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Targeted Disruption of the Mouse *Caspase 8* Gene Ablates Cell Death Induction by the TNF Receptors, Fas/Apo1, and DR3 and Is Lethal Prenatally

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Summary

Homozygous targeted disruption of the mouse *Caspase 8* (*Casp8*) gene was found to be lethal in utero. The *Caspase 8* null embryos exhibited impaired heart muscle development and congested accumulation of erythrocytes. Recovery of hematopoietic colony-forming cells from the embryos was very low. In fibroblast strains derived from these embryos, the TNF receptors, Fas/Apo1, and DR3 were able to activate the Jun N-terminal kinase and to trigger IκBα phosphorylation and degradation. They failed, however, to induce cell death, while doing so effectively in wild-type fibroblasts. These findings indicate that Caspase 8 plays a necessary and nonredundant role in death induction by several receptors of the TNF/NGF family and serves a vital role in embryonal development.

Introduction

Programmed cell death in metazoans involves a crucial role for the caspases, members of a cysteine protease family that are expressed in the living cell as inactive precursors and become activated upon death induction. Once activated, these proteases cleave a specific set of substrate proteins that act as regulators of the apoptotic mechanisms and thus set the apoptotic program in motion (reviewed in Nicholson and Thornberry, 1997; Villa et al., 1997). Several receptors of the TNF/NGF family, including Fas/Apo1 (CD95), the p55 TNF receptor (CD120a), and others, can induce programmed death in cells. Studies of the mechanisms of action of these receptors led to the identification of a member of the caspase family, Caspase 8 (MACH/FLICE), which is recruited to Fas/Apo1 and apparently also to the p55 TNF receptor through association of a duplicated N-terminal motif in this caspase, the death effector domain (DED), with a homologous motif in an adapter protein, MORT1/FADD (Boldin et al., 1996; Muzio et al., 1996). It has also been suggested that two other caspases can associate with these receptors through specific adapter proteins: Caspase 10 (Casp10) through binding to MORT1/FADD (Fernandes-Alnemri et al., 1996; Vincenz and Dixit, 1997) and Caspase 2 (Casp2) through binding to the adapter protein RAIDD/CRADD (Ahmad et al., 1997; Duan and Dixit, 1997). Evidence has also been presented suggesting that Fas/Apo1 can induce death in a less direct manner through recruitment of the adapter protein Daxx, which activates the serine/threonine kinase Jun N-terminal kinase (Yang et al., 1997).

Most of the indications that the above signaling pathways may be involved in death induction are rather indirect, being based on assessment of the functional consequence of overexpression either of proteins suspected to participate in these pathways or of their nonfunctional mutants in transfected cells. Knowledge of the in vivo functional significance of the activation of these pathways is limited. Rather solid evidence implicates the death-inducing activity of Fas/Apo1 in restricting the immune response, mainly through self-destruction of lymphocytes and also in killing cells that express foreign antigens (reviewed in Nagata and Golstein, 1995). Other than this, however, there is little knowledge of the functional significance of the cell-killing activity of the various receptors of the TNF/NGF family.

In this study, we attempted to gain more direct information about the role and functional significance of Caspase 8 (Casp8) in the cell death–inducing activity of the receptors of the TNF/NGF family by targeted disruption of the mouse *Caspase 8* (*Casp8*) gene.

Results

The Mouse *Caspase 8* cDNA and Gene

Using the human *CASP8* cDNA as a probe, we cloned the corresponding mouse cDNA, which we then used to clone the mouse *Casp8* genomic region. Detailed sequence analysis of this region and comparison with the cDNA sequence disclosed the existence of eight exons within the region: exon I encoding the first DED; exons II and III, the second DED; exon IV, the intermediate region between the DEDs and the protease region;

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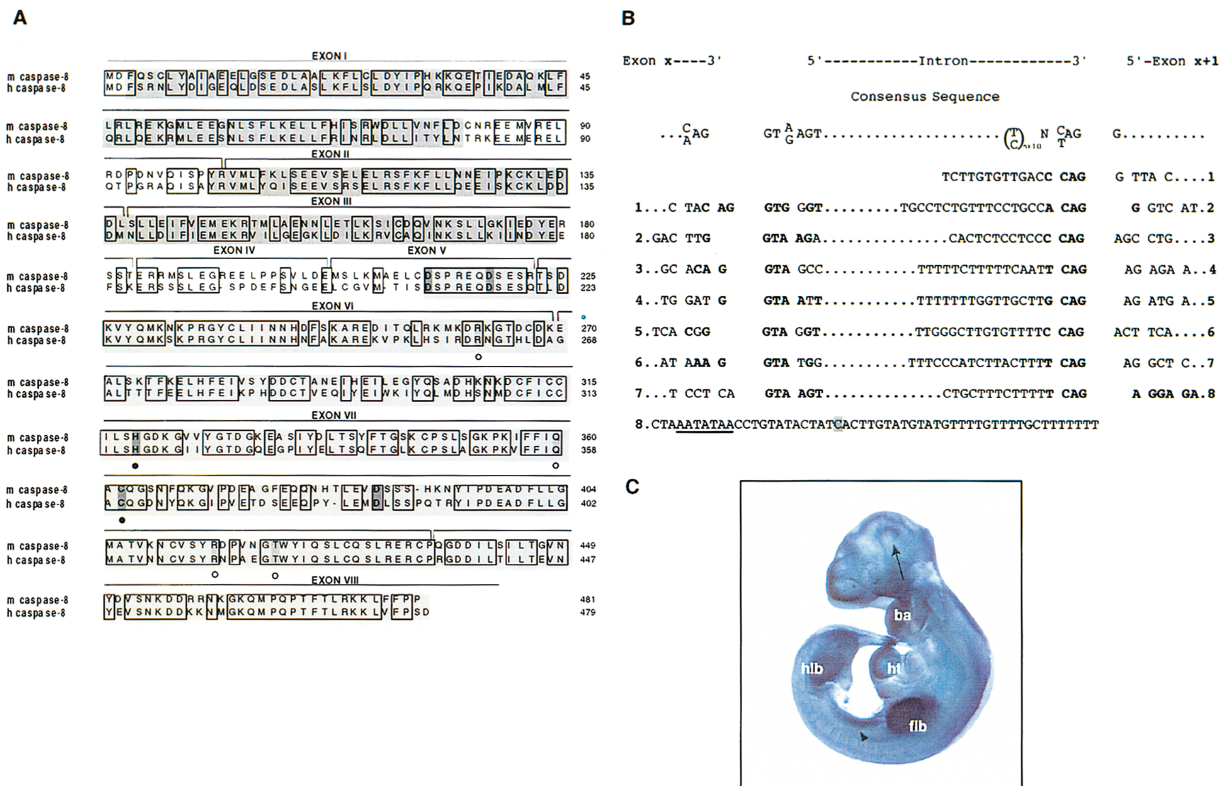

