Metaxin is required for tumor necrosis factor-induced cell death

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We used retrovirus insertion-mediated random mutagenesis and tumor necrosis factor (TNF) selection to generate TNF-resistant lines from L929 cells. The metaxin gene, which encodes a protein located on the outer membrane of mitochondria, was identified to be the gene disrupted in one of the resistant lines. The requirement of metaxin in TNF-induced cell death of L929 was confirmed by the restoration of TNF sensitivity after ectopic reconstitution of metaxin expression. Analysis of the cell death induced by other stimuli revealed that metaxin deficiency-mediated death resistance was selective to certain stimuli. Studies using deletion mutants of metaxin showed that mitochondrial association of metaxin is required for the function of metaxin. Interfering with metaxin prevented TNF-induced necrotic cell death in L929 cells and apoptosis in MCF-7 cells. Our work has thus defined a novel component in the death pathway used by TNF and some other death stimuli.

INTRODUCTION

Tumor necrosis factor α (TNF) is a proinflammatory cytokine originally identified and purified as a factor that leads to rapid hemorrhagic necrosis of an established tumor (Beutler and Cerami, 1988). TNF can induce cell death in numerous different tumor cell lines (Fiers et al., 1999). Interestingly, the morphological characteristics differ markedly among TNF-treated cell lines: KYM, MCF-7, and PC60 tumor lines die apoptotically in response to TNF treatment (Fiers et al., 1999), whereas L929 and WEHI 164 clone 13 cells die with necrotic phenotypes (Fiers et al., 1999). The typical symptoms of apoptosis such as nuclear condensation and DNA fragmentation are rarely detectable in L929 cells after exposure to TNF, and cell death is accompanied by the loss of plasma membrane integrity (Fiers et al., 1999). Moreover, the caspase inhibitor zVAD does not block TNF-induced L929 cell death, but in fact enhances TNF-induced cell killing (Vercammen et al., 1998a).

Both apoptosis and necrosis are initiated by TNF receptor I (TNF-R1) clustering (Aggarwal et al., 1987; Ashkenazi and Dixit, 1998; Kroemer et al., 1998; Fiers et al., 1999). The clustered TNF-R1 recruits the death domain (DD)-containing adaptor protein TRADD to its intracellular death domains. One function of TRADD is to bind to FADD, which in turn stimulates caspase-8 autoactivation in the apoptosis pathway. Active caspase-8 either acts via cytochrome c (cyt c) release or by the direct activation of effector caspases to execute apoptosis. Trimerization of the DD of TNF-R1 also recruits TRADD in L929 (Fiers et al., 1999). It is known that neither the known pro-apoptotic caspases including caspase-8, nor cyt c release are involved in TNF-induced L929 cell death (Fiers et al., 1999). TNF stimulates reactive oxygen species (ROS) generation in mitochondria and the necrotic death of L929 cells is dependent on mitochondrial ROS generation (Fiers et al., 1999). The intracellular molecules linked to the TNF-induced necrosis are largely unknown.

We designed a functional gene identification procedure and applied it to L929 cells to search for the molecules required for TNF-induced necrotic cell death. As a result, we identified metaxin to be required for TNF-induced death of L929. Metaxin is a protein serendipitously identified in an attempt to establish a mouse model for Gaucher’s disease by introducing an A to G mutation into the glucocerebrosidase gene (GC) using homologous recombination (Armstrong et al., 1997). Because the metaxin gene is located near the GC gene, it was disrupted by this approach which led to the death of mice during early gestation.
RESULTS AND DISCUSSION

