Published in final edited form as:
Cancer Res. 2012 May 1; 72(9): 2373–2382. doi:10.1158/0008-5472.CAN-11-3369.

Expression of the p53 target CDIP correlates with sensitivity to TNF-alpha induced apoptosis in cancer cells

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Abstract

TNFα is a pleiotropic cytokine that signals for both survival and apoptotic cell fates. It is still unclear that the dual role of TNFα can be regulated in cancer cells. We previously described an apoptotic pathway involving p53→CDIP→TNFα that was activated in response to genotoxic stress. This pathway operated in the presence of JNK activation; therefore, we postulated that CDIP itself could sensitize cells to a TNFα apoptotic cell fate, survival or death. We show that CDIP mediates sensitivity to TNFα-induced apoptosis, and that cancer cells with endogenous CDIP expression are inherently sensitive to the growth suppressive effects of TNFα *in vitro* and *in vivo*. Thus, CDIP expression correlates with sensitivity of cancer cells with TNFα, and CDIP appears to be a regulator of the p53-mediated death versus survival response of cells to TNFα. This CDIP-mediated sensitivity to TNFα-induced apoptosis favors pro-over anti-apoptotic program in cancer cells and CDIP may serve as a predictive biomarker for such sensitivity.

Keywords

p53; CDIP; apoptosis; survival; TNF-α; JNK

INTRODUCTION

Two pathways of apoptosis have been described for mammalian cells: the intrinsic mitochondrial pathway, activated in response to cellular stress, and the extrinsic death receptor pathway, activated at the cell surface by the binding of tumor necrosis factor alpha (TNFα) family cytokines to their cognate receptors (1-7). TNFα, however, can exert pleiotropic cellular effects, controlling cell proliferation, differentiation, and apoptosis, as well as regulating the host immune response (4-7).

TNFα does not generally induce apoptosis unless the NF-κB survival arm of the signal is inhibited (e.g. by blocking *de novo* protein synthesis with cyclohexamide). However, any perturbation of the TNFα→NF-κB signal would be expected to produce a cellular outcome equivalent to that of blocking protein synthesis, resulting in apoptosis rather than survival in response to TNFα. Among transcription factors, the contribution of NF-κB and p53 to cancer development and progression as well as resistance to chemotherapy is well established (8-10). Considering the deregulation of these two important factors in cancers,

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defining the crosstalk between NF-κB and p53 has been studied intensively for the molecular details and biological outcome of their interaction (8-11). There are many places for intersection and crosstalk between the p53 and NF-κB pathways, which represent ways that NF-κB can regulate p53 and vice versa (12).

Recently, we identified a novel p53 target, CDIP, and demonstrated that CDIP acts in a hitherto unknown apoptotic pathway involving p53→CDIP→TNFα, acting in certain cells in response to DNA damage (13). These findings were particularly intriguing given that in our cell systems NF-κB was not blocked, but cells nevertheless underwent apoptosis in a TNFα- and caspase 8- dependent manner. This raised the question of what was directing these cells to undergo TNFα-induced apoptosis. We hypothesized that CDIP itself could act as a sensitizing factor for a TNFα apoptotic cell fate.

Here we show that TNFα-induced apoptosis is dependent upon CDIP, and that endogenous CDIP expression in cancer cells correlates with sensitivity to the death effects of TNFα. Importantly, the pro-apoptotic effect of CDIP→TNFα, which has previously been shown to be blocked by knockdown of endogenous TNFα, is rescued by the addition of human recombinant TNFα only in CDIP expressing cells. CDIP-dependent sensitivity to TNFα-induced apoptosis is further shown to be dependent upon JNK, and occurs through a mechanism that involves an alteration in the NF-κB transcriptional program that favors pro-apoptotic over pro-survival gene expression. Overall, the identification of CDIP as an important sensitizing factor to TNFα-induced apoptosis has implications for TNFα-based cancer therapeutics, as well as autoimmune diseases resulting from excessive TNFα signaling.

Materials and Methods

Cell lines and culture conditions

U2OS, U2OS-CDIP (tet-on), IMR90-E1A, EJ, EJ-p53 (tet-off), A549, HT-1080, ME-180, A172, BT474, Calu3, LS174T, SW480, HCT116, MDAMB468, SKBR3, HCT1937, MDAMB435, H1975, AU565, SUM149, WiDr, SNU1040, T24 and RD cells were cultured in standard DMEM (Lonza, MD) containing 10% fetal bovine serum (FBS) (Invitrogen, CA), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C. All these cell lines were obtained from the American Type Culture Collection. The cells were periodically authenticated by morphologic inspection and tested Mycoplasma contamination by PCR test (MycoSensor QPCR kit, Agilent Technologies Inc.) every 6 months. In addition, cell line authentication was performed before the initiation of the study by evaluating the presence of gene mutations (i.e. p53, Ras, etc.) and specific gene expression via Q-PCR. EJ-p53 cells were additionally maintained in the presence of tetracycline (1 μg/mL), except for conditions of p53 induction. CDIP expression was induced in U2OS-CDIP cells by the addition of doxycyclin to a final concentration of 1 μg/mL. Camptothecin (25 μM) and etoposide (100 μM) were solubilized in DMSO and maintained at stock concentrations at −20°C, and used at the concentrations indicated (Sigma-Aldrich, MO). SP600125 (SP) JNK inhibitor (Calbiochem, CA) was solubilized in DMSO, maintained at a stock concentration (50 μM, −20°C), and used at a final concentration of 5 μM. N-Acetyl-L-Cysteine (NAC) was solubilized in PBS, maintained at a stock concentration (1 M, − 20°C) and used at final concentrations of 1 – 40 mM.