Figure 1. The Mouse *Caspase 8* cDNA, Its Exon Structure, and Its Embryonic Expression in Midgestation

(A) Collinear amino acid sequence alignment of the mouse and human *Caspase 8* gene. The residues are numbered to the right of each sequence. Dashes indicate gaps in the sequence to allow optimal alignment. The DED modules are shown in dark shading. The protease homology region is shown in light shading. Identical amino acids are shown in boxes. Within the region of protease homology, the residues putatively involved in catalysis are shown in dark shading and marked by closed circles below the alignment. The residues constituting the binding pocket for the carboxylate side chain of P1 Asp are less heavily shaded and are marked by open circles. The potential sites of cleavage are darkly shaded.

(B) Splice junctions of the mouse *Caspase 8* (*Casp8*) gene. Bases matching the splicing consensus sequence are noted in bold. The polyadenylation signal is underlined and the polyadenylation site is shaded.

(C) Analysis of the expression of *Casp8* in a wild-type embryo at 10.5 dpc. Whole-mount mRNA in situ hybridization. Abbreviations: ba, bronchial arches; flb, forelimb bud; hlb, hindlimb bud; ht, heart. Arrow, area of the mid-hind brain junction. Arrowhead, region of the dorsal mesentery.

exons V through VIII, the protease region; and exon VIII, also encoding the 3′ UTR (Figures 1A, 1B, and 2B). The boundaries between exons IV, V, and VI roughly corresponded to those of "blocks" 1, 2, and 3, parts of the Casp8 molecule that occur in different splice variants of the human protein in various combinations (Boldin et al., 1996). The 3′ UTR contained a noncanonical polyadenylation signal (AATATA) located 14 bp upstream of the adenylation site, followed by a stretch of GT-rich sequence, and ending with a T_7 stretch (Figure 1B).

To characterize the 5′ region of the *Casp8* gene, we isolated a P1 mouse genomic clone of about 60–70 kb that hybridized with the identified genomic sequence. This clone contained the region between exons I and VI and, about 15 kb upstream of exon I, two additional exons corresponding to two alternative 5′ UTRs of the *Casp8* cDNA. In addition, about 30 kb further upstream it contained an exon corresponding to the 3′ end of the cDNA of *CASH*α, a proteolytically inactive homolog of Casp8 and Casp10 (also having several other names, such as cFLIP and Casper), which apparently acts as a modulator of death initiation by Casp8 (reviewed in Wallach, 1997) (Figure 2A).

The mouse chromosomal location of the *Casp8* locus was determined by interspecific backcross analysis using the 94 progeny in the Jackson BSS cross. Polymorphisms were detected upon PCR amplification of C57BL/6J and *M. spretus* genomic DNAs. Linkage analysis revealed that the mouse *Casp8* gene maps to the proximal region of chromosome 1 [Centromere-*D1Mit4*-17.02 ± 3.88 cM-*Inpp1*-1.06 ± 1.06 cM-*Casp8, Cd152*-4.25 ± 2.08 cM-*Crygb*-3.19 ± 1.81 cM-*D1Mit7*].

The human *CASPASE 8* (*CASP8*), *CASPASE 10* (*CASP10*), and caspase homolog (*CASH*) genes have been localized to chromosome 2, band q33–34, telomeric to the STS marker D2S116 (Fernandes-Alnemri et al., 1996; Han et al., 1997; Rasper et al., 1998; V. M. B. and E. E. V., unpublished data). This region is homologous to that found for the mouse *Casp8* gene. It is consistent with the location of inositol polyphosphate-1-phosphatase (*INPP1*), cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*), and gamma B crystallin (*CRYGB*), the human homologs of *Inpp1*, *Cd152*, and *Crygb*, respectively, which were found adjacent to mouse *Casp8*. Comparison with the location of several different known human EST and STS markers indicated that the human

