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CD95 ligand induces motility and invasiveness of apoptosis-resistant tumor cells

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The apoptosis-inducing death receptor CD95 (APO-1/Fas) controls the homeostasis of many tissues. Despite its apoptotic potential, most human tumors are refractory to the cytotoxic effects of CD95 ligand. We now show that CD95 stimulation of multiple apoptosis-resistant tumor cells by CD95 ligand induces increased motility and invasiveness, a response much less efficiently triggered by TNFα or TRAIL. Three signaling pathways resulting in activation of NF-κB, Erk1/2 and caspase-8 were found to be important to this novel activity of CD95. Gene chip analyses of a CD95-stimulated tumor cell line identified a number of potential survival genes and genes that are known to regulate increased motility and invasiveness of tumor cells to be induced. Among these genes, urokinase plasminogen activator was found to be required for the CD95 ligand-induced motility and invasiveness. Our data suggest that CD95L, which is found elevated in many human cancer patients, has tumorigenic activities on human cancer cells. This could become highly relevant during chemotherapy, which can cause upregulation of CD95 ligand by both tumor and nontumor cells.

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Introduction

CD95 ligand (CD95L/FasL), a member of the death ligand family, which also includes TNFα and TRAIL has been viewed as an apoptosis-inducing ligand and members of this family are therefore being considered as potential antitumor reagents (Gardnerova *et al*, 2000). CD95L is expressed either on cell membranes (mCD95L) or in soluble form (sCD95L) and induces apoptosis when it triggers its cognate receptor CD95 (APO-1/Fas) (Peter *et al*, 2003). We have previously demonstrated that cells can die in different ways following CD95 activation, either dependent on (Type II) or independent of (Type I) mitochondria (Scaffidi *et al*, 1998; Barnhart *et al*, 2003). We recently found that Type I cells correspond to mesenchymal tumors, whereas Type II cells have a more epithelial phenotype (Algeciras-Schimnich *et al*, 2003). sCD95L was only cytotoxic to Type II/epithelial tumor cells and did not kill Type I/mesenchymal cells, indicating that CD95L has differential effects on tumors depending on the tumor phenotype.

Chemotherapeutic drugs can cause upregulation of CD95L and it is believed that this contributes to the elimination of tumor cells by inducing their apoptosis (Friesen *et al*, 1996). However, many tumor cells are resistant to CD95-mediated apoptosis, especially after therapy (Friesen *et al*, 1999). Although it has been recognized that CD95 stimulation of certain cells under certain conditions can cause induction of genes that have functions outside of apoptosis (Faouzi *et al*, 2001; Park *et al*, 2003) and that CD95 can promote proliferation of T cells (Alderson *et al*, 1993; Alam *et al*, 1999; Kennedy *et al*, 1999), specific tumor-promoting effects of CD95 stimulation for CD95 apoptosis-resistant tumor cells have not been reported.

We now have performed an analysis of the responses of a panel of CD95 apoptosis-resistant tumor cell lines to sCD95L, mCD95L or agonistic anti-CD95 antibodies. Our data demonstrate that a significant number of these cells respond to any stimulation of CD95 with increased motility and invasiveness through Matrigel-coated membranes. This activity of CD95 involves activation of at least five different apoptosis-independent pathways of which at least three (activation of caspase-8, NF-κB and Erk1/2) are independently required to different degrees for the increased motility and invasiveness. An analysis of 54 transcription factors demonstrated that AP1, AP2, CREB and NF-κB are activated in CD95-stimulated cells. The effect is selective for CD95 stimulation since treatment of apoptosis-resistant cells with either TNFα or TRAIL only marginally induced motility or invasiveness. Furthermore, we found that one of the genes identified in gene screens, urokinase plasminogen activator, is required for both CD95-induced motility and invasiveness. Our data suggest that elevated levels of CD95L found in cancer patients could contribute to increase tumorigenicity of tumor cells.

Results

Stimulation of CD95 on CD95 apoptosis-resistant tumor cells induces increased invasiveness

It has been previously suggested that sCD95L is very inefficient in inducing apoptosis in tumor cells (Schneider *et al*, 1998; Tanaka *et al*, 1998) and although it was shown that sCD95L can activate other nonapoptotic pathways (Ahn *et al*, 2001) the range of biological responses of tumors cells to this stimulus is unknown. It is well established that as part of carcinogenesis tumors often either inactivate the apoptosis-inducing activity of CD95 by downregulating or inactivating CD95 or its proapoptotic signaling molecules, or

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by upregulation of antiapoptotic proteins such as Bcl-2 or c-FLIP (Peter *et al*, 2003). Since increased expression of sCD95L is frequently found in cancer patients and is associated with various forms of cancer (Owen-Schaub *et al*, 2000), we asked whether CD95 stimulation of CD95 apoptosis-resistant tumor cells provokes protumorigenic responses.

To address directly this question, we performed *in vitro* invasiveness assays (Figure 1). In this assay, tumor cells were placed in the top of two chambers separated by a Matrigel-coated membrane (pore size 8 μm) in serum-free medium. The bottom chamber contained 10% FCS. The anti-CD95 stimulus (either anti-CD95 antibody or CD95 ligand) was added to both the top and bottom chambers. Any response by tumor cells in this assay was therefore the result of stimulation of surface CD95 on the tumor cell ultimately triggering a program of increased motility and invasiveness through the basement membrane mimetic Matrigel.

To test the effects of triggering CD95 on apoptosis-resistant tumor cells under controlled conditions, we first tested MCF7-Fas-Bcl-x_L(FB) breast carcinoma cells, which express high levels of CD95 (Algeciras-Schimnich *et al*, 2003) and are rendered apoptosis resistant by the antiapoptotic protein Bcl-x_L (Stegh *et al*, 2002). These cells are a model for a tumor cell that has acquired apoptosis resistance through upregulation of a Bcl-2 family member (which is frequently found in human tumors). As expected in apoptosis-sensitive MCF7-Fas control cells, CD95 stimulation did not promote invasiveness (Figure 1A) but induced apoptosis (data not shown). Unstimulated MCF7(FB) cells also exhibited low invasiveness. However, when these cells were exposed to either the agonistic anti-CD95 mAb anti-APO-1, leucine zipper-tagged CD95L (LzCD95L) or sCD95L, they migrated through Matrigel. This response was not limited to MCF7(FB) cells since it was also observed in a naturally CD95-resistant ovarian tumor cell line SK-OV-3. Again all three CD95-specific stimuli induced increased invasiveness of SK-OV-3 cells (Figure 1A). Stimulation of CD95 on apoptosis-resistant tumor cells could induce increased proliferation, which might interfere with the quantification of the invasiveness assay. To determine whether tumor cells responded to CD95 with increased proliferation, we counted MCF7(FB) cells plated in Boyden chambers under the exact conditions of an invasiveness assay and counted the total number of cells (invaded and noninvading) at different time points in the absence or presence of LzCD95L (Figure 1B). The cells did not respond to triggering with increased proliferation, indicating that the increased numbers of cells detected in an invasiveness assay on the bottom of the Matrigel-coated membrane was solely due to increased invasiveness and not accelerated growth. This result was also confirmed by performing a 3-day MTS assay and by growing and counting cells under standard conditions (data not shown). We next determined the percentage of cells that responded to stimulation of CD95 with increased motility (as determined in a migration assay, without Matrigel) and/or invasiveness (Figure 1C). We found that the percentage of cells that migrated through the membrane after CD95 stimulation increased from 2 to 12.9% and the percentage of cells invading from 0.5 to 4.8%. The increase in invasiveness induced by CD95L was not limited to MCF7(FB) or SK-OV-3 cells. A total of 14 other cell lines also responded with increased invasiveness when stimulated through CD95 (Figures 1D and 5C). Again we determined that these responses were not due to an increase in cell proliferation but due to increased invasiveness, by performing 3-day MTS proliferation assays with all tested cells (data not shown).