Cell death assays

Cell viability was determined by crystal violet staining (0.2% w/v in 2% ethanol) and Trypan blue exclusion (GIBCO-Invitrogen, CA). Apoptotic cell populations were determined by TUNEL assay (Roche, IN). Briefly, cells were trypsinized, recovered by

centrifugation at 300 x g, and fixed in 2 % paraformaldehyde-PBS for 16 hours. Cells were then washed twice with PBS and re-suspended in fresh permeabilization solution (0.1% Triton-X 100 and 0.1% Sodium citrate) for 30 minutes at room temperature. Enzymatic labeling of free 3′-OH DNA ends with dUTP-conjugated to TMR red was performed according to the manufacturer's protocol. The percentage of TUNEL positive apoptotic cells was determined by flow cytometry enumeration of fluorescent cells (FL2-H: 570 - 620 nm).

Plasmids and siRNAs

The CDIP coding sequence based on DQ167023 was tagged with haemagglutinin and expressed in pcDNA3.1. We used validated CDIP siRNA oligonucleotides (Origene Trilencer-27 Human siRNA pool SR309314) to knock down the expression of endogenous CDIP as compared to cells transfected scrambled negative control siRNA duplex (Origene Trilencer-27 Universal Scrambled Negative Control siRNA Duplex SR30004). To exclude off-target effects of the siRNA we used the three unique 27mer CDIP siRNA duplexes separately and in pool, all of which reduced CDIP expression (data not shown). Transfection conditions and vectors expressing control luciferase shRNA, and shRNAs directed against CDIP and TNFα were previously described (13).

Mouse Tumor Xenografts

Athymic NCr-*Foxn1*^{nu} female mice (Taconic Farms, NY) were given subcutaneous injections of 1×10^6 A549 and 2×10^6 RD cells in a 50% Matrigel™ (BD Biosciences, CA) solution. Once a tumor weight of 25 to 50 mg was reached mice were given IP injections of 5 μg rhTNFα (Invitrogen, CA), reconstituted in endotoxin-free 0.9% saline, every five days. Tumors weights (mg = (Length (mm) x Width $(mm)^2/2$) were measured every two days for up to three weeks.

RESULTS

CDIP expression sensitizes cells to recombinant TNFα induced cell death

Previous studies demonstrated that in response to genotoxic stress, TNFα plays an essential role in CDIP induced apoptosis (13). If CDIP acts as a sensitizing factor for recombinant/exogenous TNFα-induced cell death, then we would expect that the treatment of these cells with exogenous recombinant human TNFα (rhTNFα), without concomitant CDIP induction, would produce a cell survival rather than cell death effect. Indeed, in cells grown under normal conditions, in the absence of any p53→CDIP stress signal, rhTNFα has no effect on cell viability (Fig. 1A). To directly test whether CDIP plays a role as a TNFα sensitizing factor, we disabled the endogenous CDIP→TNFα pathway by siRNA-directed knockdown of TNFα. As expected, this lead to increased long-term viability of CDIP-expressing U2OS-CDIP "tet-on" cells in response to doxycyclin (dox) treatment, and of IMR90-E1A cells exposed to Camptothecin (CPT), which activates the p53→CDIP→TNFα pathway. However, the addition of rhTNFα to the culture was able to rescue the cell death effects of CDIP, with no apparent effect on cells lacking CDIP induction (Fig. 1B and C). We then used TUNEL assay to evaluate whether these effects on cell viability were due to apoptosis, and found that the addition of rhTNFα is able to promote apoptosis, *only* in CDIP expressing cells (Fig. 1B and C).

To further substantiate these findings we titrated down the endogenous CDIP→TNFα signal, and then tested whether exogenous rhTNFα could still rescue CDIP-dependent apoptosis in TNFα-shRNA expressing cells. The expression of TNFα transcript and TNFα-induced signaling, observed as I-κB phosphorylation, correlates with CDIP protein expression in a dose-dependent manner in both U2OS-CDIP "tet-on" and IMR90-E1A cells (Fig. S1A and B). CDIP-dependent apoptosis also showed a dose-dependent decrease as we

decreased the concentration of dox, indicating that we could control this cellular response by varying the amount of CDIP expressed (Fig. S1C). Again, the addition of rhTNFα to the culture rescued the attenuated apoptotic response of cells lacking endogenous TNFα (i.e. TNFα-siRNA transfected). Importantly, the rescue effect of rhTNFα was diminished as CDIP expression was decreased, demonstrating that CDIP is required for TNFα-induced apoptosis. If CDIP was not involved in this response, we would expect the levels of apoptosis in rhTNFα-rescued cells to remain the same regardless of any decrease in CDIP expression (Fig. S1C, dashed line). We next confirmed these findings in IMR90-E1A cells, in which the CDIP→TNFα pathway acts to induce apoptosis in response to genotoxic stress. The ability of exogenously added rhTNFα to induce apoptosis in TNFα-siRNA cells decreased in a similar fashion with decreasing amounts of CPT (Fig. S1D & E). These results support the hypothesis that CDIP expression is required for TNFα-induced apoptosis.

