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Advances in Brief

p53-dependent and -independent Regulation of the Death Receptor *KILLER/DR5* Gene Expression in Response to Genotoxic Stress and Tumor Necrosis Factor α

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Abstract

The death receptor (DR) *KILLER/DR5* gene has recently been identified as a doxorubicin-regulated transcript that was also induced by exogenous wild-type p53 in p53-negative cells. *KILLER/DR5* gene encodes a DR containing cell surface protein that is highly homologous to DR4, another DR of the tumor necrosis factor (TNF) receptor family. Both DR4 and KILLER/DR5 independently bind to their specific ligand TRAIL and engage the caspase cascade to induce apoptosis. TRID (also known as TRAIL-R3) is an antiapoptotic decoy receptor that lacks the cytoplasmic death domain and competes with KILLER/DR5 and DR4 for binding to TRAIL. In this study, we demonstrate that the DR *KILLER/DR5* gene is regulated in a p53-dependent and -independent manner during genotoxic and nongenotoxic stress-induced apoptosis. Just like other p53-regulated genes, ionizing radiation induction of *KILLER/DR5* occurs in p53 wild-type cells, whereas methyl methanesulfonate regulation of *KILLER/DR5* occurs in a p53-dependent and -independent manner. However, unlike other p53-regulated genes, *KILLER/DR5* is not regulated following UV irradiation. TNF-α, a nongenotoxic cytokine, also induced the expression of *KILLER/DR5* in a number of cancer cell lines, irrespective of p53 status. TNF-α did not alter the *KILLER/DR5* mRNA stability, suggesting that the TNF-α regulation of *KILLER/DR5* expression appears transcriptional. We also provide evidence that *KILLER/DR5* is regulated in a trigger and cell type-specific manner and that its induction by TNF-α, p53, or DNA damage is not the consequence of apoptosis induced by these agents. Unlike *KILLER/DR5*, none of the other KILLER/DR5 family members, including *DR4*, *TRID*, or the ligand *TRAIL*, displayed genotoxic stress or TNF-α regulation in a p53 transcription-dependent manner. Thus, *KILLER/DR5* appears a bona fide downstream target of p53 that is also regulated in a cell type-specific, trigger-dependent, and p53-independent manner.

Introduction

p53 is the most commonly mutated gene in a variety of human cancers (1). Although p53 has been implicated to affect a variety of cellular processes (reviewed in Ref. 2), the most extensively studied and, perhaps, undisputed roles of p53 are to induce growth arrest and to induce apoptosis (reviewed in Refs. 2–4). It is now well documented that p53 mediates its effects by modulating the expression of important cellular genes. p53 directly binds DNA in a sequence-specific manner to transactivate its downstream effector genes (reviewed in Refs. 2–4). A number of genes that are directly regulated by p53 have been identified, some of which include $p21^{WAF1/CIP1}$, *GADD45*, *MDM2*, and *BAX* (reviewed in Ref. 2). Although p53-induced cell cycle arrest is believed to be mediated, at least in part, by $p21^{WAF1/CIP1}$ (reviewed in Ref. 2), the molecular mechanisms of p53-induced apoptosis remain largely unclear. In a recent study, a number of p53-induced genes were reported; the cumulative induction of p53-induced genes was thought to mediate the p53 induction of apoptosis (5). p53 has also been shown to regulate the expression of *BAX* (6) and *Fas/APO1/CD95* (hereafter referred to as *Fas*; Ref. 7). There is, however, evidence to suggest that Fas expression is not an absolute prerequisite for p53-induced apoptosis (8). Several recent studies have also shown that p53-induced apoptosis occurs in the absence of increases in Bax mRNA and/or protein levels (Refs. 2 and 5 and references therein). Furthermore, cells from Bax-deficient mice were proficient in mounting the p53-dependent IR[2]-induced apoptotic response, suggesting that Bax was not absolutely required for p53-induced apoptosis (reviewed in Ref. 2; Ref. 9).