Identification of metaxin as a required element in TNF-induced L929 cell death

Retroviral integration can generate null alleles, resulting in diminished endogenous gene expression. Since cells with mutations in genes required for TNF-induced killing may become TNF resistant and be selected by TNF treatment, we used a retrovirus-mediated mutagenesis to study the mechanism of TNF-induced L929 cell death. The retroviral vector we used is shown in Figure 1A. Open bars represent the flanking endogenous sequences from the insertion site. There are three potential gene products that can result from viral insertion: a truncated endogenous gene, GFP, and a neo gene product. GFP protein will be expressed irrespective of the viral integration site because the viral genome contains a complete expression cassette [with a 5′-LTR promoter and viral poly(A) signal sequence close to 3′-LTR]. Hence, GFP expression can be used to assess the efficiency of infection. The expression of two other gene products is dependent on the site of insertion. If integration occurs within an intron, the upstream splicing donor (SD) of the endogenous gene will splice with a vector-encoded splicing acceptor (SA) to generate a truncated endogenous gene. If integration occurs within an exon, no splicing will occur upstream of the vector, and the transcript of the endogenous gene will directly fuse with the viral gene to end at the vector poly(A) signal (pA), generating a truncated endogenous gene. The neo transcript will join exons downstream of the insertion site by fusion or splicing events, depending on the presence of a downstream SA. If the insertion occurs within a non-gene region, or within a gene but in a wrong orientation, the endogenous gene will splice with a vector-encoded splicing donor (SD) of the endogenous gene to generate a truncated endogenous gene. The SD downstream of the neo coding sequence will splice with endogenous SA to generate a fused mRNA capable of expressing full-length Neo protein. If integration occurs within an exon, no splicing will occur upstream of the vector, and the transcript of the endogenous gene will directly fuse with the viral gene to end at the vector poly(A) signal (pA), generating a truncated endogenous gene. The neo transcript will join exons downstream of the insertion site by fusion or splicing events, depending on the presence of a downstream SA. If the insertion occurs within a non-gene region, or within a gene but in a wrong orientation, the endogenous gene will lack a poly(A) signal and will not be expressed. Thus, G418 resistance can be used to select the cells that had a viral insertion in genes. We term such an event—functional insertion. As only 2% of the total genome encodes genes, a large number of non-functional insertions were eliminated by G418 selection.

We infected ∼10⁶ cells and obtained ∼10⁴ G418-resistant clones. Ten TNF-resistant clones were isolated from the G418 resistant clones. 3′-rapid amplification of cDNA ends (RACE) of the mRNA fused with neo was used to identify the disrupted gene in each clone. The metaxin gene was identified as having been disrupted in one of the TNF-resistant lines. The junction sequence of neo–metaxin fusion mRNA is shown in Figure 1B. The pDisrup insertion was mapped to the 5′-portion of the
metaxin gene (94 bp from the starting codon) and thus caused a truncation. We analyzed the metaxin-disrupted line (termed Meta\textsuperscript{mut}) together with the other four mutation lines by northern blotting using randomly \textsuperscript{32}P-labeled double-strand DNA probe for the neo gene. As shown in Figure 1C, a common band was seen in all clones (indicated by an asterisk), which is most likely the transcript derived from the viral genome driven by the 5′-LTR. The fused neo mRNA driven by the CMV promoter from the opposite direction was of a different size, indicating that insertion of the viral genome was random. Only one fused gene was found in each of the cell lines including Meta\textsuperscript{mut} (lane 4). It is crucial to the efficiency of this protocol that multiple functional insertions in a single cell be avoided, since these events will cause difficulty in determining which disrupted gene is responsible for the functional alteration.

Northern blotting with a metaxin probe showed that the mRNA level of metaxin was significantly reduced in Meta\textsuperscript{mut} cells (Figure 1D), suggesting that a functional allele of the metaxin gene was indeed disrupted. In support of this, we found an mRNA with a larger mass detected in the metaxin line with the metaxin probe, indicating a probable metaxin-vector fusion product.