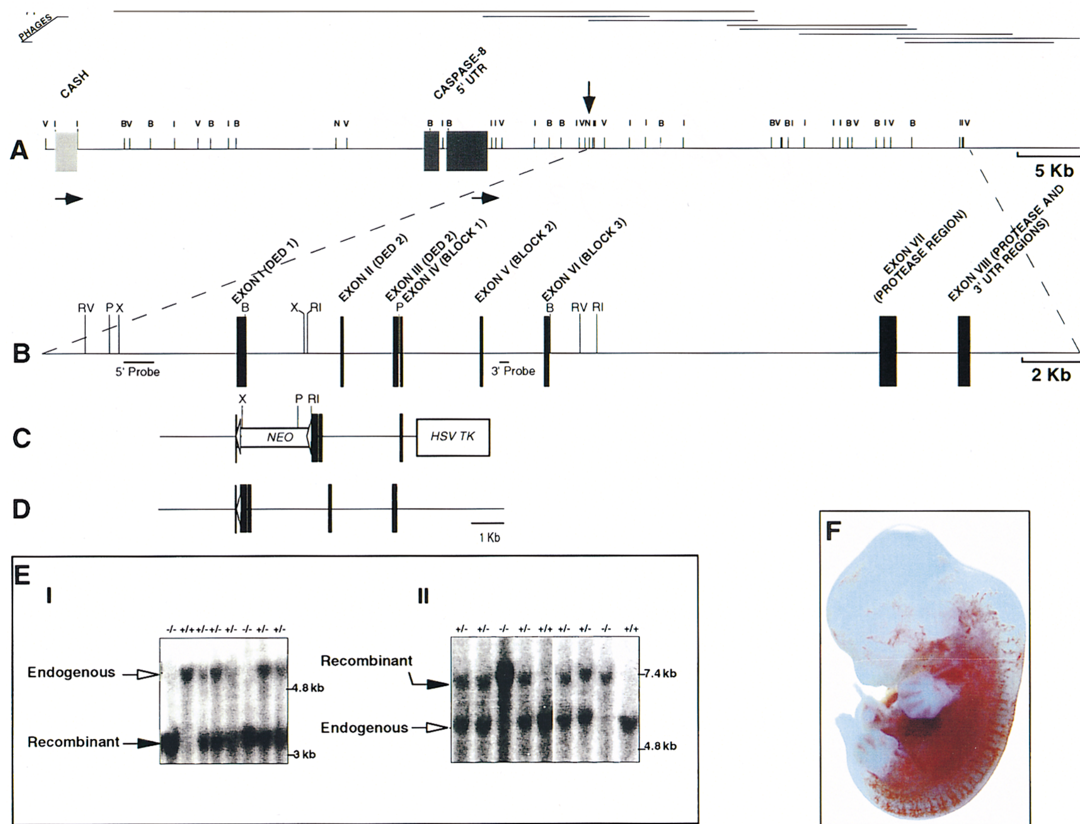

Figure 2. Targeting the *Caspase 8* Gene

(A) Analysis of the mouse *Casp8* genomic region. Aligned contig and partial restriction map of seven phage clones and a P1 clone encompassing the mouse *Casp8* genomic locus. The bottom line represents the final contig, based on alignment of the indicated clones. Boxes correspond to the genomic regions containing the 3′ end exon of CASHα (light shading), and the two 5′ untranslated exons of Casp8 (dark shading). Restriction sites are marked by vertical lines. "V," "I," "B," and "N" correspond to EcoRV, EcoRI, BamHI, and NotI, respectively. The region marked by a vertical arrow upstream of a NotI site contains additional unresolved restriction nuclease recognition sites. The orientations of the *CASH* and *Caspase 8* genes are marked by horizontal arrows.

(B) Structure of a 32.5 kb region in the mouse *Casp8* gene encompassing the coding region and 3′ UTR in the gene. The exons, deduced by comparison with the cDNA sequence, are denoted by black boxes. The positions of the endogenous and introduced restriction sites are marked by vertical lines ("RV," "P," "X," "B," and "RI" correspond to EcoRV, PvuII, XbaI, BamHI, and EcoRI restriction sites, respectively; only the restriction sites essential for the analysis are shown). The positions of the 5′ and 3′ external probes applied for Southern blot analysis of the recombination are underlined.

(C) The targeting construct designed to replace most of the first exon and the whole second exon with a floxed *neo*^{r} cassette. The positions of the *loxP* sites flanking the *neo*^{r} cassette are designated by open triangles.

(D) Structure of the mutated region in the *Casp8* gene.

(E) Southern blot analysis of the recombination in the *Casp8* gene. Analysis of XbaI-digested DNA derived from 10.5 dpc embryos obtained by crossing heterozygotic mice. (EI) Mice whose DNA contains the *neo*^{r} cassette. (EII) Mice after excision of the *neo*^{r} cassette. DNA was digested with XbaI, fractionated on 0.8% agarose gel, blotted onto a nylon membrane, and hybridized with a 5′ probe.

(F) Whole view of a 12.5 dpc *Casp8*$^{-/-}$ homozygous mutant embryo.

CASH gene is located centromerically to *CASP8*, and that the 5′ ends of both genes (as well as of the *CASP10* gene, which is located between them) are oriented toward the centromere (V. M. B., unpublished data). Presumably, the mouse *Casp8* and *CASH* genes are similarly oriented.

Transcription Pattern of *Caspase 8* in the Midgestation Mouse Embryo

Both whole-mount in situ hybridization (Figure 1C) and radioactive histological in situ hybridization (data not shown) at gestational ages between 9.5 and 12.5 days postcoitum (dpc) revealed widespread distribution of *Casp8* transcripts. Elevated signals could be detected in the heart (especially in the primitive ventricle), the surface ectoderm of the fore- and hindlimb buds, the branchial arches, and the area of the mid–hind brain junction (see arrow in Figure 1C) as well as in the region of the dorsal mesentery (Figure 1C, arrowhead).

Targeted Disruption of the *Caspase 8* Gene Results in a Lethal Embryonic Phenotype

Casp8 binds to MORT1/FADD through its two N-terminal DED motifs (Boldin et al., 1996; Muzio et al., 1996). To abolish signaling to death through this caspase, we transfected R1 embryonic stem (ES) cells with a targeting vector allowing replacement of exons I and II, which encode these motifs, with a neomycin resistance