Tumor cells most likely encounter CD95L in two different forms *in vivo*: sCD95L in the serum and membrane-bound (m)CD95L, for example, on tumor-infiltrating lymphocytes, proinflammatory cells or stromal cells. This could potentially involve cell–cell interaction between tumor cells and CD95L-expressing cells. To test whether cell-bound human mCD95L could trigger an increase in motility and/or invasiveness of CD95 apoptosis-resistant tumors, we incubated MCF7(FB) cells with a cell line expressing authentic unmodified human mCD95L (CT26L; Aoki *et al*, 2001). When mixed at a ratio of 3.3:1, CT26L cells induced maximal motility of MCF7(FB) cells (Figure 1E) although significant increase of motility was observed when mixed at a ratio of 1:1 or 6.7:1. The activity of the CT26L cells was due to the human mCD95L that they express since it could be significantly inhibited by a neutralizing anti-human CD95L mAb NOK-1. CT26L cells also induced invasiveness, which was not observed with the parental CT26 cells that do not express human CD95L (Figure 1E). In summary, any form of stimulation of CD95 tested, including sCD95L and physiological, unmodified mCD95L, can induce an increase in motility and invasiveness of CD95-resistant tumor cells.

To test directly the form of CD95L that tumor cells might encounter *in vivo*, we incubated MCF7(FB) cells with human peripheral blood mononuclear cells (PBMCs) (Figure 1F). Resting PBMCs did not show an effect on the invasiveness of the tumor cells. However, when the tumor cells were mixed with PBMCs that had been activated with phytohemagglutinin (24 h) followed by stimulation with interleukin 2 (IL-2) for 5 days, which selectively activates T lymphocytes, an increase in invasiveness could be detected (Figure 1F). This activity of the activated PBMCs was primarily due to CD95L since it could substantially be blocked by the CD95L-neutralizing antibody.

The majority of CD95 apoptosis-resistant tumor cells respond to anti-CD95 stimulation with activation of NF-κB

Two independent Affymetrix gene screens of MCF7(FB) cells stimulated through CD95 identified a number of NF-κB target genes that were upregulated in CD95-stimulated tumor cells (see Figure 6B). Electromobility shift assays (EMSAs) confirmed activation of NF-κB in MCF7(FB) cells when stimulated through CD95 by both the agonistic anti-CD95 antibody anti-APO-1 and two CD95 ligand preparations (Figure 2A). Activation of NF-κB was first detectable 1 h after CD95 stimulation and it progressively increased reaching levels almost as high as those induced by TNFα (Figure 2A). Activation of NF-κB was likely a direct effect as CD95-induced activation of NF-κB was not prevented by cycloheximide pretreatment (Supplementary Figure 1A). Supershift analysis identified the canonical p50/p65 heterodimer as the predominant complex activated by CD95 (Supplementary Figure 1B).

To assess the significance of the activation of NF-κB in CD95-resistant tumor cells, we monitored NF-κB activation in cell lines of the antitumor drug screening panel of the NCI (NCI60). We previously determined that the majority of these

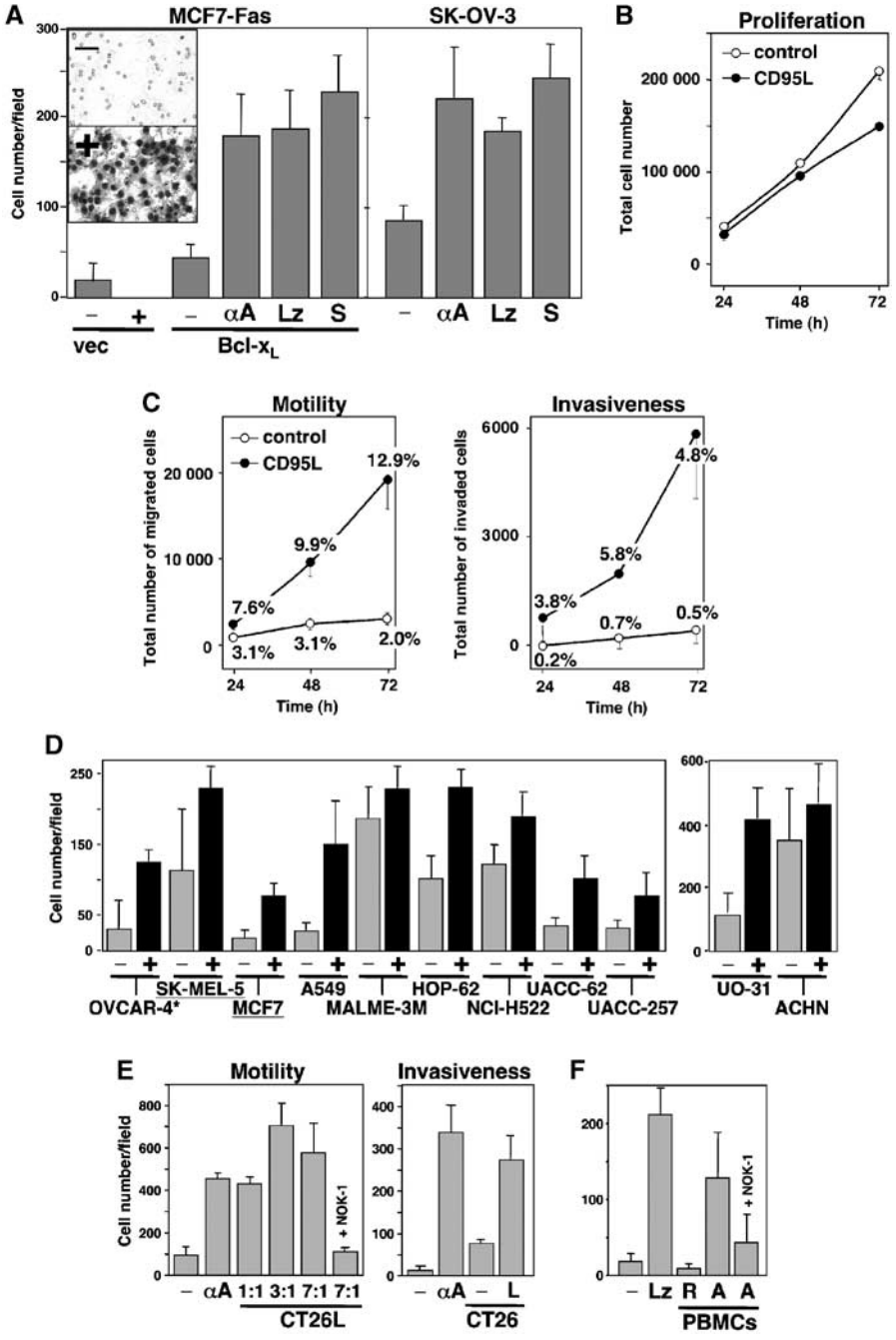


Figure 1 Stimulation of CD95 in CD95 apoptosis-resistant tumor cells induces invasiveness. (**A**) A 3-day *in vitro* invasiveness assay of MCF7(FB) and SK-OV-3 cells incubated with anti-APO-1 (αA), LzCD95L (Lz) or sCD95L (S).The inset shows fixed and stained cells that migrated through a Matrigel-coated membrane unstimulated (−) or stimulated (+) with anti-APO-1. (**B**) Cell proliferation in MCF7(FB) cells following stimulation with LzCD95L was determined by counting cells each day following trypsinization. (**C**) Increase in the total number of migrating (left) and invading (right) MCF7(FB) cells upon stimulation with LzCD95L. Percentages of cells compared to the total number of cells in the well are given. (**D**) Invasiveness assay of apoptosis-resistant cells (left panel) following stimulation with anti-APO-1 (asterisk), LzCD95L (underlined) or sCD95L. Two apoptosis-sensitive Type I tumor cell lines that are resistant to sCD95L also responded with increased invasiveness (right panel). Cell proliferation was controlled by MTS assay to ensure that the increase in cell number was not due to the increase in proliferation. Selected cell lines were also counted as described in (B). (**E**) Invasiveness (left) and motility (right) assay of MCF7(FB) cells incubated with anti-APO-1 (αA), CT26L or CT26 cells at the ratios 1:1, 3.3:1 (labeled as 3:1) and 6.7:1 (labeled as 7:1). The ratio of CT26 to MCF7(FB) cells in the invasiveness assays was 6.7:1. (**F**) PBMCs that had been activated for 16 h with PHA, followed by 6 days with IL-2 (A), or were not stimulated with either PHA or IL-2 (R) were cocultured with MCF7(FB) cells at a 6.7:1 ratio in an invasiveness assay. Where indicated, the PBMCs were preincubated with NOK-1. NOK-1 was maintained in the culture at 10 μg/ml.