The cell viability after TNF treatment of wild-type parental L929, Meta\textsuperscript{mut}, Meta\textsuperscript{mut} cells transfected with metaxin expression vector (termed reconstituted), and Meta\textsuperscript{mut} cells transfected with empty vector (vector-Meta\textsuperscript{mut}), is shown in Figure 1E. Parental L929 cells were very sensitive to TNF-induced cell killing, while Meta\textsuperscript{mut} cells were resistant to TNF-induced cell death. Expression of metaxin in Meta\textsuperscript{mut} cells regained the TNF sensitivity to almost the same level as wild-type L929, while vector control had no effect. Thus metaxin is required for TNF-induced L929 cell death.

The TNF resistance of the metaxin-deficient line

To further evaluate TNF resistance in the Meta\textsuperscript{mut} line, we analyzed the TNF sensitivity of parental, Meta\textsuperscript{mut}, reconstituted, and vector-Meta\textsuperscript{mut} lines using several different criteria. Conversion of the yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the dark-blue formazan measures the activity of various dehydrogenases and quantifies the reductive capacity of the cell. This assay has been widely applied to the measurement of the cytolytic response to TNF. We used the MTT assay to compare TNF-induced cell death in the four cell lines (Figure 2A). Meta\textsuperscript{mut} and vector-Meta\textsuperscript{mut} lines had a smaller reduction in their capacity to convert MTT compared to the parental and reconstituted lines, supporting the conclusion that metaxin mutation promotes resistance to TNF-induced cell death.

Translocation of phosphatidylserine (PS) from the inner part of the plasma membrane to the outer layer is a common early event in apoptosis. Annexin staining of PS was also observed in TNF-treated L929 and may represent a change in TNF-induced necrosis of L929 (Fiers et al., 1999). Uptake of propidium iodide (PI) is an indicator of a loss in cell-membrane integrity which is widely used as a measurement of cell death. We used annexin and PI staining to analyze the death profile of the four lines in response to TNF treatment. As shown in Figure 2B and C, metaxin deficiency reduced TNF-induced annexin V and PI staining. Ectopical expression of metaxin in Meta\textsuperscript{mut} cells restored TNF sensitivity.

To address whether new protein synthesis is involved in metaxin mutation-mediated TNF resistance, we treated cells with TNF in the presence of the translation inhibitor cycloheximide (CHX). CHX enhanced cell killing by TNF in both normal and metaxin-deficient cells (Figure 2D and C). As observed without CHX, a higher survival rate was seen in metaxin-deficient cells, suggesting that metaxin mutation-mediated death resistance is independent from new protein synthesis.

Meta\textsuperscript{mut} line is selectively resistant to some death stimuli

To establish whether Meta\textsuperscript{mut} is selectively resistant to different death triggers, we examined its sensitivity to several death stimuli. As shown in Figure 3, Meta\textsuperscript{mut} was resistant to TNF-, ceramide-, londiamine- and sodium nitroprusside (SNP)-induced cell death. In contrast, Meta\textsuperscript{mut} was equally sensitive to \textsubscript{2}O\textsubscript{2}-induced cell killing and was even more sensitive to staurosporine and etoposide. Both parental and Meta\textsuperscript{mut} lines were insensitive to dexamethasone, serum starvation, UV- and \textgamma-irradiation (data not shown). Expression of Fas renders L929 cells sensitive to Fas ligation-induced cell killing (Vercammen et al., 1998b). We transfected fas cDNA into both parental and Meta\textsuperscript{mut} cells and selected the clones that express Fas as described (Vercammen et al., 1998b). Anti-Fas antibody treatment caused very quick cell death within 3 h, which was not influenced by metaxin mutation (Figure 3, bottom right panel). The different sensitivities of Meta\textsuperscript{mut} cells to different death stimuli supported the idea that there are multiple ways of cell death (Fiers et al., 1999) and demonstrated that metaxin mutation-mediated TNF resistance is not due to a general promotion of cell survival, but an impairment of the death pathway used by TNF.
Mitochondrial association is essential for metaxin function