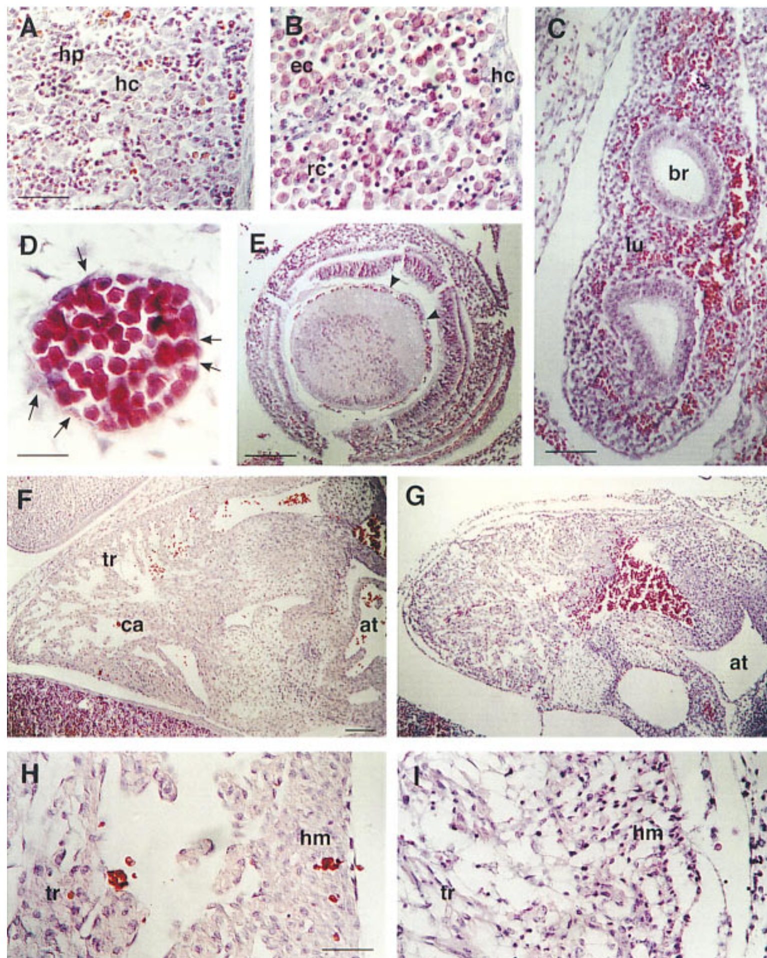

Figure 3. Histological Study of the *Caspase 8*$^{-/-}$ Embryos

Liver of (A) wild-type and (B) mutant (*Casp8*$^{-/-}$) embryos at 11.5 dpc. (C) Lung of a mutant embryo at 10.5 dpc. (D) Intersomitic blood vessel of mutant embryo at 11.5 dpc. (E) Eye of mutant embryo at 12.5 dpc (note hyperemia in the lens capsule). Heart of (F) wild-type and (G) mutant embryos at 11.5 dpc. Heart of (H) wild-type and (I) mutant embryos (high magnification) at 12.5 dpc. Abbreviations: at, atrium of the heart; br, bronchus; ca, camera (common ventricle) of the heart; ec, erythrocytes; hc, presumptive hepatocytes; hm, presumptive heart muscle cells; hp, hematopoietic precursors; lu, lung; rc, round cells (lymphocytes?); tr, trabeculae of the heart ventricule. Arrows in (D), endothelial cells; arrowheads in (e), capsula lentis. Scale bars: (A and B), 40 μm; (C), 100 μm; (D), 20 μm; (E), 200 μm; (F and G), 100 μm; (H and I), 40 μm.

(*neo*^{r}) cassette (Figures 2B, 2C, and 2D). Of 186 recombinant ES clones examined, 2 were positive for homologous recombination. Germline competent chimeras were generated by aggregation of the recombinant ES cells with morula-stage embryos, and progeny from heterozygous parents (*Casp8*$^{+/-}$) were examined.

Although the heterozygous mice appeared phenotypically normal, no homozygous (*Casp8*$^{-/-}$) mice could be detected in their intercross, whereas the *Casp8*$^{+/-}$ and *Casp8*$^{+/+}$ genotypes were represented in a ratio of 2 to 1, indicating that the homozygous disruption of *Casp8* leads to prenatal death. Southern analysis of the genotypes of embryos collected between 9.5 and 11.5 dpc (Figure 2E) disclosed normal Mendelian segregation ratios. Until day 10.5, no gross morphological abnormalities could be detected. At day 11.5, however, 40% of the *Casp8*$^{-/-}$ embryos displayed the abnormal phenotype described below, and at day 12.5 almost all *Casp8*$^{-/-}$ embryos examined had this phenotype and some were found dead or close to death.

The most salient feature of the abnormal phenotype of the *Casp8*$^{-/-}$ mutant was marked hyperemia in the abdominal area (Figure 2F). In most mutant embryos this was accompanied by hyperemia in the superficial capillaries and other blood vessels, mainly in the umbilical and trunk area, including those in the intersomitic and interdigital blood vessels and to a lesser degree the face, head, and even the lens of the eye (Figure 3). Most mutant embryos were somewhat smaller than their wild-type littermates, but they were normally shaped and formed according to their gestational age. Histological examination revealed extensive erythrocytosis in the liver at day 10.5–13.5 dpc. Most of this organ was occupied by fully formed embryonic erythrocytes. Normal embryonic liver tissue, with its characteristic early hematopoietic elements, and early liver cells were visible only in the cortical area (compare Figures 3A and 3B). Hyperemia similar to that seen in the liver was observed in most major blood vessels and in many organs (for example, the lung [Figure 3C]); it was also seen in the intersomitic (Figure 3D) and interprevertebral blood vessels, mesenchymal spaces, the retina, and under the capsule of the lens (Figure 3E). Examination at high magnification revealed that the erythrocytes were contained in larger and smaller blood vessel areas (Figure 3D), suggesting hyperemia and/or congestion rather than outright bleeding.

Detailed histological observation also revealed that the heart had developmental abnormalities. Although the heart was not appreciably larger than normal, the developing ventricular musculature was thin and in some cases not different from early mesenchyme. The trabeculae were thin and disorganized (compare Figures 3F and 3G as well as Figures 3H and 3I).

To further investigate the nature of the hyperemia, we assessed the numbers of hematopoietic precursors in

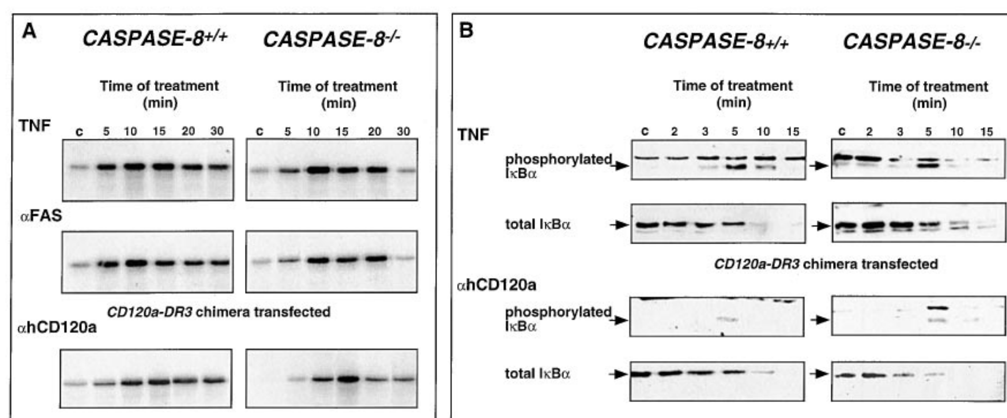

Figure 4. Noncytocidal Effects of the TNF Receptors, Fas/Apo1, and DR3 in Wild-Type and *Caspase 8*$^{-/-}$ Mice

(A) Time course of c-Jun phosphorylation. (B) Time course of IκBα phosphorylation and degradation. Cells were stimulated with 2000 U/ml of human TNFα or monoclonal anti-Fas or (in the case of cells transfected with the CD120a-DR3 chimera) anti-CD120a antibodies for the indicated time periods, or they were left unstimulated (c). They were then collected, lysed, and either subjected to immunoprecipitation by antibodies raised against JNK1 followed by in vitro kinase reaction using recombinant GST-c-Jun as substrate or analysed by Western blotting using phosphospecific anti-IκBα or phosphorylation state–independent anti-IκBα antibodies.

the normal and mutant 11.5 dpc embryos. Whereas the cells of normal embryos yielded greater than 500 colonies (mostly myeloid) per input of 5×10^4 cells in the in vitro test, the same cell input yielded fewer than 10 colonies on average in the mutants (see Experimental Procedures). Disruption of the *Casp8* gene thus appears to result in a dramatic primary or secondary depletion of the hematopoietic precursor pool.