cell lines are resistant to CD95-mediated apoptosis, and many of them express high levels of surface CD95, suggesting that they developed mechanisms to withstand apoptotic CD95 stimulation (Algeciras-Schimnich *et al*, 2003; data not shown). A total of 13 of these cell lines were selected randomly and examined for NF-κB activation upon CD95 triggering. Most cell lines (11/13, 85%) activated NF-κB in response to one or more of the CD95-specific stimuli with

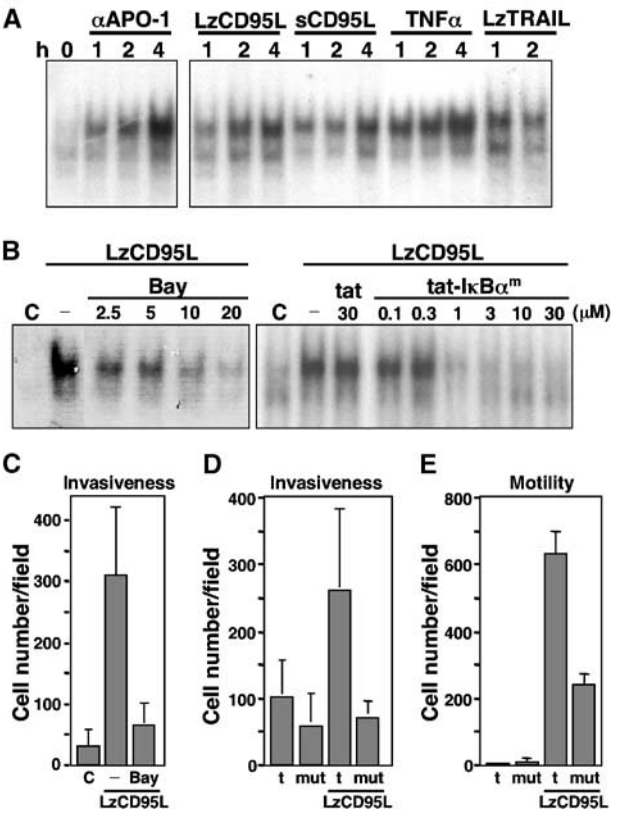


Figure 2 CD95-mediated activation of NF-κB contributes to *in vitro* invasiveness. (**A**) EMSA analysis of NF-κB activation of MCF7(FB) cells stimulated through CD95 or cells treated with TNFα or LzTRAIL. (**B**) EMSA analysis of NF-κB activity of cell extracts of MCF7(FB) cells stimulated with LzCD95L for 4 h and after pretreatment with BAY 11-7082 or either GST-TAT-IκBα^{mut} or GST-TAT control (tat). (**C**) MCF7(FB) cells were treated with 2.5 μM BAY 11-7082 and subjected to invasiveness assay. The NF-κB inhibitor was maintained in the culture for the duration of the assay. (**D**) Invasiveness assay and (**E**) migration assay of MCF7(FB) cells incubated with GST-TAT-IκBα^{mut} (mut) or GST-TAT control (t).

only two lines exhibiting no CD95-induced NF-κB complexes (Supplementary Figure 2A).

Our recent analysis of the NCI60 cells demonstrated that Type I but not Type II cells (both of which are apoptosis sensitive to crosslinked agonistic anti-CD95 antibodies or highly aggregated CD95L) are resistant to the toxic effects of soluble CD95L (sCD95L) (Algeciras-Schimnich *et al*, 2003). We now demonstrate that sCD95L induces NF-κB activation in the prototype Type I tumor cell lines SKW6.4 and H9 (Supplementary Figure 2B), and in seven of eight cell lines randomly picked from the 11 NCI60 we identified as Type I cells (Supplementary Figure 2C). In summary, the majority of all CD95 apoptosis-resistant cell lines tested, and even the CD95-sensitive Type I cell lines stimulated with sCD95L, from a variety of histological origins responded to CD95 stimulation with activation of the NF-κB pathway. It has been demonstrated previously that the NF-κB pathway can block apoptosis induced by death receptors as well as promote tumor progression (Orlowski and Baldwin, 2002) and NF-κB has been shown to be activated in cells after triggering of CD95 (Ponton *et al*, 1996; Packham *et al*, 1997). However, these studies focused on only a few cell lines and the functional relevance of this activation remained unknown. Our data now suggest that activation of NF-κB may be involved in the increased invasiveness of CD95-stimulated tumor cells.

CD95-induced invasiveness requires activation of NF-κB

We next tested whether the CD95-induced invasiveness was dependent on activation of NF-κB. Incubation of MCF7(FB) cells with the NF-κB inhibitor Bay 11-7082 (Mori *et al*, 2002) or a cell-permeable glutathione *S*-transferase (GST) fusion protein of a nondegradable IκBα mutant protein (GST-TAT-IκBα^{mut}) blocked activation of NF-κB (Figure 2B) and CD95-induced invasiveness (Figure 2C and D). A migration assay demonstrated that activation of NF-κB was also at least in part responsible for increased motility (Figure 2E).

CD95L is more potent in inducing invasiveness than TNFα or TRAIL

The data so far suggested that activation of NF-κB was involved in CD95-induced motility and invasiveness. CD95 triggering resulted in activation of the canonical NF-κB p50/p65 heterodimer that is also activated by TNFα stimulation (Figure 2A; Tang *et al*, 2001). However, in both the EMSA analysis and gene reporter assays using a κB-driven CAT reporter construct, TNFα was more active in inducing NF-κB than CD95L (Figure 3A) without toxicity (as monitored by MTS assays, data not shown). We therefore tested the activity of TNFα and the CD95L-related death ligand TRAIL in inducing invasiveness of MCF7(FB) cells (Figure 3B). TNFα and TRAIL only marginally induced invasiveness in these cells. When compared side by side, both TNFα and LzCD95L induced activation of the IκB kinase complex as evidenced by activation of its subunit IKKβ (Figure 3C). The activation of NF-κB through CD95 was somewhat delayed compared to TNFα-induced NF-κB activation, suggesting a different pathway upstream of the IKK complex. We identified NEMO to be an essential component in the pathway of both death receptors since a peptide that blocks interaction of NEMO with the IKK complex (May *et al*, 2000) efficiently prevented activation of NF-κB as evidenced by both EMSA (Supplementary Figure 1C) and an ELISA-based assay that detects activated p65 (Supplementary Figure 1D). We therefore determined the role of the receptor proximal component of the TNF pathway, RIP, in CD95-mediated activation of NF-κB by testing RIP-deficient Jurkat cells. RIP was required for TNFα-induced activation of NF-κB but dispensable for CD95-induced NF-κB activation (Figure 3D). In summary, our data indicate that NF-κB activation is required but not sufficient for tumor cells to demonstrate increased motility and invasiveness, suggesting involvement of other pathways that either emanate in the pathway somewhere upstream of the IKK complex or that act in parallel to the NF-κB pathway.

