediated cell death. Our strategy has relied on two critical factors: the choice of a human tumor cell line that does not respond to TNF-α or anti-Fas mAb and that is deficient in Fas and TNFR expression, and the creation and expression of fusion proteins that bear a common extracellular domain that can be triggered by the same natural ligand. This combination has revealed that the three receptors have both common and distinct functional characteristics.

Engagement of receptors by specific ligands, or cross-linking by specific mAb, usually causes receptor dimerization or oligomerization which promotes recruitment of mediators that trigger a signaling cascade. It has been proposed that the extracellular domain of cell surface receptors primarily serve to facilitate ligand-induced clustering of the cytoplasmic domain which is responsible for signal release (42). Our results show that replacement of the extracellular domain of Fas and TNFRs by that of different molecules of the same receptor family does not abrogate transduction of lethal signals upon stimulation with corresponding ligands, indicating that expression of the extracellular domain of Fas and TNFRs is not required for triggering cell death. However, appropriate ligand-mediated clustering appears to be critical, as anti-CD40 mAb S2C6, which is costimulatory with inducers of B cell activation (39), but recognizes an epitope distinct from that bound by gp39 (Clement, M.-V., and I. Stamenkovic, unpublished results), had no effect on the MC40-Fas and -TNFR transfectants even after extensive cross-linking by secondary antibody. Similarly, replacement of the extracellular domain of Fas by that of the hyaluronate receptor CD44 generated fusion proteins that could not induce cytotoxicity in MC cells after antibody or ligand-mediated cross-linking. The CD40 ligand, gp39, on the other hand, induced cytokinetic signals in all three transfectants, as did TNF-α in MCTNFp55-Fas cells. The TNF-α molecule has been shown to possess three receptor binding sites, which induce the formation of TNFR trimers upon engagement (43), and it has been suggested that gp39 may have a similar effect on the CD40 receptor (36). These observations support the notion that the CD40, Fas, and TNF receptors may share similar oligomerization requirements for appropriate ligand-mediated signaling.

Stable transfectants expressing CD40-Fas, TNFRp55-Fas, and CD40-TNFR fusion proteins have helped demonstrate that the transmembrane/intracellular domains of Fas, TNFR p55, and TNFRp75 can each mediate cell death independently. However, several distinctive functional properties have emerged. Whereas Fas-dependent signals induced cell death within hours of stimulus, in the most sensitive clones, detection of TNFR-dependent cytotoxicity required 2-3 d of exposure to ligand. The two CD40-TNFR fusion proteins displayed comparable cytokinetic signaling in MC cells, but TNFRp55-dependent cytotoxicity was enhanced by inhibitors of protein synthesis, whereas TNFRp75-related signals were unaffected. The kinetics of Fas-dependent cytotoxicity was not significantly altered by cycloheximide in the most sensitive clones, suggesting that the observed death rate might reflect maximal efficiency of Fas-dependent cytotoxicity in these cells. However, the threshold of sensitivity to Fas-mediated cell death in partially resistant clones was reduced by addition of cycloheximide. Thus, in MC melanoma cells, Fas and TNFRp55-associated cytokinetic signaling may be subject to the regulatory action of inhibitory intracellular proteins whereas the cytolytic function of TNFRp75 appears to be independent of similar control mechanisms.

Finally, our model suggests that the efficacy of Fas and TNFR cytokinetic signals may be dependent on a critical level of receptor surface expression. Intermediate levels of CD40-Fas and low levels of CD40-TNFR expression were associated with the most rapid and extensive killing in response to the CD40 ligand, whereas cells displaying high levels of fusion protein expression were partially resistant, and were rendered sensitive only in the presence of cycloheximide. Sensitivity of tumor cells to lethal signals from endogenous TNFRs and Fas is known to be highly variable (24, 44, 45), consistent with the possibility that the activity of TNFR and Fas may be subject to cell type-specific regulation. The results presented here are consistent with the notion that the stoichiometry of the interaction between receptors, inhibitors of receptor function and second messengers that constitute the signaling cascade, may provide such a regulatory mechanism. Cell surface receptor signal transduction requires ligand-mediated receptor clustering that is thought to be necessary for any given signaling threshold to be reached. Optimal signal transduction presumably requires interaction between a critical fraction of receptors forming the ligand-mediated cluster and specific second messenger molecules. Limitation of interactions between second messenger molecules and receptors by any number of possible mechanisms, including low steady state second messenger abundance, high turnover, or competitive binding by other molecules, would predict that receptor overexpression may result in receptor saturation of the messenger pool, and the presence of excess “free” receptors. Since ligand engagement of receptors is random, the fraction of receptors, within ligand-induced aggregates, which communicate with appropriate messengers would be reduced, and signaling efficiency potentially lowered. A simple explanation for ineffective cytokinetic signaling by transfectants expressing high levels of Fas and TNFR fusion proteins, might therefore be a receptor/messenger imbalance. Removal of inhibitor molecules by blockers of protein synthesis, on the other hand, may reduce the signaling threshold, and partially restore triggering of TNFRp55 and Fas-associated death signals despite suboptimal receptor-messenger interaction. Because receptor, second messenger, and receptor inhibitor stoichiometry is likely to vary among cell types, it is conceivable that efficient cytokinetic might require high receptor expression in some cells and low expression in others. The model presented here may provide a means to help elucidate some of the mechanisms that regulate TNFR/Fas-mediated cell death.

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