The recombination event by which *Casp8* was disrupted involved the introduction of a *neo*^{r} gene flanked by two *loxP* recombination target sequences into the mouse genome (see Experimental Procedures). To confirm that the observed phenotype resulted from deficient *Casp8* expression and not from distortion of the expression patterns of other genes by the *neo*^{r} gene or its control elements, we excised the *loxP*-flanked insert by mating mice heterozygous for the recombination with *PGK-Cre*^{mat} (Lallemand et al., 1998) transgenic mice (Figure 2E, II). Comparison of *neo*$^+$ and *neo*$^-$ mutants revealed no differences in their gross phenotype or in the kinetics of their prenatal death.

To confirm that the recombination effect was restricted to *Casp8*, we evaluated the embryonic expression of *CASH*, a gene located upstream of *Casp8* (Figure 2A). The expression of *CASH*, which could be discerned both in the embryos and in the ES cells, seemed unaffected by the homologous recombination, while *Casp8* expression was abolished (data not shown).

Cultured Fibroblasts Derived from the *Caspase 8*$^{-/-}$ Mice Respond Normally to Noncytocidal Effects of the TNF Receptors, Fas/Apo1, and DR3

To assess the functional consequences of the *Casp8* mutation at the level of the individual cell, we established continuous fibroblast strains from wild-type and *Casp8*$^{-/-}$ embryos at 10.5 dpc. From each of the strains we also derived cell lines expressing a chimeric receptor comprised of the extracellular domain of the human p55 TNF receptor (CD120a) and the intracellular domain of DR3, a DD-containing receptor of the TNF/NGF family that induces cell death by a mechanism that seems closely related to that of CD120a (Chinnaiyan et al., 1996; Kitson et al., 1996; Marsters et al., 1996a; Bodmer et al., 1997).

Gene activation by TNF and DR3 involves stimulation of the transcription factors NF-κB and AP1 through protein kinase cascades that lead to phosphorylation of the AP1-component Jun (reviewed in Kyriakis and Avruch, 1996) and the NF-κB inhibitory protein IκB (reviewed in Stancovski and Baltimore, 1997). Activation of Fas/Apo1 also leads to enhanced phosphorylation of Jun (Latinis and Koretzky, 1996; Goillot et al., 1997; Lenczowski et al., 1997; Yang et al., 1997). As shown in Figure 4, these effects could be induced in the *Casp8*$^{-/-}$ fibroblasts just as effectively as in the wild-type cells. In cells derived from both sources, treatment with TNF, as well as ligation of Fas/Apo1 or the CD120a-DR3 chimera, induced increased Jun N-terminal kinase activity within 5 min of stimulation (Figure 4A); in addition, TNF treatment or CD120a-DR3 ligation induced phosphorylation of IκBα, which was promptly followed by IκBα degradation (Figure 4B).

Cultured Fibroblasts Derived from the *Caspase 8*$^{-/-}$ Mice Are Resistant to Death Induction by the TNF Receptors, Fas/Apo1, and DR3 but Are Sensitive to Death-Inducing Agents that Act from within the Cell

Wild-type (*Casp8*$^{+/+}$) cells were killed by human TNF when it was applied to these cells in the presence of the protein-synthesis blocker cycloheximide. They were also killed by antibody cross-linking of Fas/Apo1. In addition, the wild-type cells expressing the CD120a-DR3 chimera were killed by cross-linking these chimeric molecules using antibodies to human CD120a. In contrast, cells derived from the *Casp8*$^{-/-}$ embryos were completely resistant to death induction by these agents (Figure 5). They were equally resistant to killing by mouse

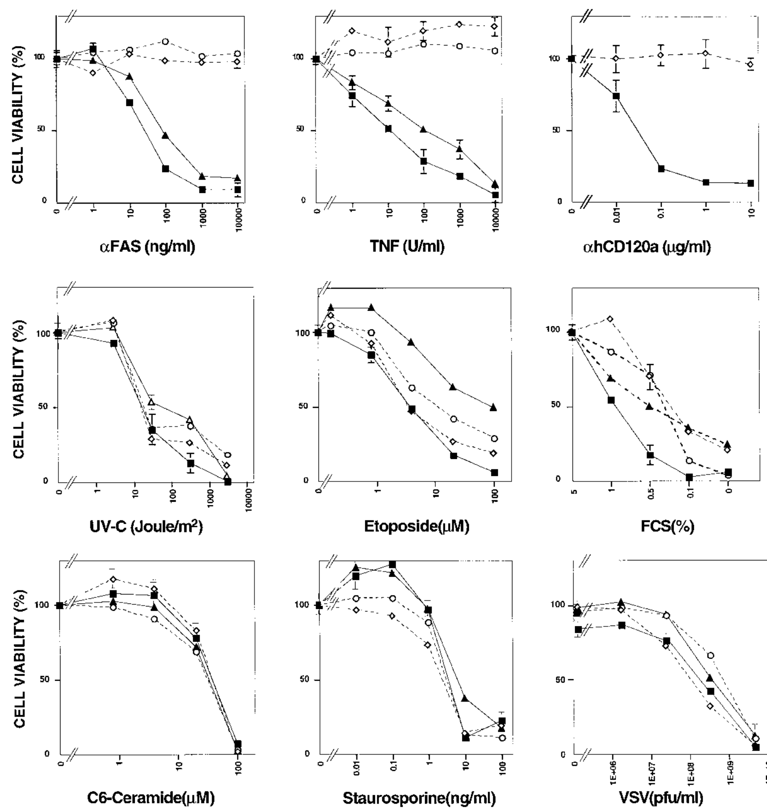

Figure 5. Caspase 8 Is Required for Death Induction by Fas/Apo1, TNF, or DR3 but Not for Its Induction by UV Irradiation, Etoposide, Ceramide, Staurosporine, Serum Starvation, or VSV

The figure shows the proportions of cells that remained viable following incubation of wild-type (filled squares and triangles) and *Casp8*$^{-/-}$ fibroblasts (open circles and diamonds) with the indicated reagents. Means ± SD of duplicate representative examples from a series of at least seven experiments are shown.