Recently, *KILLER* gene was identified in a subtractive hybridization screen as a novel transcript that was induced by doxorubicin only in cells with wild-type p53 status (10). Overexpression of wild-type p53 transgene in cells lacking wild-type p53 caused induction of endogenous *KILLER* expression, suggesting that *KILLER* gene might be a novel downstream effector of p53 (10). *KILLER* gene was also independently identified by other groups as DR5, TRAIL-2, and TRICK2 (hereafter referred to as *KILLER/DR5*; Refs. 11–15). *KILLER/DR5* codes for a cell surface protein of M_r 45,000 that contains a cytoplasmic death domain and shows a high degree of homology with members in the TNFR family (11–15). *DR4* is another DR gene that is closely related to KILLER/DR5 (11–15). The protein products of both the *KILLER/DR5* and *DR4* genes have been shown to mediate the apoptotic effects of TRAIL by activating the downstream caspase cascade (11–15). TRAIL belongs to TNF cytokine family of death-inducing ligands (16) and specifically binds to KILLER/DR5 and DR4 but does not bind to TNFR or Fas (11–15). Another molecule that is highly homologous to KILLER/DR5 but lacks the cytoplasmic death domain was also identified as an antagonist decoy receptor (11–13, 15). The decoy receptor named TRID (11), DcR1 (12), or TRAIL-R3 (Ref. 13; hereafter referred to as TRID) was found to be selectively expressed in normal tissues and appears to blunt the apoptotic effects of TRAIL by competing with KILLER/DR5 (and DR4) for binding to TRAIL (11–13). *KILLER/DR5*, by contrast, appears to be selectively expressed in cancer cells and is thought to mediate TRAIL-induced apoptosis in cancer cells (11–13). When overexpressed, KILLER/DR5 has also been shown to induce apoptosis in a ligand-independent manner (11–15).

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[2] The abbreviations used are: IR, ionizing radiation; DR, death receptor; TNF, tumor necrosis factor; TNFR, TNF receptor; MMS, methyl methanesulfonate; ActD, actinomycin D; CHX, cycloheximide; DAPI, 4′-diamidino-2-phenylindole; NFκB, nuclear factor κB.

Evidence of p53-dependent regulation of *KILLER/DR5* gene expression would directly link p53 to caspase signaling cascades. However, more studies are needed to determine whether or not p53 is absolutely required for *KILLER/DR5* induction in response to genotoxic or nongenotoxic stresses. We undertook this study to further explore the dependence of *KILLER/DR5* induction on p53 in response to genotoxic and nongenotoxic stresses. We also investigated the genotoxic and nongenotoxic stress regulation of KILLER/DR5 family members *DR4* and *TRID* and their ligand *TRAIL*. Here, we report that IR induction of *KILLER/DR5* expression predominantly occurs in p53-dependent manner, whereas the MMS regulation of *KILLER/DR5* occurs in a p53-independent manner as well. TNF-α, a nongenotoxic cytokine, also enhanced the expression of *KILLER/DR5* in a p53-independent manner. Unlike *KILLER/DR5*, none of the other family members, including *DR4*, *TRID*, and the ligand *TRAIL*, displays a pattern of regulation similar to that of *KILLER/DR5* in response to genotoxic stresses or TNF-α treatment.

Materials and Methods

Materials. MMS was purchased from Aldrich (Milwaukee, WI). TNF-α, ActD, and CHX were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Lines and Cell Culture. The following human cell lines were used in this study, and their origin has been described previously (10, 17, 18): MCF-7 and SkBr3 breast carcinoma cells; RKO, HCT116, and SW480 colon carcinoma cells; A549, H1299, and H460 lung carcinoma cells; ML-1 myeloid leukemia cells; HL60 promyelocytic leukemia cells; PA1 and SKOV3 ovarian cancer cells; and 2184D normal lymphoblastoid cells.

Cells were regularly maintained in DMEM or in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (Intergen, Purchase, NY). HCT116 Neo/E6, PA1 Neo/E6, H460 Neo/E6, and SW480 cell lines were maintained as described previously (19). The 2184D cell line was maintained in RPMI 1640 supplemented with 15% fetal bovine serum, 1% penicillin, and 1% streptomycin.

Cells were exposed to IR or UV irradiation or treated with chemical agents essentially as described previously (17, 18). Briefly, for IR treatment, cells were exposed to a ^{137}Cs source, MMS was used at 100 μg/ml, and UV irradiation was at 14 J/m^2. Cells were harvested 4 h after exposure to IR, UV, or MMS and 10 h after continuous treatment with 0.3 μg/ml doxorubicin.