Endogenous CDIP expression correlates with sensitivity to TNFα-induced apoptosis

If CDIP were acting to sensitize cells to TNFα-mediated apoptosis, we would expect to see some level of endogenous CDIP expression in cancer cells that are inherently sensitive to the anti-proliferative effects of TNFα. We obtained a panel of cancer cell lines: a total of 22 human cancer cell lines, including nine lines with wt-p53 and thirteen lines with mutant p53, and investigated whether there was any relationship between endogenous CDIP expression and sensitivity to TNFα-induced death. First, CDIP protein expression levels were evaluated by western blot analysis (Fig. 2A). CDIP wasabundantly expressed in most of wt-p53 containing cell lines, including U2OS, HT1080, ME-180, and A172, and lesser amounts in A549, H1975, LS175T, but very little in HCT116 and BT474 (Fig. 2A). However, CDIP levels were relatively lower in most of mutant p53 containing cell lines but RD cell line. We then divided several lines from this panel of cells into CDIP little-expressing (Fig. 2B) and CDIP-expressing cells (Fig. 2C), and tested their growth response to rhTNFα after 2 days of treatment. The viability of these cells after treatment with rhTNFα did in fact reflect their CDIP status, as rhTNFα exerted a more pronounced anti-proliferative effect on cell lines expressing endogenous levels of CDIP protein while there are few exceptions (Fig. 2B and C). The contribution of CDIP to this effect was directly assessed by knockdown of CDIP in these rhTNFα sensitive lines, followed by trypan blue staining to measure cell death up to 3 days after treatment. The once sensitive ME-180 and RD cells were rendered insensitive to TNFα killing upon knockdown of endogenous CDIP (Fig. 2D). Conversely, we introduced CDIP into the TNFα-insensitive A549 and SW480 cell lines by transfection with a CDIP expression vector, and measured viability after rhTNFα treatment. CDIP expression lead to an increase in the cell death response of both A549 and SW480 cells to rhTNFα, in comparison to empty vector control treated cells (Fig. 2E). These findings lend further support to our hypothesis that CDIP expression correlates with sensitivity to acts to TNFα.

CDIP activates the JNK-dependent TNFα apoptotic pathway

We next investigated the effect of CDIP expression on pathways known to act as conduits of TNFα survival and apoptotic signaling. During the TNFα-mediated survival response, JNK is activated in a transient manner, and activation is terminated within one hour of TNFα stimulation (4, 14). Conversely, during the TNFα-mediated apoptotic response, JNK is activated in a sustained manner, and this sustained JNK activity is known to be required for an apoptotic cell fate (14, 15). After activation of the CDIP→TNFα pathway, U2OS-CDIP cells showed late JNK phosphorylation (Fig. 3A and Fig. S2). Moreover, induction of CDIP→TNFα downstream of p53 activation (i.e. in response to DNA damage, or induced p53 expression) resulted in a similar pattern of late JNK phosphorylation (Fig. 3B). Treatment with recombinant human TNFα (rhTNFα) alone (i.e. in the absence of CDIP activation) produced no such late JNK phosphorylation (Fig. S2). This absence of sustained JNK phosphorylation was observed despite an increase in phosphorylated I-κB, indicating

that the cells were responsive to rhTNFα. CDIP→TNFα signaling also produced an increase in I-κB phosphorylation, confirming that the TNFα pathway was in fact activated in response to dox-induced CDIP→TNFα signal. Interestingly, when compared to cells treated with rhTNFα alone cells with CDIP→TNFα had lower FLIP expression (Fig. S2). Since FLIP is a known NF-κB survival target, we supposed that CDIP might be influencing TNFα survival signaling at the level of NF-κB target gene expression (Fig. S2).

The effect of CDIP on late JNK phosphorylation was next assessed directly by siRNA directed knockdown of CDIP. In comparison to control siRNA treated cells with DNA damage-induced p53→CDIP→TNFα activation, knockdown of CDIP resulted in a block of JNK phosphorylation (Fig. 3C). The contribution of JNK to the CDIP→TNFα apoptotic signal was determined using a well-established chemical inhibitor of JNK (16-18). Pretreatment of U2OS-CDIP cells and IMR90-E1A cells with a JNK-selectiveinhibitor resulted in a complete block of CDIP-dependent apoptosis (Fig. 3D and E). U2OS parental cells were not affected by dox or JNK inhibitor treatments, supporting the conclusion that these effects were specific to CDIP activation and JNK inhibition. In addition, CDIP-induced apoptosis was completely unaffected by pretreatment of cells with other MAPK inhibitors (Fig. S4). Together, these results show that activation of CDIP→TNFα induces a TNFα apoptotic signal that is dependent upon JNK, which is consistent with previous reports demonstrating that JNK is required for the TNFα apoptotic cell fate decision (13-19).

NF-κB target gene expression is influenced by CDIP

To test whether CDIP could influence the NF-κB transcriptional program in response to TNFα treatment we used the expression PCR arrays to analyze NF-κB target-gene expression under two conditions: 1) activation of CDIP→TNFα, which leads to an apoptotic cell fate, and 2) treatment with rhTNFα alone, which is a condition of cell survival (Fig. S4A). There were two NF-κB targets that displayed differential expression under these conditions. Interleukin-8 (IL-8) was specifically up-regulated in response to CDIP→TNFα, whereas TNF Receptor Associated Factor 1 (TRAF1) was specifically up-regulated in response to TNFα treatment alone (Fig. S4B). To confirm and expand these results, real-time-PCR was performed for additional NF-κB targets. When CDIP was not expressed we observed a specific up-regulation of some NF-κB targets known to play a role in cell survival, such as SOD2 (Fig. S4B). A sandwich ELISA assay confirmed that the CDIP→TNFα-induced increase in IL-8 transcript also resulted in an increase in secreted IL-8 (Fig. S4C).