Requirement for the Erk signaling pathway for CD95-induced invasiveness

CD95 activates the three major MAP kinase pathways under certain conditions (Toyoshima *et al*, 1997; Desbarats *et al*, 2003). Western blot analyses using pairs of antiphospho site-directed antibodies and their corresponding panspecific antibodies against the three major components of the MAPK pathways, Erk1/2, JNK1/2 and p38, determined that all three MAP kinases are activated upon stimulation of CD95 in MCF7(FB) cells (Figure 4A). Treatment of MCF7(FB) cells

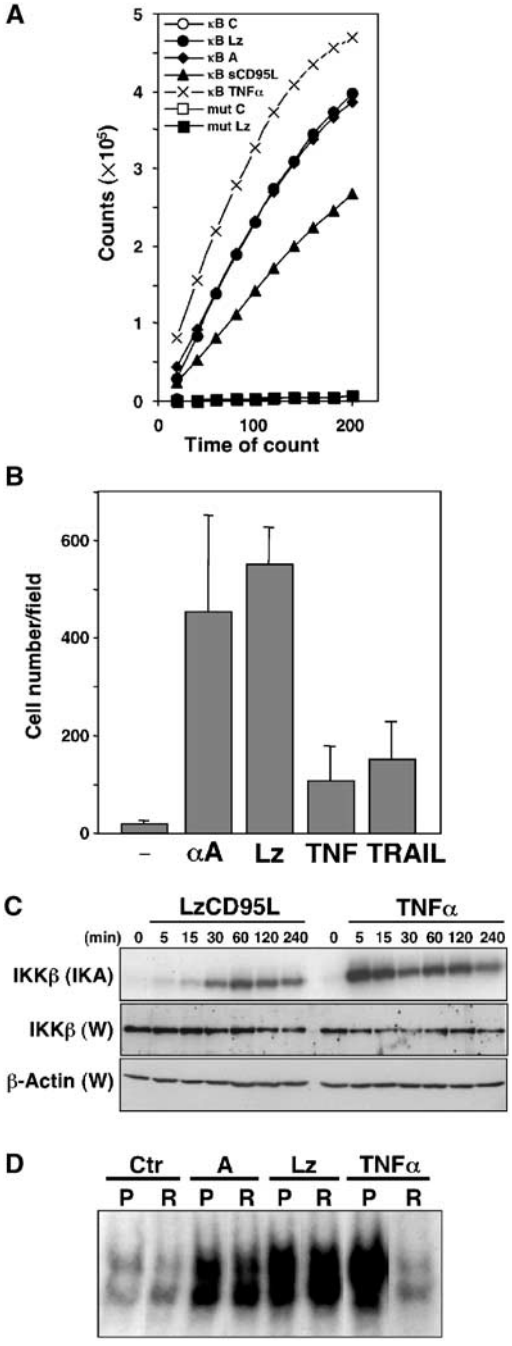


Figure 3 CD95L is more efficient than TNFα or TRAIL in inducing increased motility and invasiveness of MCF7(FB) cells. (**A**) CAT reporter gene analysis of MCF7(FB) cells transfected with an NF-κB promoter (κB) or mutated NF-κB promoter (mut) and treated with LzCD95L (Lz), anti-APO-1 (A), sCD95L or TNFα (for 4 h) and assayed over time. (**B**) A 3-day invasiveness assay of MCF7(FB) cells stimulated with 1 μg/ml LzCD95L, 1000 U/ml TNFα or 1 μg/ml LzTRAIL. (**C**) MCF7(FB) cells were incubated for the indicated times with LzCD95L or TNFα. Cell lysates were subjected to IKKβ immunoprecipitation followed by *in vitro* kinase assay (IKA) using GST-IκBα as the substrate. Phosphorylated IκBα was resolved and exposed to film. W, Western blot. (**D**) Jurkat cells deficient for RIP expression stimulated with either anti-APO-1/zVAD-fmk (A), LzCD95L/zVAD-fmk (Lz) or TNFα for 4 h were subjected to EMSA. P, parental Jurkat cells; R, RIP-deficient cells.

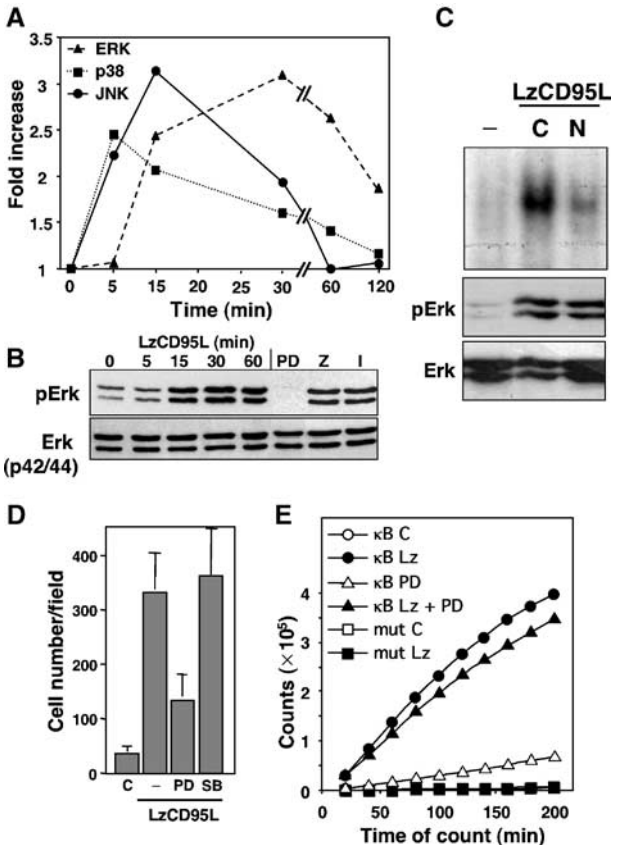


Figure 4 All three MAP kinase pathways are activated by LzCD95L in MCF7(FB) cells and activation of Erk is involved in CD95-induced invasiveness. (**A**) MCF7(FB) cells were stimulated with LzCD95L for indicated times and lysates of the cells were subjected to Western blot analysis for phosphorylation of indicated MAP kinases. Ratios of intensity of phosphorylated versus unphosphorylated kinase bands were determined by densitometry. (**B**) Kinetics of phosphorylation of p42/p44 Erk in LzCD95L-treated MCF7(FB) cells. As indicated (30 min stimulated), cells were preincubated with the following inhibitors: 20 μM PD98059 (PD), 40 μM zVAD (Z) or 40 μM zIETD (I). (**C**) MCF7(FB) cells were preincubated for 3 h with 140 μM of control peptide (C) or NEMO binding domain (NBD) peptide (N) and then stimulated for 2 h with LzCD95L. Nuclear extracts were purified and subjected to an EMSA (top panel). In parallel, cells were lysed and phosphorylation of Erk was analyzed by Western blotting (bottom panels). (**D**) Invasiveness assay of MCF7(FB) cells preincubated with PD98059 or p38 inhibitor SB203580. Effectiveness of the SB inhibitor was tested using phosphospecific antibodies (not shown). (**E**) CAT reporter assay of LzCD95L-stimulated MCF7(FB) cells following preincubation with PD98059.