RNA Extraction and Northern and Dot-Blot Hybridization. RNA extraction was as described by Chomczynski and Sacchi (20) with modifications. Northern and quantitative dot blot hybridizations were performed as described previously (17, 18, 21). The signals were quantitated using a PhosphorImager, and the values for the relative mRNAs were subjected to quantitation, as described previously (18, 21).

cDNA Probes. The following cDNA probes were used: (*a*) a partial-length cDNA representing the 3′ end of human *KILLER/DR5* (10); (*b*) a human *DR4* cDNA representing the complete coding sequence; and (*c*) human expressed sequence tags representing *TRID* (clone 504745) and *TRAIL* (clone 322703). The expressed sequence tag clones were obtained from IMAGE Consortium, LLNL, and were sequenced to confirm their authenticity. Human *DR4* cDNA was cloned using reverse transcriptase-PCR from total RNA isolated from HCT116 Neo cell line treated with 0.3 μg/ml doxorubicin for 10 h. The following oligonucleotides were used in PCRs: 5′-GCACAAGCTTATGGCGCCACCACCAGCTAG-3′; 5′-CGCGGATTCTCACTCCAAGGACACGGCAGA-3′.

PCR conditions were as follows: 35 cycles of 45 s at 94°C/1 min at 66°C; 1 cycle of 2 min at 72°C; and 1 cycle of 5 min at 72°C. This PCR product, following restriction by *Bam*HI and *Hin*dIII, was subcloned into the mammalian expression vector pCEP4 (Invitrogen).

Stable Transfection. MCF-7 and A549 cells were plated at a density of 1×10^5 cells/100-mm plates 24 h prior to transfection. Cells were transfected using Lipofectamine reagent (Life Technologies, Inc., Gaithersburg, MD) per manufacturer's instructions. The CrmA expression vector (kindly provided by Dr. V. Dixit, University of Michigan, Ann Arbor, MI) carry the CrmA coding region under the control of a cytomegalovirus promoter and have a neomycin resistance dominant selection marker. For mock transfection, the same expression vector, lacking the CrmA insert, was used. Selection was carried out in G418 (500 μg/ml), and the resistant colonies were selected after approximately 4 weeks. Pooled clones as well as isolated colonies were selected for further studies.

Analysis for Apoptosis. Cells were analyzed for morphological features of apoptosis, as described recently (22). Briefly, cells were fixed with methanol, washed with PBS, and stained with DAPI solution. Apoptotic cells exhibiting characteristic morphological features of apoptosis were identified using an Olympus fluorescent microscope. To determine the apoptotic index, cells exhibiting apoptotic features were directly counted in several randomly selected fields, and the results were expressed as the number of apoptotic nuclei over the total number of nuclei counted.

Results and Discussion

To gain insight into the mode of DNA damage-mediated p53-dependent and -independent regulation of *KILLER/DR* gene expression, we monitored the effects of IR, MMS, and UV irradiation on *KILLER/DR5* gene expression in a panel of cell lines with varying p53 status. We have previously demonstrated that these agents positively modulate the expression of p53-regulated genes, such as *MDM2*, *GADD45*, *p21*$^{WAF1/CIP1}$, and *BAX*. (17, 18). Furthermore, IR induces their expression only in p53 wild-type cells, whereas their regulation by MMS and UV occurs in both a p53-dependent and -independent manner (17, 18). As outlined in Table 1, the IR enhancement of the *KILLER/DR5* mRNA levels occurred predominantly in p53 wild-type cells. Fig. 1*A* depicts a representative Northern blot showing IR-mediated increases in *KILLER/DR5* mRNA levels in 2184D lymphoblastoid cells. The MMS effect, by contrast, was noted both in p53 wild-type and mutant cells (Fig. 1*B*), as has been reported for other p53-regulated genes (17, 18). Surprisingly, UV irradiation did not induce *KILLER/DR5* mRNA levels in any of the cell lines tested, irrespective of p53 status (Fig. 1*B*).