Metaxin is a protein of 317 amino acids (aa) with an extramitochondrial domain (aa 1–271), a transmembrane domain (aa 272–294) and an Aps/Glu rich C-terminal domain inside the outer membrane of mitochondria (Figure 4A) (Armstrong et al., 1997). Deletion of the C-terminal domain (ΔC) did not affect the mitochondrial location of metaxin, while further deletion of transmembrane domain (ΔTM/C) disrupts its mitochondrial localization (Armstrong et al., 1997). We examined whether the C-terminal intra-mitochondrial domain and the transmembrane domain are required for the function of metaxin in TNF-induced cell death. As shown in Figure 4B, full-length metaxin reconstituted TNF sensitivity of Metαmut cells. While MetαC had a partial reconstitution effect, MetaΔTM/C did not restore the TNF sensitivity in the Metαmut line. We also over-expressed wild-type and truncated metaxin mutants in parental L929 cells. metaxin and MetaαC did not have a significant effect on TNF-induced parental L929 cell death (Figure 4C); however, transfection with MetaΔTM/C led to TNF resistance in the parental cells (Figure 4C). The dominant-negative effect by the over-expression of MetaΔTM/C lacking its mitochondria-targeting sequence is likely to be elicited by binding to metaxin-interacting proteins and subsequently disrupting the function of the complex. Since MetaΔTM/C can act as a dominant-negative mutant, we tested whether over-expression of MetaΔTM/C in MCF-7 and MCF-7.3.28 [a cell line generated from MCF-7 by transfection of caspase-3 cDNA (Janicke et al., 1998)] cells influenced TNF-induced apoptosis. Indeed, MetaΔTM/C expression inhibited TNF-induced MCF-7 and MCF-7.3.28 cell death (Figure 4D and data not shown). Thus, metaxin is required for TNF-induced apoptosis and necrosis.

Since free radical generation in mitochondria has an essential role in TNF-induced L929 cell death, we measured mitochondrial ROS levels in TNF-treated MetaTM/C and parental lines. The ROS levels of MetaTM/C and parental cells were similar at rest; ROS induction profiles in response to TNF stimulation were also comparable over a period of 24 h (data not shown). In addition, similar inhibition of TNF-induced cell death by the free radical scavenger butylated hydroxyanisole (BHA) was observed between the parental and MetaTM/C cells; GSH/GSSG contents are also similar between these two lines (data not shown). Hence, metaxin should function at a position downstream of free radical generation.

Cell death is controlled by both death and survival pathways. We have determined by using electro-mobility shift assay and reporter gene assay that TNF-induced NF-κB activation was comparable between the parental and MetaTM/C cells (data not shown). The PI3K-Akt pathway is another well-studied survival pathway, but its role in TNF-induced cell death is unclear. We found that PI3K inhibitor LY294002 did not enhance, but rather inhibited TNF-induced L929 cell death (data not shown). The effect of LY294002 in both parental and metaxin-mutated cells was the same (data not shown). In addition, TNF did not induce any detectable change in Akt phosphorylation in either parental or metaxin-mutated cells. Thus, we did not have evidence to suggest that the survival pathway was affected by metaxin mutation at this moment.