with LzCD95L induced activation of the Erk1/2 MAP kinases, which was completely prevented by the MEK1/2 inhibitor PD98059 (Figure 4B). Interestingly, addition of caspase inhibitors did not inhibit Erk activation, suggesting that these events occur independently. This conclusion is consistent with the finding that activation of Erk through CD95 is independent of its death domain (DD) (Desbarats *et al*, 2003). When we treated MCF7(FB) cells with the NEMO-inhibiting peptide, which resulted in a profound inhibition of NF-κB activation in CD95-stimulated cells (Figure 4C, see also Supplementary Figure 1C), no effect was seen on activation of Erk, indicating that CD95-induced activation of Erk is also independent of activation of NF-κB. To determine the functional consequences of Erk activation, we tested the effects of PD98059 in the *in vitro* invasiveness assay (Figure 4D). PD98059 treatment significantly inhibited invasiveness, indicating that like activation of NF-κB Erk

activation contributes to CD95-induced invasiveness. The p38 inhibitor SB203580 had no effect on invasiveness (while it completely abolished activation of p38, data not shown), excluding a role of p38 in the CD95-induced invasiveness (Figure 4D). A contribution of JNK kinases to the *in vitro* invasiveness could not be determined since inhibition of JNK reduced viability of these cells (as assessed by an MTS assay, data not shown). Additionally, we did not detect activation of PI3 kinase in CD95-stimulated MCF7(FB) cells, and the PI3 kinase inhibitors wortmannin and LY294002 did not inhibit CD95-induced invasiveness (data not shown). To test whether the MAP kinase pathway acts independently or in conjunction with NF-κB, we determined the effects of PD98059 on an NF-κB-driven CAT reporter assay. PD98059 caused only a minor reduction of reporter gene activity (Figure 4E).

Involvement of active caspase-8 in CD95-induced invasiveness

We recently demonstrated that MCF7(FB) cells process caspase-8 despite their resistance to CD95-mediated apoptosis (Stegh *et al*, 2002). Active caspase-8 could therefore play a role in the increased invasiveness observed upon CD95 stimulation. Treatment of these cells with the oligo-specific caspase inhibitor zVAD-fmk or the caspase-8 selective inhibitor zIETD-fmk completely blocked CD95-induced invasiveness in MCF7(FB) cells (Figure 5A). Inhibition of caspases had no effect on CD95-induced increase in NF-κB DNA binding (data not shown). Furthermore, it also did not prevent NF-κB-dependent transcriptional activation (Figure 5B), suggesting that caspase-8 acts downstream or in parallel to the transcriptional activation induced by activated NF-κB.

To determine the contribution of both the MAP kinase pathway and caspase-8 activation to the increase in invasiveness in untransfected tumor cells, we pretreated six of the NCI60 cells with either PD98059 or zIETD-fmk followed by stimulation with sCD95L (Figure 5C). In all cases, we detected increased invasiveness, which was blocked by inhibition of MAP kinases. In contrast, inhibition of caspase-8 significantly inhibited invasiveness of most but not all cells. Invasiveness of EKVX and TK-10 cells was critically dependent on activation of caspase-8, whereas in untransfected MCF7 cells caspase-8 activation did not play a significant role in this process. In summary, multiple pathways are activated and at least three of them, activation of caspase-8, NF-κB and Erk1/2, independently contribute to the response of apoptosis-resistant tumor cells to CD95 stimulation to varying extents. This stimulation results in increased motility and invasiveness, indicating a high degree of complexity of regulation of this process.

Transcription factors activated by CD95

Our data suggest that CD95-mediated invasiveness of tumor cells involves differing degrees of at least three different signaling pathways. These pathways appear to act independently, at least at the level at which their activation was monitored. These data can be interpreted in two ways: the three pathways could either act independently resulting in activation of multiple transcription factors regulating expression of multiple genes or, at the other extreme, a very limited set of transcription factors may regulate expression of a few genes with complex promoter regulation, requiring activation of more than one signaling pathway. To determine which transcription factors are activated in CD95-stimulated cells, we performed a Transignal Protein/DNA array analysis (Bafica *et al*, 2004) with MCF7(FB) cells stimulated with LzCD95L (Figure 6A). Of the 54 transcription factors assayed, four were found to be activated in a CD95-dependent fashion. Consistent with our analysis of the signaling pathways, we detected the activation of the AP1, AP2 and NF-κB transcription factors. In addition, we detected activation of CREB. Activation of AP1 and CREB was transient peaking at 4 h, whereas activation of NF-κB was more sustained.

CD95 stimulation of CD95 apoptosis-resistant MCF7(FB) cells results in transcriptional upregulation of antiapoptotic and potentially tumorigenic genes

To test whether triggering of CD95 induced transcriptional upregulation of genes that would be consistent with a protumorigenic nature of the response observed with apoptosis-resistant tumor cells and the activation of a limited set of transcriptional activators, we treated MCF7(FB) cells for 8 h with anti-APO-1 and subjected their mRNA to a gene array analysis using the Affymetrix U133A/U133B gene chip set covering 33 000 distinct human genes (data not shown). Of these genes, 121 were induced greater than two-fold and the induction of nine of these genes was confirmed by real-time and/or RT–PCR (Supplementary Figure 3). LzCD95L was similar in its gene-inducing activity (Supplementary Figure 3B). To confirm the results of this screen, we performed a second independent screen under the same conditions using a different clone of MCF7(FB) cells (with similar ability to invade when stimulated through CD95). This second screen was performed with only chip A since chip B did not contain any of the known genes activated by CD95 (data not shown). When the data of the two independent chip analyses were compared, only 17 distinct genes were upregulated more than two-fold in both analyses (Figure 6B). Of these 17 genes, 11 were known NF-κB target genes, again supporting the important role of NF-κB activation of nonapoptotic signaling of CD95 in apoptosis-resistant tumor cells. When the threshold was lowered to genes that were at least 1.5-fold upregulated in both screens, 56 genes were found to be induced by CD95 stimulation, with 27 genes known targets of NF-κB (Supplementary Figure 4). To determine the contribution of the activation of the MAK kinase and NF-κB pathways on gene activation more comprehensively, the second screen was also performed with cells treated with the inhibitors of these two pathways (Figure 6B). Inhibition of the MAP kinase pathway affected expression of a few genes consistent with the detection of AP1 activation (Figure 6A). However, induction of most genes was severely reduced by inhibiting activation of NF-κB, indicating that NF-κB was responsible for most of the transcritionally induced CD95-responsive genes. We did not find evidence for a major requirement of caspases for transcriptional regulation, as neither zVAD nor zIETD significantly inhibited induction of CD95-induced genes (Figure 6C).

Urokinase plasminogen activator (uPA) is involved in CD95-induced invasiveness

Among the genes specifically induced in CD95-stimulated cells in both screens was uPA, one of the best-established marker proteins of tumor motility and invasiveness (Kim *et al*,