Inhibition of ROS and IL-8 impairs TNFα apoptosis downstream of CDIP

In view of the evidence that CDIP→TNFα-induced apoptosis requires JNK, the identification of superoxide dismutase 2 (SOD2) as a survival target was noteworthy. SOD2 is an anti-oxidant enzyme and known NF-κB target (20, 21) that functions to counteract the deleterious effects of intracellular ROS. Since ROS accumulation leads to sustained JNK activity in cells undergoing TNFα-induced apoptosis (22), it is likely that ROS contributes to the late JNK phosphorylation we observed in cells undergoing CDIP→TNFα-dependent apoptosis. To investigate the role of SOD2 and IL-8 modulation in CDIP→TNFα-dependent apoptosis, we pre-treated U2OS-CDIP cells with N-Acetyl-L-Cysteine (NAC), a chemical anti-oxidant and an IL-8 blocking antibody, both alone and in combination. The cells were then assessed for ROS production and apoptosis after inducing CDIP expression. ROS levels were induced under conditions of CDIP expression, an effect that was abrogated by pretreatment with NAC (Fig. 4A, B and C). While blocking of IL-8 had some ability to attenuate the apoptotic response (Fig. S5), blocking ROS completely abrogated apoptosis (Fig. 4D). As expected for a JNK-dependent apoptotic process, the treatment of cells with

NAC produced a pronounced decrease in phosphorylated JNK, despite the presence of a TNFα apoptotic signal (Fig. 4E). Notably, cells that are refractory to TNFα-induced apoptosis showed no detectable levels of phosphorylated JNK (Fig. 4E). Together, these results demonstrate a primary role for ROS in CDIP→TNFα-dependent apoptosis, accounting for the sustained JNK activation and JNK dependency of this apoptotic response. To further determine if ROS production was linked to SOD2 we first asked whether SOD2 protein expression changed with CDIP expression and found that SOD2 protein levels were inversely correlated with CDIP expression (Fig. 5A). Moreover, SOD2 activity was protective against CDIP-induced apoptosis (Fig. 5B and C). Overall, this suggests a CDIP→TNFα-dependent apoptotic pathway that involves ROS generation via suppression of the anti-oxidant SOD2.

TNFα limits the growth of CDIP-positive tumors *in vivo*

In our previous screen we identified various tumor cell lines that either displayed high levels of endogenous CDIP expression or no detectable levels of CDIP expression (Fig. 2B and C). Using two such cell lines we examined the ability of TNFα to limit tumor growth using an *in vivo* mouse tumor xenograft model. Athymic mice were given subcutaneous injections of RD (positive for CDIP expression) and A549 (negative for CDIP expression) cells. Once the tumors had reached a significant size rhTNFα was administered by intraperitoneal injection every five days and tumor growth was monitored for up to three weeks. After sixteen days, the growth of RD tumors in mice given rhTNFα injections was significantly lower compared to control treated mice. Conversely, the growth of A549 tumors was not significantly different between mice treated with and without rhTNFα (Fig. 6A and B). These data lend further support to our hypothesis that CDIP sensitizes cancer cells to TNFα-mediated apoptosis, and suggests that TNFα may be effective in limiting the growth of CDIP positive tumors *in vivo*.

DISCUSSION

The TNFα cell fate decision-making process is not completely understood. While the survival arm of this pathway is well characterized and known to require NF-κB up-regulation of pro-survival target genes, and the termination of a JNK-dependent pro-apoptotic signal, less is known about the apoptotic arm. In this study, we identified CDIP as a key-sensitizing factor to TNFα-mediated apoptosis in various cancer cells. We provided evidence that cells with endogenous CDIP expression were inherently sensitive to TNFα-induced death; conversely, cells that have no or little detectable basal CDIP expression were refractory to TNFα killing *in vitro* and *in vivo*.

Our analysis of NF-κB target gene expression showed that a program of survival gene expression (i.e. SOD2, TRAF1, A1, c-IAP1 and 2) is activated when TNFα has no apoptotic effect, and that an alternate pro-apoptotic program, involving the up-regulation of IL-8 (23) and the attenuated induction of survival targets is activated when TNFα induces apoptosis. This is consistent with a previous report showing that NF-κB-dependent TRAF and c-IAP expression virtually disappears in cells rendered susceptible to TNFα-induced apoptosis by the expression of an I-κB super-repressor (24). In addition, we found that CDIP→TNFα-induced apoptosis is associated with late JNK phosphorylation, and that JNK activity is required for this pro-apoptotic TNFα signal. Given these results, the identification of SOD2 as an NF-κB survival target was particularly interesting. SOD2 is an anti-oxidant enzyme responsible for the conversion of superoxide radicals (O_2-) to H_2O_2 leading to their eventual elimination through peroxidases, which convert H_2O_2 to H_2O. ROS is known to inhibit JNK phosphatases leading to the sustained JNK activation required for TNFα-induced death (22). Our observed up-regulation of SOD2 would be expected to lower intracellular ROS, or maintain it at levels below a threshold required for JNK apoptotic activity, and is therefore

consistent with a TNFα-mediated survival response. In addition, a recent study demonstrated a deeper understanding of the cross talk between NF-kB and p53 showing that p53 significantly contributes to nuclear NF-κB activity induced during S-phase arrest and remarkably also in response to TNFα (11).