TNF (not shown). The *Casp8*$^{-/-}$ cells could, however, be killed by various other death-inducing agents including UV irradiation; the protein kinase inhibitor staurosporine; the topoisomerase inhibitor etoposide, C6-ceramide, which is believed to act as an intracellular mediator of some signaling pathways for cell death; and the cytopathic virus vesicular stomatitis virus (VSV). They were also killed by serum deprivation (Figure 5).

Discussion

The resistance of *Casp8*$^{-/-}$ fibroblasts to death induction by several receptors of the TNF/NGF family, despite their normal responses to other, noncytocidal effects of the same receptors, implies that Casp8 plays a central and nonredundant role in the death-induction mechanism. This finding may appear to be at variance with studies suggesting that death induction by the receptors can also occur through other signaling pathways, namely, activation of Casp10 and Casp2 in a manner similar to the activation of Casp8 (reviewed in Villa et al., 1997) and activation of the Jun N-terminal kinase through the adapter protein Daxx (Yang et al., 1997). This apparent discrepancy could be accounted for in a number of possible ways: (1) although these other death-inducing pathways might contribute to death induction, their contribution perhaps depends on a permissive role of Casp8; (2) these alternative pathways might act in a cell-type-specific manner, having little involvement in death induction in fibroblasts, yet important roles in other cells; or (3) these pathways might not mediate death induction by the TNF receptors or Fas/Apo1, and the data suggesting otherwise are based on their involvement in death induction by other receptors, which act similarly to those utilizing Casp8 in their death-inducing function. Tests employing overexpression of dominant negative mutants of signaling molecules to assess the involvement of such molecules in signaling are prone to such misinterpretation, as illustrated by the conflicting reports on the mechanisms of death induction by TRAIL/Apo2-L, a ligand related to TNF. Some of these studies suggested that the receptors triggered by TRAIL/Apo2-L employ MORT1/FADD and Casp8 in death induction (Chaudhary et al., 1997; Schneider et al., 1997; Walczak et al., 1997; Wajant et al., 1998), while others pointed to the involvement of Casp10 and some adapter protein(s) other than MORT1/FADD (Marsters et al., 1996b; Pan et al., 1997; Yeh et al., 1998). Unfortunately, the fibroblast lines established in the present study showed little death in response to TRAIL/Apo2-L, precluding their use for assessing the involvement of Casp8 in death induction by TRAIL/Apo2-L.

While pointing to a crucial role for Casp8 in death induction by receptors of the TNF family, our data indicate that this caspase is not essential in death induction by agents like etoposide or staurosporine. These agents employ caspases in their death-inducing effects (Jacobsen et al., 1996; Martins et al., 1997; Bossy-Wetzel et al., 1998), yet they apparently function through activation of intracellular mechanisms such as those triggered by cytochrome c upon its release from damaged mitochondria to the cytosol (Li et al., 1997).

Our findings also indicate that Casp8 is not involved in the induction of phosphorylation and degradation of

IκBα, nor in the induction of Jun N-terminal kinase activation by the TNF receptors, Fas/Apo1, or DR3. Studies have indeed delineated caspase-independent signaling pathways through which these receptors can stimulate the protein kinases involved in these two effects (see Ting et al., 1996; Yeh et al., 1997; Kelliher et al., 1998, and references cited therein). Nevertheless, there is also evidence suggesting that Jun N-terminal kinase activation by Fas/Apo1 can occur in a way that does depend on caspase activation (Chen et al., 1996; Lenczowski et al., 1997; Roulston et al., 1998). Again, while clearly excluded from operating in fibroblasts, such a Casp8-dependent signaling pathway may well be found to act in other cells.

The in vivo consequences of the targeted disruption of the *Casp8* gene are quite different from those observed for knockout mutations of ligands or receptors known to employ Casp8 in their death-inducing pathways. Disruption of the genes of the TNF receptor Fas/Apo1 or its ligands had pronounced effects on immune functions that operate in the adult. Yet, in contrast to the lethal prenatal effect of *Casp8* disruption, ablation of the expression of these ligands or receptors had almost no effect on the development of the mice, the only exception being defective Peyer's patch organogenesis in mice with targeted disruption of the p55 TNF receptor (CD120a) (reviewed in Gruss and Dower, 1995; Matsumoto et al., 1997). This apparent discrepancy implies that, apart from signaling for the effects of the TNF receptors, Fas/Apo1, and DR3, Casp8 also mediates signaling by other stimuli that play vital roles in embryogenesis. One candidate for such a stimulus is the ligand for DR3 (Marsters et al., 1998), a receptor shown conclusively in this study to involve Casp8 in its signaling for death.

The *Casp8*$^{-/-}$ embryonic phenotype is characterized by two salient features: impaired heart muscle development and congested accumulation of erythrocytes. These features resemble the recently reported phenotype of mice with targeted disruption of the *MORT1/FADD* gene (Yeh et al., 1998; Zhang et al., 1998). *MORT1/FADD*$^{-/-}$ embryos died at about the same age as the *Casp8*-deficient mice. Moreover, like the *Casp8*-deficient embryos, they exhibited impaired heart muscle development and congested accumulation of erythrocytes. Conceivably, the death of the *MORT1/FADD*$^{-/-}$ embryos can be accounted for by the failure to activate Casp8.

The mechanism underlying the midgestation lethal *Casp8*$^{-/-}$ phenotype remains to be clarified. Congestion and edema frequently accompany heart failure. In our case, however, no generalized edema was observed. Moreover, a similar failure of midgestation heart development in the targeted mutation of *neuregulin* was not accompanied by congestion or hyperemia (Meyer and Birchmeier, 1995). Defects such as those observed in the *Casp8*$^{-/-}$ mice could be due to abnormal angiogenesis. However, despite detailed examination, we could not discern any defects in the small or large blood vessels of the mutants. Indeed, the *MORT1/FADD*$^{-/-}$ embryos were found to exhibit normal expression of *flk-1*, a major regulator of early angiogenesis (Yeh et al., 1998). Thorough investigation of additional markers of blood vessel formation should fully clarify whether or not defective blood vessel formation contributes to the phenotype. The extensive hyperemia could also have originated from a hematopoietic defect. Some kind of hematopoietic abnormality whose nature has yet to be clarified is indeed indicated by the dramatic decrease of hematopoietic precursors in the *Casp8*$^{-/-}$ mice. We cannot, however, exclude the possibility that this hematopoietic defect was secondary to poor circulation, nor can we exclude the possibility that some additional, less easily discernible consequence(s) of the disruption of the *MORT1/FADD* or *Casp8* genes contributed to the death of the embryos. Assessment of the effect of *MORT1/FADD* disruption on the development of lymphocytes indicated that this adapter protein contributes not only to the regulation of death of these cells, but also to the induction of their growth (Zhang et al., 1998). Likewise, it may well be that the lethal effect of *MORT1/FADD* or *Casp8* disruption on these embryos results not from insufficient death induction, but from deficient induction of some other effect (perhaps growth stimulation) through the MORT1/FADD–Casp8 signaling pathway.