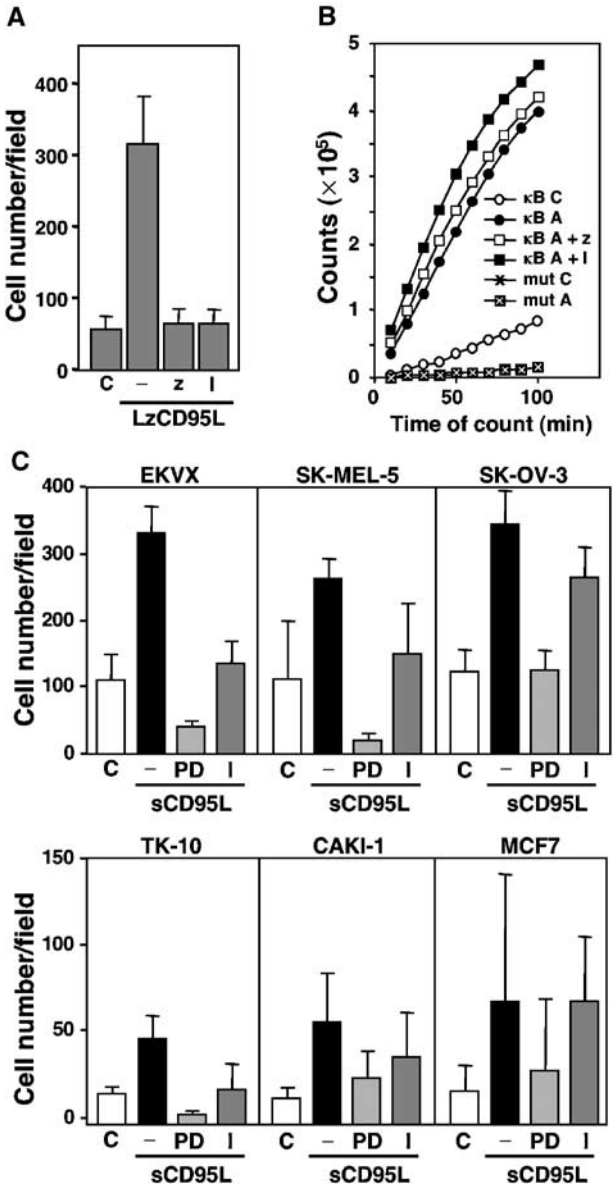


Figure 5 CD95-induced invasiveness requires caspase-8 activity. (**A**) Invasiveness assay of MCF7(FB) cells in the presence of zVAD-fmk (z) or zIETD-fmk (I). (**B**) κB-driven CAT reporter assay of MCF7(FB) cells preincubated with zVAD-fmk (z) or zIETD-fmk (I). (**C**) Invasiveness assay of NCI60 cells stimulated through CD95 in the presence of 20 μM PD98059 (PD) or 40 μM zIETD-fmk (I).

1998). We also detected the uPA precursor mRNA in these cells (Figure 6B). We therefore tested both the activity of a neutralizing anti-uPA antibody as well as a selective uPA inhibitor on CD95-induced invasiveness and motility of MCF7(FB) cells (Figure 7). Both uPA inhibitory reagents profoundly blocked CD95-induced motility and invasiveness, suggesting that uPA is a major mediator of the novel activity of CD95 in CD95 apoptosis-resistant tumor cells.

Discussion

In this report, we have demonstrated that stimulation of CD95 apoptosis-resistant tumor cells by any form of CD95L triggers signaling pathways that can increase their motility and invasiveness. This activity of CD95 is not limited to certain forms of cancers, since tumor cells representing malignancies from ovary (OVCAR-4, SK-OV-3), breast (MCF7), skin (MALME-3M, SK-MEL-5, UACC-62, UACC-257), lung (A549, EKVX, HOP-62, NCI-H522) and kidney (ACHN, CAKI-1, TK-10, UO-31) all showed similar responses to CD95 stimulation. CD95 ligand has not been previously reported to have this effect on tumor cells. The only report linking CD95 with migrational activity came from an analysis of renal tubular epithelial cells treated with agonistic anti-CD95 antibodies (Jarad *et al*, 2002). However, this form of migration was shown to depend on CD95-induced expression of $\alpha_v\beta_8$ integrin (which was not found to be induced in MCF7 cells, also used in our study) and correlated with migration to vitronectin.

We determined that at least three pathways are activated by CD95, which elicit the nonapoptotic responses in tumor cells: activation of caspase-8, Erk1/2 and NF-κB. These pathways regulate gene expression, increased invasiveness and increased motility. We found that caspase-8 activity is required for the increased invasiveness of certain but not all tumor cells, and inhibition of caspase-8 completely blocked CD95-induced invasiveness in some cells without interfering with the activation of NF-κB or any of the MAP kinase pathways, Erk1/2 (Figure 6A), JNK or p38 (data not shown), in MCF7(FB) cells. Although we cannot completely exclude a role for caspase-8 in the induction of genes, our analysis rather suggests that caspase-8 acts downstream of gene induction and might be involved in the migration of tumor cells (Watanabe and Akaike, 1999).

Activation of Erk1/2 through CD95 has recently been shown to be required for neurite outgrowth of certain neurons (Desbarats *et al*, 2003), and we now demonstrate that inhibition of this pathway substantially inhibits CD95-induced invasiveness. Interestingly, like caspase-8, inhibition of Erk1/2 did not interfere with activation of NF-κB, the third pathway triggered by CD95 that is important for CD95-induced invasiveness, nor did inhibition of NF-κB have any effect on activation of Erk. The data presented therefore indicate that these three major pathways act in parallel. Gene chip analyses suggested a protumorigenic activity of NF-κB through transcriptional activation of genes, consistent with the role of NF-κB in tumor development (Lin and Karin, 2003). However, like the other pathways, activation of NF-κB was found to be required yet not sufficient to mediate the nonapoptotic effects of CD95.

Our data suggest that CD95 activates NF-κB through the canonical pathway of activation of the IKK complex (Figure 3C and Supplementary Figure 1C and D) and degradation of IκBα (not shown). It was therefore surprising that the related death ligand TNFα, which activates NF-κB more efficiently than CD95L, and TRAIL (which also induced NF-κB activation in MCF7(FB) cells; Figure 2A) induced invasiveness in CD95 apoptosis-resistant cells much less efficiently than CD95L. This result is consistent with a model in which multiple signaling pathways must be activated in parallel (and not NF-κB alone) to induce the maximal effect and could be due to the differences in upstream signaling events that link the death receptors to the IKK complex. We found a difference in the activation of NF-κB between the two death receptors by confirming the requirement of RIP for NF-κB activation by TNFα (Kelliher *et al*,

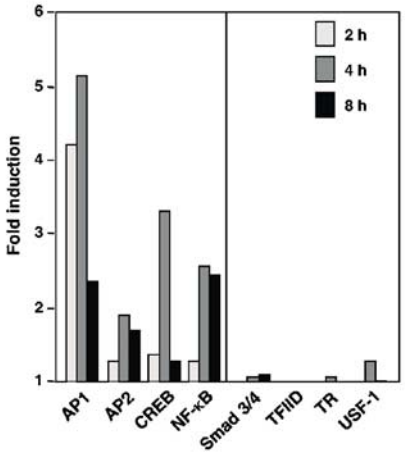
1998) but demonstrating that RIP, at least in Jurkat T cells, is not required to activate NF-κB through CD95.

The CD95 apoptosis resistance in the tumor cells in our study likely represents a multitude of different mechanisms. Tumors can acquire resistance to CD95 stimulation by upregulation of antiapoptotic Bcl-2 family members (Scaffidi *et al*, 1998), upregulation of the caspase-8 protease inactive homolog c-FLIP$_L$ (Tschopp *et al*, 1998), or by silencing or deleting the caspase-8 gene (Mandruzzato *et al*, 1997; Teitz *et al*, 2000). CD95 apoptosis resistance may also be dependent on mutations that render CD95 inactive or on the downregulation of CD95 expression (Muschen *et al*, 2000; Bullani *et al*, 2002; Reichmann, 2002). CD95 is primarily viewed as an apoptosis-inducing receptor. However, if this were the sole function of the receptor, one would expect to find tumors that either delete the CD95 gene or completely block its expression by promoter hypermethylation. However, a complete loss of CD95 expression has not been reported, suggesting that tumors derive an advantage from maintaining expression of CD95.