The importance of ROS to the CDIP→TNFα-mediated apoptotic response is supported by the observation that treating cells with NAC, a chemical anti-oxidant, resulted in an almost complete block of CDIP-dependent apoptosis, and an attenuation of JNK phosphorylation. Further, if ROS is critical for tipping the balance in favor of TNFα-mediated apoptosis in CDIP expressing cells over survival in CDIP lesser expressing cells treated with rhTNFα, we would expect to see some difference in intracellular ROS levels between these conditions. Indeed, cells undergoing CDIP→TNFα-mediated apoptosis showed elevated ROS levels compared to CDIP non-expressing cells that survived in response to TNFα.

Together, these results extend our previous work that identified a novel CDIP→TNFα pro-apoptotic pathway acting downstream of p53 (13), and provide an explanation for why cells with an activated CDIP→TNFα signal follow an apoptotic cell fate. In our current model (Fig. 6*C*), NF-κB survival gene expression downstream of TNFα is suppressed in CDIP expressing cells in favor of IL-8 expression, which allows the apoptotic cell fate to prevail. Conversely, cells that do not express CDIP respond to TNFα by activating a program of NF-κB survival target gene expression. In particular, SOD2 acts to maintain intracellular ROS levels below a threshold required for JNK activation and apoptosis, while other NF-κB survival targets also likely contribute to this survival cell fate. Deregulated TNFα signaling contributes to a number of disease pathologies (i.e. cancer, sepsis, diabetes, autoimmune disease); therefore, identifying factors that modulate TNFα cell fate decision-making is of great therapeutic value. To this end, we are currently employing a chemical genetic approach to identify CDIP activators and suppressors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grants CA085681, CA078356, and CA80058. L B-E is supported by an MGH ECOR Fund for Medical Research Fellowship.

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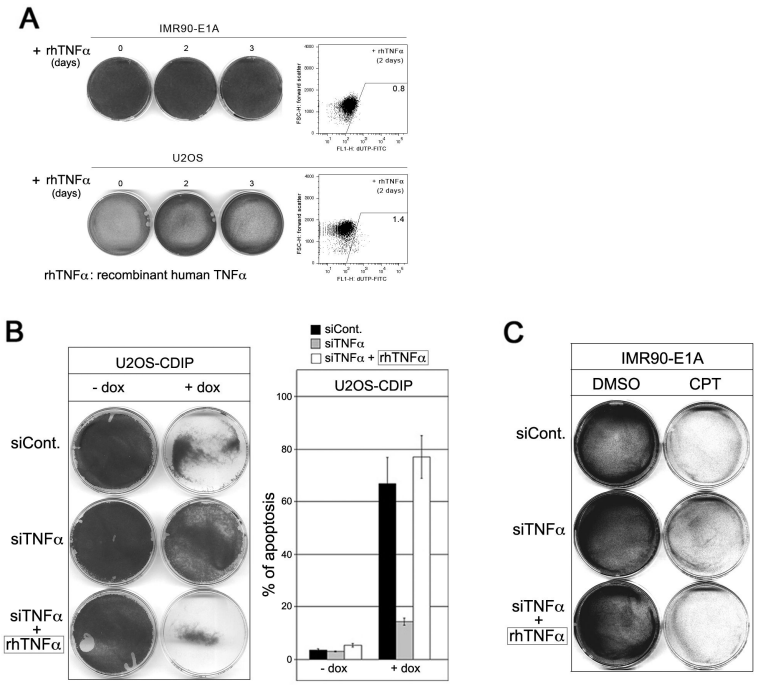

Figure 1.

Sensitivity to exogenous/recombinant TNFα-induced apoptosis is dependent upon CDIP.

A, IMR90-E1A and U2OS cells were treated with recombinant human TNFα (rhTNFα) for up to 3 days, and viability was assessed by crystal violet staining at the time points indicated. Apoptosis following 2 days of treatment with rhTNFα was determined by TUNEL assay followed by enumeration of dUTP-FITC positive cells by flow cytometry (FL1-H: dUTP-FITC).

B, USOS-CDIP cells were transfected with control luciferase- (Cont.-) and TNFα-targeting (TNFα-) siRNA for 24 hours, treated with doxycyline (dox) (1 μg/mL), and supplemented with either 5 ng/mL recombinant human TNFα (rhTNFα) or an equivalent amount of BSA. Long-term viability was assessed by crystal violet staining 72 hours after drug treatments. TUNEL assay was used to determine apoptosis 2 days following treatments and is shown as the percentage of dUTP-FITC positive cells +SEM (n=3).

C, IMR90-E1A cells were transfected as in (B), and treated with CPT (0.5 μM) for 24 hours, and supplemented with either 5 ng/mL recombinant human TNFα (rhTNFα) or an equivalent amount of BSA.