We also investigated whether the expression profiles of related molecules such as *DR4*, *TRID*, and their ligand *TRAIL* in response to DNA damage. Unlike *KILLER/DR5*, the expression of which is induced by doxorubicin (10), *DR4* and *TRID* expression was not regulated by doxorubicin in the majority of the cell lines (Fig. 1*C*). Doxorubicin, nevertheless, altered the *DR4* expression in HCT116 Neo and E6 cells (Fig. 1*C*). The doxorubicin effect on the *DR4* induction was more pronounced in HCT116 Neo cells than in the E6-transfected counterparts (Fig. 1*C*). Doxorubicin also enhanced the *TRID* mRNA levels in PA-1 cells (Fig. 1*C*). The molecular basis and the physiological significance of the cell type-specific regulation of *DR4* and *TRID* by doxorubicin remain unclear and require further investigation. *TRID* mRNA induction by IR, albeit less pronounced, was also noted in some cell lines (see below), whereas *TRAIL* and

Table 1 *IR enhancement of KILLER/DR5 gene expression occurs in a p53-dependent manner: comparison with the effect on other p53-regulated genes*

IR induction of apoptosis occurs early in nonadherent cells, whereas it is a late effect in adherent cells. In adherent cell lines such as MCF-7, A549, RKO, and H1299, apoptosis was determined approximately 48–72 h post-IR (20 Gy). Cells were stained with DAPI, and those exhibiting the morphological features of apoptosis were identified by using a fluorescent microscope, as described in "Materials and Methods."

Cell line	*p53* status	Apoptosis	Fold induction of relative mRNA				
			DR5	*GADD45*[a]	*p21*^{WAF1a}	*BAX*[b]	*MDM2*[a]
ML-1	wt/wt[c]	+	6.9	9.9	48.0	4.7	11.5
MCF-7	wt/wt	+	6.7	4.6	8.5	2.0	2.8
A549	wt/wt	+	3.1				
RKO	wt/wt	+	3.1	3.1	3.1	0.7	2.2
HL60	−/−	+	0.9	1.0	2.0	1.2	1.3
U937	mut/−	+	1.1				
H1299	−/−	+	2.1	1.3	0.6	1.3	0.9

[a] These values were taken from Ref. 17.
[b] These values were taken from Ref. 18.
[c] Wt, wild-type; mut, mutated.