Our understanding of the in vivo significance of cell death lags significantly behind our knowledge of the mechanisms involved in this process. The current state of knowledge of the cell death–inducing function of TNF is a pertinent example. We have known for more than 30 years that TNFα and LTα (TNFβ) can cause death of cells. Yet, while many other activities of these pleiotropic cytokines have been well placed in a physiological context, there is not yet even the slightest hint of the types of in vivo situations in which the direct cytocidal effect of TNF is manifested. This function of TNF, like most other known activities of the TNF ligand family, presumably contributes to immune defense, but the way in which it does so is unknown. The targeted disruption approach provides a valuable tool for bridging this gap between molecular and physiological understanding, as it allows genes found to participate in the death process to be used as molecular probes for assessing the in vivo occurrence and functional consequences of the death process. The obligatory and specific role of Casp8 in death induction by receptors of the TNF/NGF family endows this molecule with particular value as such a probe. This value could not be fully exploited in the present study, since the prenatal death of the knockout embryos precluded analysis of the involvement of the Casp8 enzyme in functions manifested in the adult mice. Restricting the disruption of the *Casp8* gene to specific time periods during development or specific tissues (e.g., by creating mice chimeric for the recombination or by conditional disruption of the gene in a developmental stage-specific or cell lineage–specific manner) should allow further progress both in elucidating the role of this protein in embryonic development and in studying its function in the adult mouse.

Experimental Procedures

Isolation of the Mouse *Caspase 8* cDNA

The mouse *Casp8* cDNA was isolated from a λgt11 oligo(dT)-primed library derived from LPS-treated BAM3 mouse macrophage cells (kindly provided by S. Nagata; Watanabe et al., 1992) by screening

with a fragment of the human *CASP8* cDNA. The inserts from the positive phages were subcloned into pBluescript vector and sequenced by the dideoxy chain-termination method. Sequence alignment of the mouse and human *Casp8* cDNAs was performed, and their homology was evaluated using the alignment program in Gene-Assist 1.1 b4 (Perkin-Elmer, Applied Biosystem Division).

Cloning of the Mouse *Caspase 8* Gene and Adjacent Regions

A 129/Sv mouse genomic library (Stratagene) was screened with a mouse *Casp8* cDNA probe. Of 1 million clones screened, 7 overlapping clones encompassing the full coding and the 3′ UTR regions in the *Casp8* gene were isolated. Their inserts were subcloned into the pBluescript vector using appropriate restriction enzymes and sequenced in both directions with dye terminators on ABI377. The Sequencher program (version 3.0 for Macintosh) was used for analysis of the sequences and for final assembly of the sequence contig. To define the exon–intron boundaries of the *Casp8* gene, its cDNA sequence was compared with that of the genomic contig using the alignment program in GeneAssist 1.1 b4 (Perkin-Elmer, Applied Biosystem Division). To obtain genomic clones that contain the 5′ UTR region, we screened a mouse genomic library in P1 (MP1 Mouse P1 library number 703, Resource Center, Berlin-Charlottenburg, Germany) using a fragment at the 5′ end of the sequence isolated from the phage library as a probe (the "5′ probe" in Figure 2B). Further restriction nucleotide mapping of this clone and analysis of its structure were done by Southern DNA hybridization, using specific oligonucleotides of T_7, *SP6*, *CASH*α, or *Casp8* cDNA as probes.

Genetic Mapping of the *Caspase 8* Gene

The chromosomal location of the mouse *Casp8* gene was determined by linkage analysis with the BSS Backcross DNA Panel (Jackson Laboratory, Bar Harbor, ME). The panel consists of 94 genotyped progeny derived from a (C57BL/6JEi × SPRET/Ei)F1 × SPRET/Ei backcross (Jackson BSS) (Rowe et al., 1994). The allele pattern of *Casp8* was compared using Map Manager (Manly, 1993) to those of the approximately 3200 previously mapped loci in the Jackson BSS cross, and the exact position determined by minimizing double crossovers (http:www.jax.org/resources/documents/cmdata/).

For allele detection, *Casp8* PCR primers (5′-GGTGATCTGAGTTT GATCTCTGGAACACAT-3′ and 5′-CCCGTGACTCACTGTCTTGTTC TCTT-3′) were used to amplify mouse genomic DNA purified from C57BL/6JEi and SPRET/Ei inbred strains (by denaturation for 45 s at 94°C, annealing for 1 min at 57°C, extension for 50 s at 72°C, 33 cycles, followed by further extension for 5 min at 72°C). PCR amplification of C57BL/6JEi DNA identified a 396 bp fragment and amplification of SPRET/Ei DNA identified a 310 bp fragment resolved on a 2% agarose gel. In the BSS cross, the presence or absence of the 396 bp C57BL/6JEi-specific fragment was followed in the backcross mice. Homology data were retrieved from the Human Genome Database (GDB; http://www.gdb.org/) and the Mouse Genome Database (MGD; http://www.informatics.jax.org/).

Construction of a *Caspase 8* Targeting Vector and Production of Knockout Mice

A targeting vector was constructed in the pPNT vector (Samuel Lunenfeld Research Institute, Toronto, Canada) by replacing a 4.8 kb fragment of *Casp8* encompassing the first two coding exons with a PGK-*neo^r^*-polyadenylate (poly A) cassette bordered by *loxP* sites (derived from the pLoxpNeo vector, Samuel Lunenfeld Research Institute, Toronto, Canada). The construct contained two DNA stretches derived from the 129 mouse genome: a 2.4 kb fragment placed 5′ of the neo^r cassette and a 3.6 kb fragment, encompassing exons III, IV, and V, placed 3′ of the neo^r cassette. The neo^r gene was introduced in the opposite transcriptional orientation to *Casp8* (Figure 2C).