We have identified the serine protease uPA as a candidate gene for regulating the increased motility and invasiveness of CD95-stimulated tumor cells. After binding to the uPA receptor (uPAR), uPA cleaves plasminogen forming plasmin, which can cleave extracellular matrix proteins, and activates a number of metalloproteinases in turn resulting in the cleavage of further proteins such as collagen (Duffy, 2004). It has been shown that uPA is absolutely required for intravasation, the most important step in the multistep process of tumor metastasis (Kim *et al*, 1998). It has been recognized that uPA can also induce increased motility in tumor cells (Yebra *et al*, 1996). uPA has also been shown to be a predictor for metastasis formation in various human cancers such as breast, stomach, colorectal, bladder, ovary, endometrium and brain cancer (Schmitt *et al*, 1997; Duffy *et al*, 1999). Given the fact that we detected increased invasiveness in numerous CD95 apoptosis-resistant tumor cells derived from multiple tissues, uPA is an excellent candidate gene for the general regulation of CD95-induced invasiveness. Furthermore, uPA is a well-established NF-κB target gene (Hansen *et al*, 1992; Newton *et al*, 1999), consistent with the absolute requirement of NF-κB activation for CD95-mediated invasiveness. Future experiments will address the question of whether uPA is generally activated in CD95-stimulated tumor cells.

Our data provide the mechanistic basis for a recent report that demonstrated a tumor-promoting function of CD95. Increased growth of a lung carcinoma cell line stably expressing CD95 was observed *in vivo* in syngeneic mice when compared to *gld* mice lacking expression of functional CD95L (Lee *et al*, 2003). The tumor-promoting activity of CD95 required the CD95 intracellular domain and therefore likely

A



B

Accession no	Fold ind. scr. 1/scr. 2	Gene	% induction in the presence of PD	Bay
NM_000201.1	8.0/13.9	**ICAM-1**	93.5	0.8
AI078167	4.0/7.0	**IκBα**	81.3	33.0
AI738896	5.3/6.5	**A20**	100.0	18.9
NM_002198.1	4.6/5.7	**IRF1**	107.1	18.9
AL050388.1	6.1/4.6	**MnSOD** (mito)	162.5	35.3
U69563	2.3/4.6	Rho6/Rnd1	43.6	24.8
NM_002658.1	4.3/4.3	**uPA**	70.6	46.6
U83981	2.0/4.3	GADD34	37.8	35.5
NM_005195.1	3.5/4.0	CEBPδ	81.2	30.8
NM_004428.1	3.3/4.0	**Ephrin A**	70.8	26.8
U37546.1	3.7/3.3	**cIAP2**	161.5	53.5
NM_015887.1	2.6/3.0	Hypoth. prot.	66.0	30.8
NM_003407.1	2.0/3.0	Tristetraprolin	87.7	43.8
NM_004556.1	2.6/2.8	**IκBε**	75.6	46.6
NM_030952.1	2.8/2.8	**SNARK**	81.5	30.9
NM_002341.1	3.3/2.5	**LTβ**	115.0	46.7
U71088.1	3.5/2.0	MEK5c	57.5	40.5
K03226.1	nd/7.5	**Prepro uPA**	37.9	21.7

C

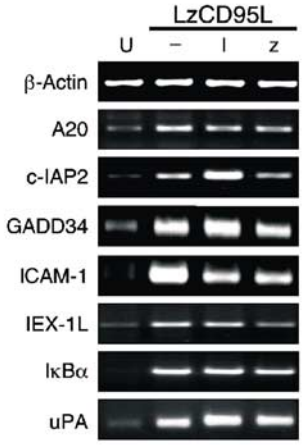


Figure 6 CD95 stimulation activates a transcriptional program. (**A**) MCF7(FB) cells were stimulated with LzCD95L as indicated and subjected to a Transignal DNA/Protein array to determine activation of transcription factors. The assay was performed three times and representative data are shown. Left, transcription factors that were activated in all three assays; right, transcription factors that were not significantly activated in any of the three assays. (**B**) MCF7(FB) cells were stimulated with anti-APO-1 and protein A for 8 h followed by isolation of RNA and gene chip analysis. The screen was performed twice (scr. 1 and scr. 2) using different MCF7(FB) clones. Only genes induced more than two-fold in both screens are shown. Prepro uPA could not be detected in the first screen due to a bad chip area. If a gene appeared more than once only the more highly induced signal is shown. Cells were treated with PD98059 and BAY 11-7082 in the second screen and percent induction of genes in the presence of these inhibitors is indicated. Genes that are inhibited more than 25% are shown in pink and more than 50% in red. (**C**) Semiquantitative RT–PCR of 8 h stimulated MCF-7(FB) cells treated with 40 μM zIETD-fmk (I) or zVAD-fmk (z) for the genes indicated. PCR products were subjected to agarose gel electrophoresis.

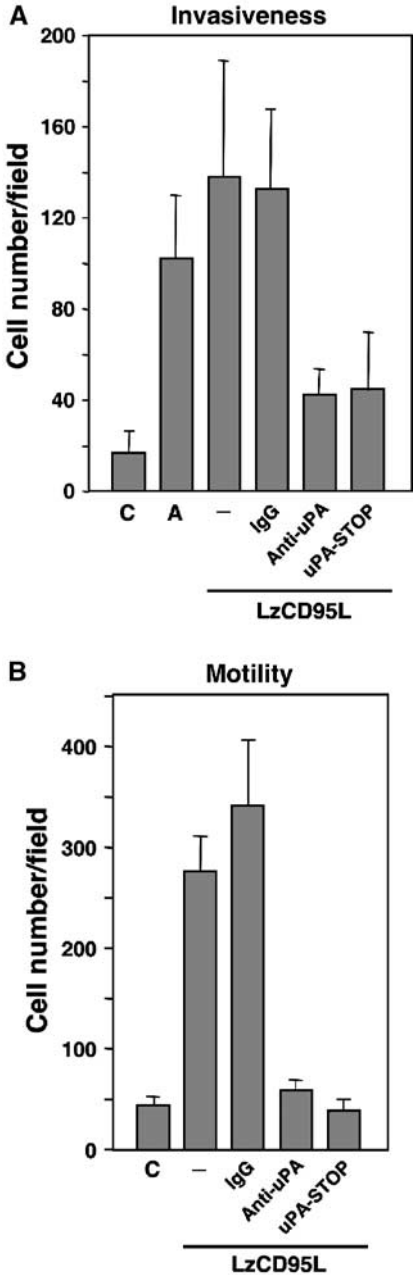


Figure 7 Urokinase plasminogen activator is a mediator of CD95-mediated invasiveness. Invasiveness (**A**) and motility (**B**) assay of MCF7(FB) cells incubated simultaneously with 1 μg/ml of LzCD95L and 20 μg/ml anti-uPA mAb or 20 μM uPA-STOP. The anti-uPA mAb was added each day. C, control; A, anti-APO-1; IgG, control IgG. The experiments shown are representative of three independent experiments. A 3-day MTS assay was performed to control for cell viability in the presence of these reagents, and no toxicity was observed (data not shown).

involved CD95 signaling pathways. Interestingly, no further CD95 stimulation was involved beyond that naturally occurring in the mice, consistent with the idea that tumor cells *in vivo* are likely exposed to CD95L that is expressed on tumor-infiltrating lymphocytes or in the serum. In a preliminary experiment, we demonstrated that CD95L produced by activated human PBMCs (>95% T cells) can induce invasiveness of apoptosis-resistant tumor cells. Increased CD95L levels have been reported to be associated with many human cancers, and elevated levels of CD95L can even serve as prognostic markers for disease progression and overall survival of patients (Supplementary Figure 5).