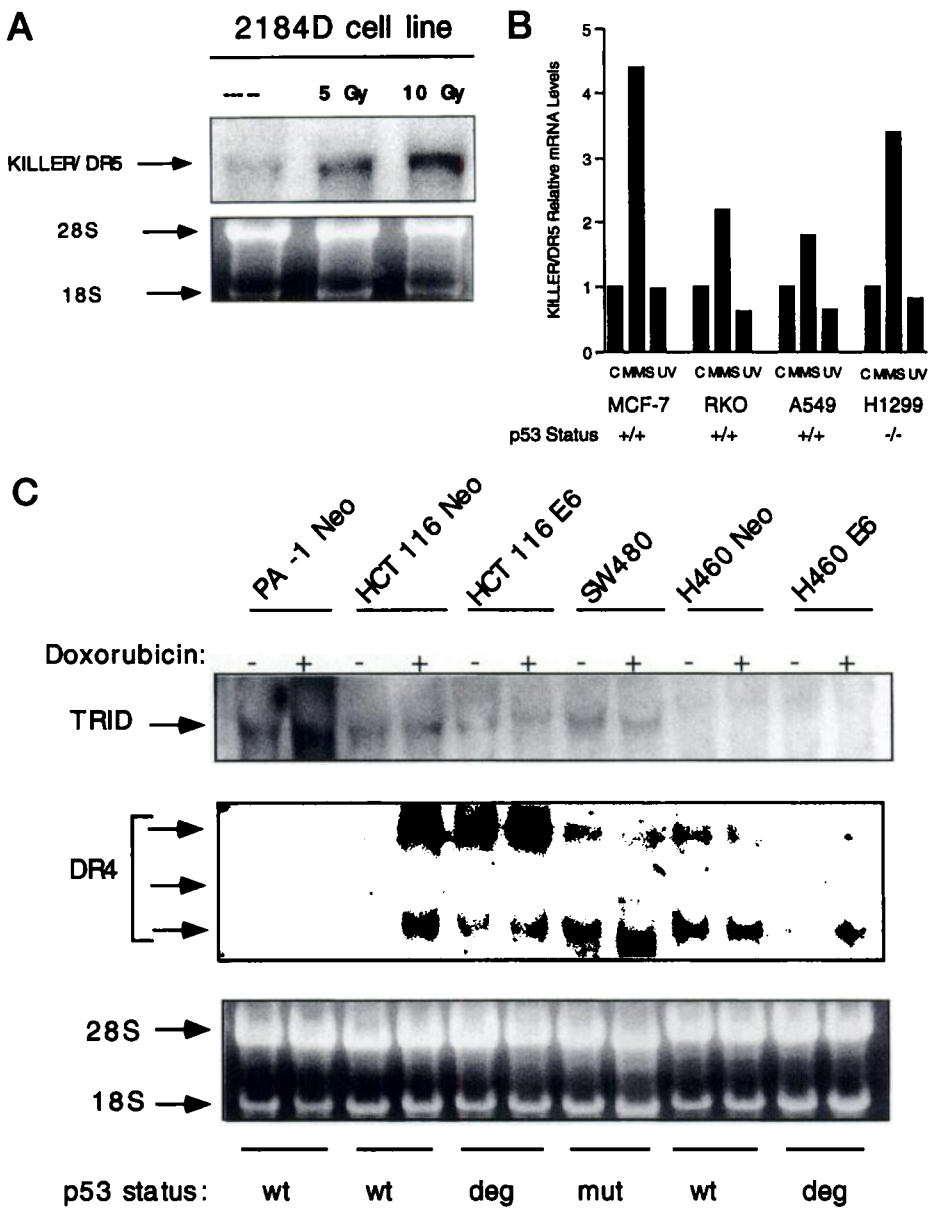

Fig. 1. The effect of genotoxic agents on KILLER/DR5, TRID, and DR4 expression in human cancer cell lines. *A*, IR regulation of KILLER/DR5 in 2184D lymphoblastoid cells. Logarithmically growing 2184D lymphoblastoid cells were either untreated (−) or exposed to 5–10 Gy of IR and harvested at 10 h. RNA extraction and Northern hybridizations were performed as described in "Materials and Methods." An ethidium bromide staining of the same gel shows RNA integrity and equal loading. *B*, MMS and UV irradiation effect on KILLER/DR5. Logarithmically growing p53 wild-type (MCF-7, RKO, and A549) and p53-null (H1299) cells were treated with MMS (100 μg/ml) or exposed to UV (14 J/m^2) and harvested 4 h later. RNA extraction and quantitative dot-blot hybridization were performed as described in "Materials and Methods." Values are expressed relative to untreated controls, each of which was given an arbitrary value of 1. Experiments were repeated to confirm the reproducibility of results. *Columns*, representative results of one experiment. *C*, doxorubicin effect on the *TRID* and *DR4* mRNA expression in human cancer cells. Cells were either left untreated or treated with an apoptosis-inducing concentration of (0.3 μg/ml) of doxorubicin. Northern hybridizations were performed as described in "Materials and Methods." Ethidium bromide staining of the gel shows RNA integrity and comparable loading in each lane.

DR4 were not regulated by IR or other DNA-damaging agents (data not shown).

Fig. 2 shows the kinetics of IR-induced *KILLER/DR5*, *TRID*, and *TRAIL* mRNA accumulation in ML-1 cells. As is shown, the IR-induced increases in the *KILLER/DR5* mRNA level occurred 2 h postirradiation, reached maximum by 4 h, and declined thereafter, with the levels approximately 2-fold above the baseline by 24 h. The IR-induced TRID mRNA accumulation, although less pronounced, was also noted within 2 h, and the maximal effect occurred by 8 h and declined thereafter (Fig. 2). IR, on the other hand, did not alter the expression of TRAIL in these cells (Fig. 2). The kinetics of IR-induced apoptosis was also monitored in these cells (Fig. 2*B*); cells exhibiting characteristic morphological features of apoptosis (chromatin condensation and nuclear fragmentation; Fig. 2*C*) were identified following staining with DAPI and using fluorescent microscopy. The percentage *KILLER/DR5* response to 20 Gy of IR is plotted against the total number of cells exhibiting apoptotic features (Fig. 2*B*) to demonstrate that the onset of IR-mediated increase in the *KILLER/DR5* expression precedes IR-induced apoptosis. In the solid tumor cell lines, MCF-7, A549, and RKO, which carry wild-type p53, the induction of *KILLER/DR5* expression was noted 4 h post-IR, but clear morphological features of apoptosis became evident only after 48–72 h. *KILLER/DR5* expression was also noted to precede apoptosis induced by adenovirus-mediated p53 infection of ovarian cancer cell line SKOV3.[3] Taken together, these findings suggest that the IR induction of *KILLER/DR5* expression occurs in a p53-dependent manner, precedes IR-induced apoptosis, and does not appear to be the secondary effect of IR-induced apoptosis.