R1 embryonic stem (ES) cells (Nagy and Rossant, 1993) were transfected with the targeting vector linearized with NotI. Recombinant ES cell clones were selected by the positive-negative technique (Mansour et al., 1988). The genotype of positively selected clones was evaluated by Southern analysis using genomic DNA probes from regions upstream of the 5′ arm and downstream of the 3′ arm of the targeting construct (Figure 2B).

Chimeric mice were produced by aggregation as described (Nagy and Rossant, 1993). Germline transmission in mice generated by mating chimeric males with MF1 females was detected by coat color and reconfirmed by Southern analysis of tail DNA.

In a fraction of the population, the "floxed" neo^r cassette was deleted by mating $Casp8^{+/-}$ mice with the early "deleter" transgenic mouse strain Cre^{mat} (Lallemand et al., 1998).

Whole-Mount mRNA In Situ Hybridization and Histological Analysis

Whole-mount mRNA in situ hybridization in 10.5 dpc embryos was performed as described (Conlon and Herrmann, 1992) using single-strand digoxigenin-UTP-labeled mouse *Casp8* sense and antisense RNA probes. Radioactive in situ hybridization was as described previously (Orr-Urtreger et al., 1993). Histological investigations were in Paraplast sections (7 μm) and H-E staining.

In Vitro Hematopoietic Colony Assay

Semisolid cultures were established in order to determine the levels of hematopoietic progenitors in wild-type and $Casp8^{-/-}$ embryos. Yolk sacs and total embryos were dissected, mechanically disrupted, and filtered through 15 μm nylon mesh. For determination of the genotype of the embryos, DNA was isolated from embryonic limbs and subjected to Southern analysis as described above. Cell viability was determined by trypan blue staining. Samples of 5×10^4 viable cells were plated in RPMI medium containing 0.9% methylcellulose (Sigma, St. Louis, MO), 30% fetal calf serum (FCS), 5×10^{-5} M β-mercaptoethanol, 50 ng/ml SCF, 100 ng/ml FLT3 ligand (Immunex, Seattle, WA), 6 U/ml erythropoietin (EPO, Orto Bio Tech, Don Mills, ON, Canada), and murine IL-3 (culture medium of the IL-3-producing cell line X63/O [kindly provided by Dr. M. Oren] applied at a dilution of 1:100) in 6 CM culture dishes. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and scored 7 days later for myeloid, erythroid, mixed, and blast colonies identified according to morphological criteria. More than ten embryos of each kind, obtained from seven pregnancies, were examined.

Establishment of Embryonic Fibroblast Cell Strains

Fibroblasts derived from 10.5 dpc embryos by trypsinization (Todaro and Green, 1963) were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM), supplemented with 10% FCS, nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell strains from individual embryos of mice whose genome did not contain the neo^r cassette were established by their transformation with an SV40 T antigen–expressing retroviral vector (Almazan and McKay, 1992).

To assess the effect of DR3 signaling in the T antigen–transformed fibroblasts, a cDNA encoding a CD120a-DR3 chimera, produced by fusion of the extracellular domain of human CD120a (amino acids 1–168) with the transmembrane and intracellular domains of human DR3 (amino acids 197–417), was subcloned into the pBabe-Hygro retroviral vector (Morgenstern and Land, 1990) and transfected into the BOSC 23 retroviral packaging line (Pear et al., 1993). Supernatants of these cells were applied to the T antigen–transformed fibroblasts, followed by selection for hygromycin-resistant colonies, as described (Morgenstern and Land, 1990). All tests of DR3 function were performed using the CD120a-DR3 chimera–expressing cells

In Vitro JNK1 Assay

Cells (4×10^5) were seeded into 6 cm plates and, after overnight incubation, were treated with human TNFα (2000 U/ml; Genentech, South San Francisco, CA), the Jo2 anti-mouse Fas/Apo1 antibody (2 μg/ml; Pharmingen, San Diego, CA), or (when using cells expressing the CD120a-DR3 chimera) monclonal antibodies against the human CD120a (20 μg/ml of the antibodies produced by clones 18 and 20 [Engelmann et al., 1990] at a 1 to 1 ratio) for the indicated time periods. They were then lysed, JNK1 was immunoprecipitated from the cell lysate using the anti-JNK1 C-17 polyclonal antibody (Santa Cruz Biotechnology, CA), and in vitro kinase reaction using purified glutathione S-transferase GST-c-Jun (5–89) as substrate

was performed as described (Kerkhoff and Rapp, 1997). ^{32}P incorporation into the GST-c-Jun (5–89) was determined by SDS-PAGE analysis followed by autoradiography.

Western Blot Analysis of TNF- and DR3-Induced IκBα Phosphorylation and Degradation

Cells were seeded and treated with human TNFα or anti-human CD120a for the indicated periods as described above for the in vitro JNK1 assay. The cells were then lysed in SDS-PAGE lysis buffer and analyzed by SDS-PAGE followed by Western blot analysis, using phosphospecific anti-IκBα (Ser32) or phosphorylation state–independent anti-IκBα antibodies (New England Biolabs, Beverly, MA) and the ECL kit (Amersham, UK).

Cytotoxicity Assays

The cytocidal activity of tested agents was determined as described (Wallach, 1984). Briefly, 12 hr prior to assay cells were seeded in 96-well plates at a density of 2.5×10^4 cells/well. Human recombinant TNF and mouse recombinant TNF (Genentech, South San Francisco, CA), the Jo2 anti-Fas/Apo1 monoclonal antibody, and the mouse anti-p55 TNF receptor monoclonal antibodies 18 and 20, at a 1 to 1 ratio (Engelmann et al., 1990), were applied to the cells in the presence of cycloheximide (50 μg/ml). Ceramide (C_6-D-erythroceramide; Matreya, PA), etoposide (Sigma), staurosporine (Sigma), VSV (Indiana strain, ATCC VR-158 grown on WISH cells), and UV-C treatment were applied to the cells in the absence of cycloheximide. After treatment for 12 hr (for TNF, anti-Fas, anti-CD120a, and staurosporine) or 24 hr (for all others), cell viability was assessed by measuring the uptake of the dye neutral red (Finter, 1969).

To assess the death resulting from serum deprivation, cells were washed three times with phosphate-buffered saline and then incubated for 48 hr with DMEM supplemented with various FCS concentrations.

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