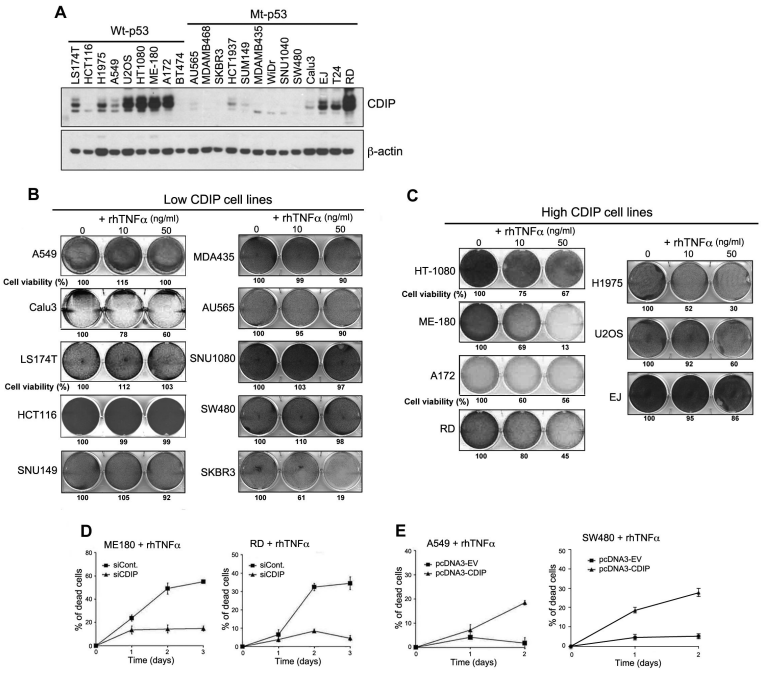

Figure 2.

CDIP expression in various cancer cells correlates with sensitivity to exogenous recombinant TNFα-induced apoptosis.

A, endogenous CDIP protein expression was examined in a total of 22 human cancer cell lines grown under normal conditions, by western blot analysis. β-actin was used as a loading control. Cell lines were grouped based on p53 status (wt-p53 and mutant p53) as indicated.

B, C, cells were treated with recombinant rhTNFα as indicated and long-term viability was assessed by crystal violet staining 48 hours post-treatment. Cells lines are grouped based on CDIP expression (B: low, and C: high). The plates were scanned by densitometer and quantitated. Cell viability of each plate was shown by a percentage after the normalization to the control (no TNFα treated).

D, the effect of CDIP knockdown in cells sensitive to TNFα death (ME-180, and RD) was assessed by transfection with either control luciferase- (Cont.-) or CDIP-targeting siRNA for 24 hours, followed by treatment with rhTNFα (10 ng/mL). Cell viability up to 3 days post-rhTNFα treatment was determined by trypan blue exclusion and is shown as the mean percent increase in dead cells relative to similarly transfected BSA-treated control cells +SEM (n=3).

E, two TNFα insensitive cell lines, A549 and SW480, were transfected with either control pcDNA3 empty vector (pcDNA3-EV) or CDIP expressing pcDNA3 constructs, respectively, followed by treatment with rhTNFα (10 ng/mL). Cell viability was determined by trypan blue exclusion and is shown relative to transfected control BSA-treated cells.

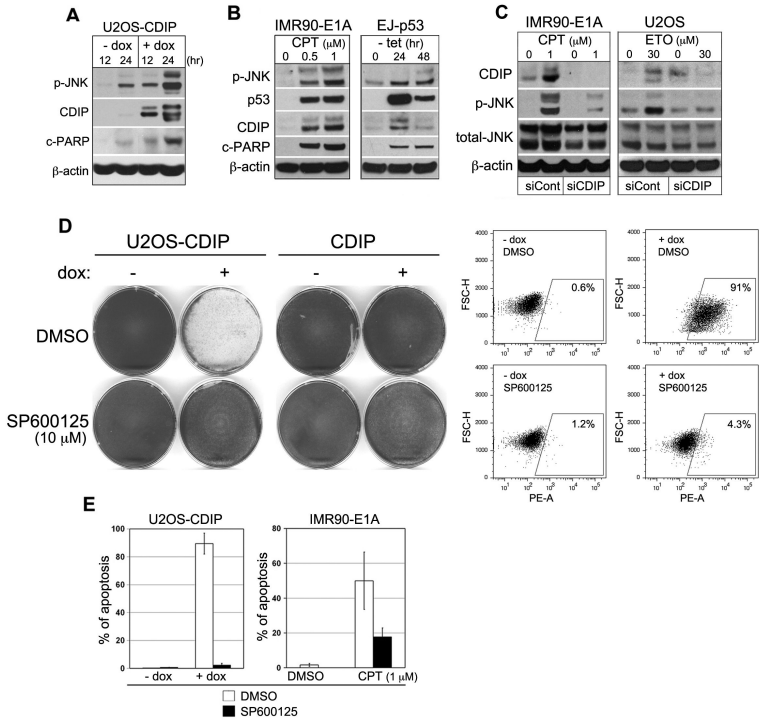

Figure 3.

CDIP-induced apoptosis requires prolonged JNK activity.

A, U2OS-CDIP cells were either left untreated or treated with dox (1 μg/mL) to induce CDIP expression for the time indicated. Active JNK was detected by Western blot analysis of p46 and p54 JNK dually phosphorylated at Thr183 and Tyr185. Western blot analysis was also performed with antibodies specific for CDIP and cleaved PARP (clv-PARP), to confirm CDIP expression and induction of apoptosis, respectively.

B, IMR90-E1A, and EJ-p53 tet-off cells were cultured with camptothecin (CPT), and in the absence to tetracycline to induce p53 expression, respectively. Phospho-JNK, phospho-p53, CDIP, and cleaved PARP were detected by Western blot analysis.