An updated database search revealed that three proteins from Caenorhabditis elegans, Drosophila and fission yeast, shared sequence similarity with metaxin in the extra-mitochondrial domain, transmembrane domain, and Asp/Glu rich C-terminal residues. The hypothetical protein F39B2.11 from C. elegans (DDBJ/EMBL/GenBank accession No. T21992), CG9393 gene product from Drosophila (AAF54402), and a metaxin homolog from fission yeast (T40446), share 34, 30 and 27% identity, respectively, with metaxin in their extra-mitochondrial domain. The function of these proteins has not been studied. Metaxin also shares a low degree of sequence similarity with TOM37, a yeast protein which is located in the mitochondrial outer membrane and is a receptor subunit for general import pore (GIP) (Gratzer et al., 1995). Armstrong et al. (1997) found that anti-metaxin antibody, when preincubated with mitochondria, partially inhibited the uptake of pre-adrenodoxin into mitochondria. Thus, metaxin was proposed to be a component of a pre-protein import complex in the outer mitochondrial membrane. However, the high degree of identity (24%) only occurs between the N-terminal halves of TOM37 and metaxin; TOM37 has an N-terminal mitochondrial targeting sequence, while metaxin has a C-terminal targeting sequence (Armstrong et
We found the TOM37-disrupted yeast strain obtained from Dr N. Pfanner was as sensitive as the parental strain to Bax-induced cell death (data not shown). Thus, metaxin may have a different function from that of TOM37. Supporting this, anti-metaxin 2 antibody did not have an effect on pre-adrenodoxin uptake (Armstrong et al., 1999). Despite the function of metaxin in pre-protein import still needing further investigation, the possibility that the TNF resistance in the Meta mut line is due to inefficient protein transport into mitochondria cannot be completely excluded. However, we have found no difference between parental and metaxin-mutant cells in ATP levels, mitochondrial succinate dehydrogenase enzyme complex (complex II) activity, creatine kinase activity, mitochondrial quantity or the major protein amount and composition in the mitochondria (data not shown). The selective resistance of metaxin-mutant cells to certain death stimuli, but not to others (Figure 3), further supports the idea that metaxin deficiency-mediated death resistance does not result from a generalized deleterious effect of cellular physiology, but a specific defect of a cell death pathway used by TNF.

METHODS

Cell culture. L929 murine fibrosarcoma cells from ATCC were cultured in RPMI-1640 media as described (Vercammen et al., 1998a). Stable cell lines derived from L929 cells were established either by retrovirus infection or plasmid transfection by GenePorterTM transfection reagent (GTS Inc., San Diego, CA). Selection of transfected clones was performed by adding 1 mg/ml G418 or 10 μg/ml blasticidin S (Invitrogen, Carlsbad, CA) to the culture medium.

Plasmid construction. The pDisrup retroviral vector was constructed based upon MMLV retroviral vector pLNCX as backbone. The splicing donor and acceptor were designed according to human adenovirus type 2 major late mRNA intron sequence. Details of the plasmid as well as its construction are available upon request. metaxin and fas cDNA were subcloned into the expression vector pcDNA6 (Invitrogen) with blasticidin resistance. Metaxin truncation was made as described by Armstrong et al. (1997).

Northern blotting. The entire coding region of the G418-resistant gene was used as a probe in the detection of neo-fusion mRNA. metaxin cDNA was used for detecting metaxin mRNA levels in L929 and Meta mut lines. The probes were labeled by Prime-It kit (Stratagene, La Jolla, CA) with [α-32P]dCTP.

3′-RACE. The portion of endogenous gene fused with neo gene was amplified by a 3′-RACE technique. Total RNA was isolated and reverse transcription was performed with an RT primer (5′-CCAGTGACAGGTGACGACCTGACCCCAAGGC(T)17-3′). A nested PCR was performed with the RT product by separately using primers P1/Q1 (5′-ATGAGCTAGCTTTGCT-3′/5′-CCA\_GTGACAGGTGACGACGGAGG-3′) and P2/Q2 (5′-GACGGTTCCTTCTG\_ACTAGCTAGGAGGAGGACCTGACCCCAAGGC-3′). P1 and P2 are located in the neo resistance gene while Q1 and Q2 are on
the anchor sequence of QT. The PCR fragments were subcloned into the TA-cloning vector (Stratagene) and sequenced.

**Cell death/survival assays.** Mouse recombinant TNF was used with a concentration of 100 ng/ml unless otherwise indicated. Plasma membrane integrity was determined according to the exclusion of PI (Vercammen et al., 1998a). Annexin V-FITC kit (Roche Molecular Biochemicals) was used for measuring PS flipping according to the manufacturer’s protocol, and MTT assay was carried out as described previously (Vercammen et al., 1998a).

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