Elevated levels of CD95L produced by tumor or nontumor cells in cancer patients are currently thought to promote tumor growth in two ways: (1) Upregulation of CD95L by tumor cells has been implicated in killing attacking cytotoxic T lymphocytes (CTLs) resulting in specific immune suppression. This has been referred to as the 'tumor strikes back' theory (Walker *et al*, 1997). However, in many cases, this theory could not be confirmed, suggesting that tumors may benefit from elevated CD95L in ways other than through facilitating immune escape (Green and Ferguson, 2001). Furthermore, the systemic increase of soluble CD95L in the serum of many cancer patients would not be consistent with a specific immune suppression. (2) Increased levels of sCD95L in the serum of cancer patients has been suggested to antagonize the cytotoxic effects of membrane-bound CD95L, which could be used by CTLs to eliminate tumor cells (Suda *et al*, 1997; Mizutani *et al*, 2001). This model is based on reports that demonstrated that sCD95L is orders of magnitude less active than mCD95L (Schneider *et al*, 1998; Tanaka *et al*, 1998). However, we recently demonstrated that sCD95L is cytotoxic to Type II/epithelial tumor cells (Algeciras-Schimnich *et al*, 2003) and only Type I/mesenchymal cells are resistant to the toxic effects of sCD95L. We also demonstrated that Type I cells are much less sensitive to the apoptosis-inducing activities of mCD95L. An important question that had not been addressed is therefore whether CD95L has other activities on both tumor cells that are either completely apoptosis resistant or Type I tumor cells (which are viewed as the most CD95 apoptosis-sensitive cells since they are highly apoptosis sensitive to artificially aggregated CD95L or agonistic anti-CD95 Abs but in fact are virtually resistant to the effects of physiological sCD95L and have low sensitivity to mCD95L; Algeciras-Schimnich *et al*, 2003). We have now demonstrated that two of the most CD95-positive Type I tumor cell lines, H9 T cell leukemic cells and the B lymphoblastoid cell line SKW6.4, respond to stimulation by sCD95L with activation of NF-κB and identified four CD95-sensitive adherent Type I cells (ACHN, CAKI-1, TK-10, UO-31; interestingly all renal carcinomas) that respond to sCD95L with increased invasiveness (see Figures 1D and 5C).

Our data demonstrate for the first time that both soluble and membrane-bound CD95L elicit biological effects on multiple CD95 apoptosis-resistant tumor cells. They also suggest that tumors gain a direct advantage from an increase in CD95L levels by triggering multiple apoptosis-independent pathways resulting in upregulation of antiapoptotic and tumorigenic genes and/or an increase in motility and invasiveness. In humans, elevated levels of sCD95L have also been reported in cancer patients in response to chemotherapy (Kanda *et al*, 1999; Bewick *et al*, 2001), and widely used chemotherapeutic drugs such as adriamycin and 5-fluorouracil have been shown to cause secretion of active sCD95L by human tumor cells *in vitro* (Friesen *et al*, 1996) and a systemic increase of CD95L in mice *in vivo* (Eichhorst *et al*, 2001), respectively. Future experiments will investigate the possible tumorigenic activity of CD95L that is produced in response to exposure to chemotherapeutic drugs for CD95 apoptosis-resistant tumors. Our study could therefore affect the way tumor therapy is currently performed.

Materials and methods

Cells, reagents and general procedures

MCF7-Fas vector (MCF7(FV)) and MCF7-Fas-Bcl-x_L (MCF7(FB)) cells were cultured as described (Stegh *et al*, 2002). All other cell lines were cultured as described previously (Algeciras-Schimnich *et al*, 2003). The anti-CD95 mAb anti-APO-1 has been used before (Algeciras-Schimnich *et al*, 2003). The MAPK inhibitors, PD98059 and SB203580, and the caspase inhibitors, zIETD-fmk and zVAD-fmk, were purchased from Calbiochem (San Diego, CA). All other chemicals used were of analytical grade and were purchased from Sigma or Molecular Probes. Generation of sCD95L and LzCD95L and apoptosis assays were performed as described previously (Algeciras-Schimnich *et al*, 2003). TNFα was purchased from Peprotech and used at a concentration of 1000 U/ml. When inhibitors were used, cells were preincubated for 30 min (zVAD-fmk, zIETD-fmk, PD98059, SB203580) or preincubated for 2 h (GST-TAT-IκBα^{mut} or GST-TAT) and then incubated for 16 h with sCD95L or anti-APO-1 in 96-well plates. All inhibitors used were tested for toxicity on all cells used by performing an MTS assay as described (Algeciras-Schimnich *et al*, 2003) after a 3-day incubation.

In vitro invasiveness and motility assay

Biocoat Matrigel invasion chambers (BD Biosciences) containing 8 μm pore size positron emission tomograph membranes were cultured in 24-well plates. After rehydration of the Matrigel, 75 000 cells were added to the top chamber in serum-free media. The bottom chamber was filled with serum-containing media. Stimulus (anti-APO-1 antibody or CD95 ligand, TNFα, or LzTRAIL) or control supernatants were added to both chambers of each well. Cells were cultured for 72 h at 37°C in a 5% CO_2, humidified incubator. To quantify invasion, cells were removed from the top side of the membrane mechanically using a cotton-tipped swab and invading cells were fixed with methanol and stained with Giemsa stain and five representative fields were counted for each insert. For experiments in which cells were incubated with inhibitors, cells were plated with inhibitors for 2 h prior to addition of stimulus. GST-TAT-IκBα^{mut} and GST-TAT were added each day at 10 μM. To test for uPA involvement, MCF7(FB) cells were incubated simultaneously with 1 μg/ml of LzCD95L and either uPA-STOP (20 μM) or an anti-uPA B-chain mAb (20 μg/ml), two inhibitors of uPA activity (American Diagnostica Inc., Stamford, CT). CT26 cells were fixed for 5 min in 2% paraformaldehyde and washed extensively prior to addition to wells. PBMCs were isolated from buffy coats purchased from Life Source (Glenview, IL). Erythrocytes were removed by centrifugation on a gradient of lymphocyte separation media (BioWhittaker). Resulting PBMCs were then washed and incubated for 16 h with 2 μg/ml phytohemagglutinin (PHA)-L (Sigma) followed by 6 days with 25 U/ml recombinant IL-2 (Sigma). Resting cells were maintained in RPMI with 10% FBS. For antibody blocking experiments, stimulator cells were preincubated with NOK-1 (Pharmingen) for 45 min and cultures were maintained with 10 μg/ml NOK-1 for the duration of the experiment. For migration assays, Transwell insert chambers with 8 μm pores (Costar) were used. Cell proliferation in assays was determined by collection of cells from invasiveness chambers at different times with trypsin and counting using hemocytometer. Proliferation was also determined by plating and counting cells in 96-well plates. Finally, proliferation of all cell lines was also determined by incubating cells with MTS reagent (Promega) for 3 days according to the manufacturer's protocols, and absorbance was determined at 490 nm using a kinetic microplate reader (Molecular Devices, Menlo Park, CA).

In vitro kinase assay

MCF7(FB) cells were incubated with stimulus (1 μg/ml LzCD95L or 1000 U/ml TNFα) for the indicated time. *In vitro* kinase assays were performed as described previously (Mercurio *et al*, 1997). Briefly, cells were washed and lysed in lysis buffer (as above) containing 300 mM NaCl. Immunoprecipitation of 200 μg of protein was performed for 16 h at 4°C with anti-IKKβ antibodies. Immunoprecipitates were incubated with 2 μg of GST-IκBα for 30 min at 30°C in the presence of 1 μCi of [γ-^{32}P]ATP in kinase buffer (Mercurio *et al*, 1997). The reaction was then resolved on a 12% polyacrylamide gel, dried and exposed to film. Western blots were performed as control and blotted with antibodies for IKKβ and β-actin.

Transcription factor screen

Transcription factor activation was determined using the Transignal DNA/Protein array kit (Panomics, Redwood City, CA). MCF7(FB) cells were stimulated with LzCD95L as indicated followed by isolation of nuclear extracts according to the manufacturer's protocols.

Additional methods are described in Supplementary Figure 6.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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