C, CDIP-dependent JNK phosphorylation was assessed in cell lines transfected with control siRNA (Cont.-shRNA) or siRNA directed against CDIP. CDIP→TNFα was induced in IMR90-E1A cells by treatment with CPT (24 hours), and in U2OS cells by treatment with etoposide (ETO, 36 hours). Knockdown of CDIP was confirmed by Western blot analysis of CDIP expression, and the effect of this on phospho- and total-JNK expression was determined by Western blot analysis.

D, U2OS-CDIP and U2OS parental cells were pretreated with either SP600125 (JNK inhibitor) or DMSO (carrier) for 1 h, and cell viability was assessed 40 hours after the addition of dox. Under the same conditions, the percent of apoptotic U2OS-CDIP cells was determined by TUNEL assay, followed by enumeration of dUTP-TMR positive cells by flow cytometry (FL2-H: dUTP-TMR).

E, the percentage of TUNEL positive U2OS-CDIP cells (in repeat experiments) and IMR90E1A cells after pre-treatment with SP600125, followed by treatment with CPT, is shown as the mean +SEM (n=3).

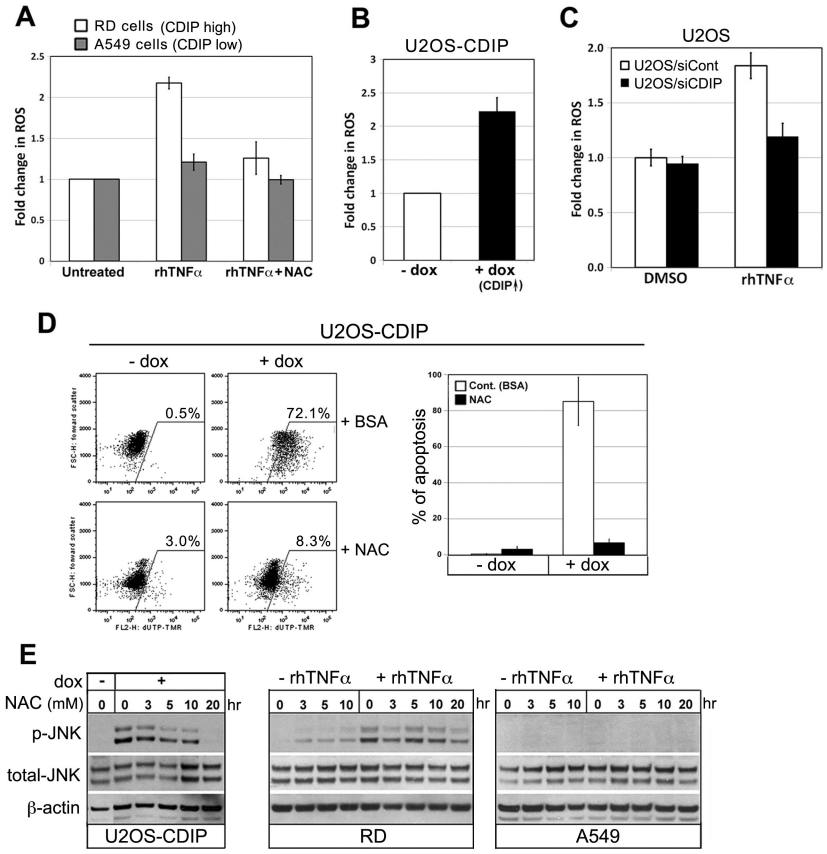

Figure 4.

CDIP-induced apoptosis is linked to ROS and activated JNK.

A, intracellular ROS was measured by DCFDA fluorescence staining 24 hours after treatment of RD (CDIP positive) or A549 (CDIP negative) with rhTNFα alone and in combination with NAC, and is shown by as the mean fold increase or decrease relative to untreated cells +STDV (n=3).

B, CDIP expression was induced in U2OS-CDIP cells (+ dox), and ROS levels were determined as in (A).

C, U2OS cells were transfected with control siRNA (siCont) or siRNA directed against CDIP (siCDIP), and ROS levels were determined as in (A) in the presence and absence of rhTNFα.

D, apoptosis was assessed in U2OS-CDIP cells (+ dox, 72h) cultured in the presence and absence of NAC +SEM (n=3).

E, the expression levels of phosphorylated (phos-) and total (tot-) JNK were assessed by Western blot analysis in different cell lines as follows: U2OS-CDIP cells treated with either rhTNFα (5 ng/mL) or dox (1 μg/mL) for 20 hours, RD (CDIP positive) and A549 cells (CDIP negative) treated with either rhTNFα or an equivalent amount of BSA (- TNFα) for 24 hours. β-actin protein expression served as a loading control.

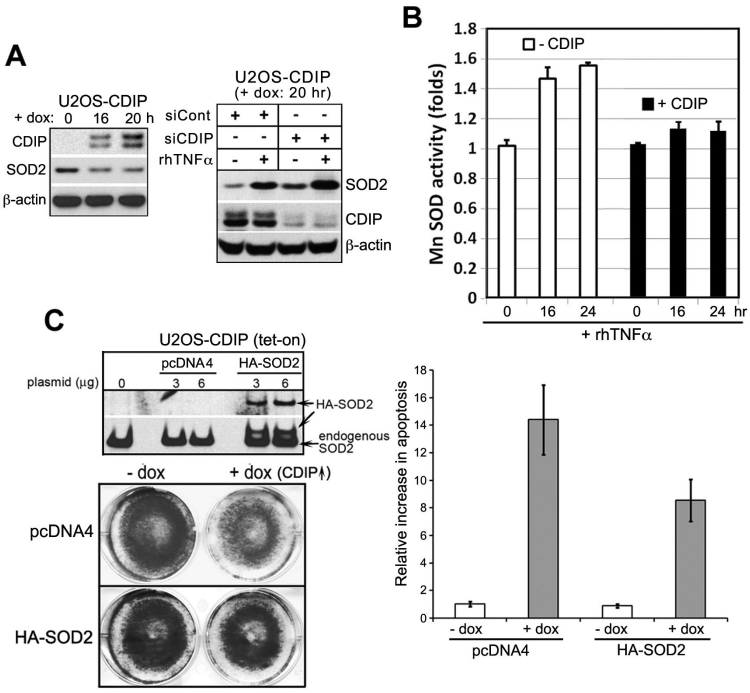

Figure 5.

CDIP expression correlates with a decrease in SOD2 expression, and SOD2 expression inhibits CDIP-dependent cell death.

A, SOD2 protein levels were assessed by Western blot analysis in U2OS-CDIP cells (+ dox, transfected with siCont or siCDIP RNA or with/without rhTNFα treatment.

B, U2OS-CDIP cells were cultured with/without dox in the presence of rhTNFα. SOD2/Mn SOD activity assay was performed as suggested by vendor (Millipore Inc: HCS232).

C, the SOD2 open reading frame with an amino-terminus HA tag was cloned into pcDNA4, and then introduced into U2OS cells along with empty vector (EV) pcDNA4, which served as a control. Sequential western blot analysis was performed with anti-HA and anti-SOD2 antibodies (Santa Cruz) to detect exogenous SOD2 protein expression. U2OS-CDIP cells were transfected with a pcDNA4-SOD2 (SOD2) expression vector or empty pcDNA4 vector as a control (EV), and 24 hours post-transfection the cells were treated with dox to activate CDIP expression. Viability was assessed with crystal violet staining and apoptosis was determined using TUNEL assay followed by FACs enumeration of TUNEL positive cells, and is shown as the an increase in the TUNEL positive population relative to cells that were left untreated (- dox).

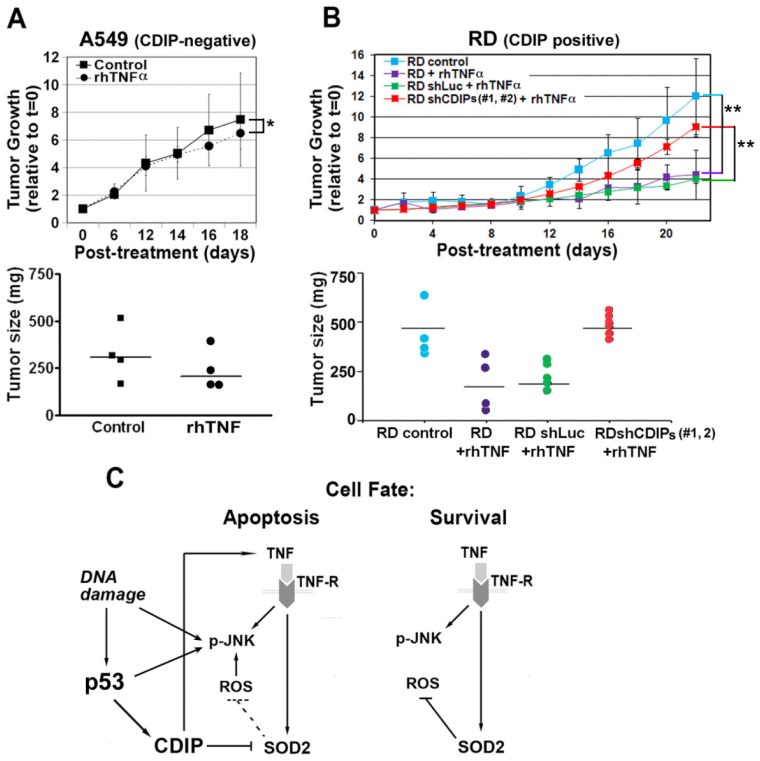

Figure 6.

Response of mouse tumor xenografts to rhTNFα, and current model of CDIP-mediated cell fate decision-making in response to rhTNFα.

A, growth of A549 tumor xenografts (little endogenous CDIP expression) and B, RD (endogenous CDIP expression) and stable control (shLuc) or CDIP knock-down (shCDIP) tumor xenografts in athymic mice treated with and without 5 μg of rhTNFα, every five days over the time course indicated. Lower panels indicated tumor sizes for A549 (left, n=4) and RD (right, n=4) at day eighteen following treatment with rhTNFα. Lines indicate the median weights, *$p<0.5$ (non-significant) and **$p<0.02$ (significant).

C, in cells with endogenous, or induced CDIP expression (i.e. by p53 in response to DNA damage), NF-κB survival gene expression downstream of TNFα is suppressed in favor of pro-apoptotic gene expression (e.g. IL-8). Cancer cells then respond to elevated ROS, which facilitates a prolonged JNK activation and an apoptotic cell fate. Conversely, under conditions of TNFα stimulation alone, a program of NF-κB survival target gene expression is activated. For example, SOD2 acts to maintain intracellular ROS levels below the threshold required for JNK activation and apoptosis. Other NF-κB survival targets act to reinforce this effect, allowing the survival cell fate to prevail.