TNF-α is a nongenotoxic cytokine, and it induces apoptosis via TNFR1 and engages caspase cascade via adaptor molecules FADD and FLICE1 (caspase 8; Ref. 23). Dominant-negative versions of FADD or FLICE1 independently block the apoptotic signals originating at TNFR1 (23). TNF-α is not a ligand for KILLER/DR5, and at least three studies have shown that the dominant-negative version of FADD does not block the KILLER/DR5-induced apoptosis (11–13). Walczak *et al.* (14) have, however, shown that the dominant-negative FADD did, indeed, block KILLER/DR5-induced apoptosis. On the

[3] Unpublished data.

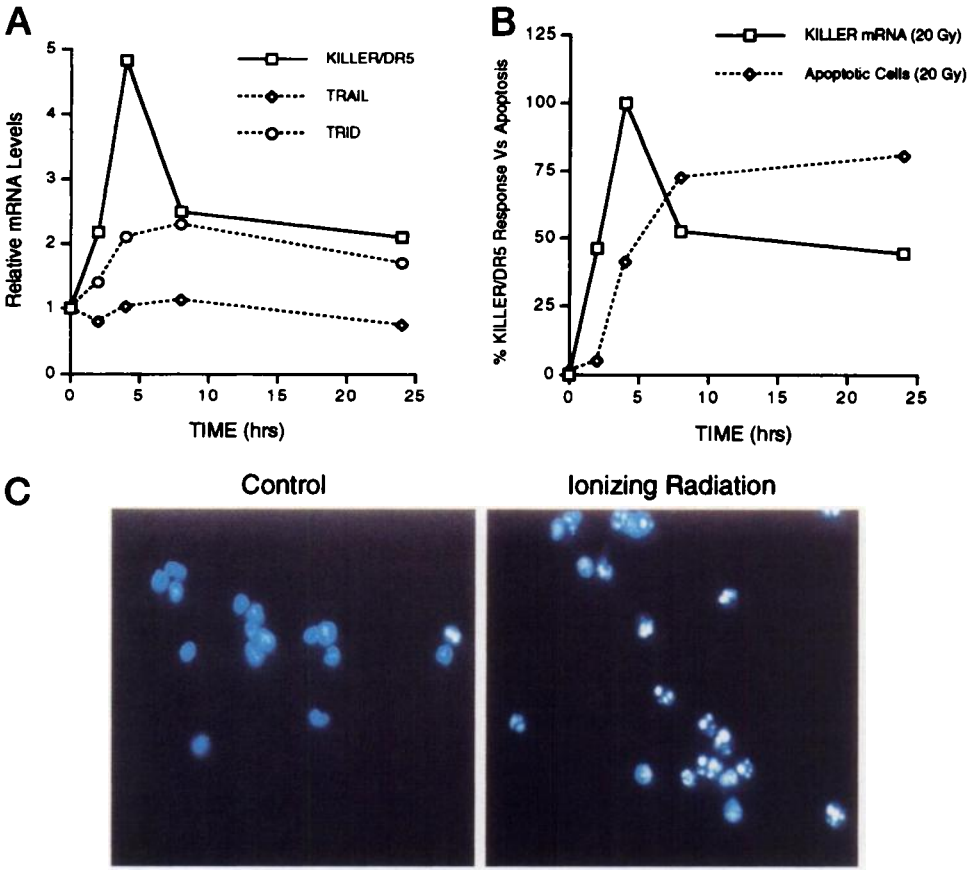

Fig. 2. *A*, time course of IR effect on *KILLER/DR5*, *TRID*, and *TRAIL* expression in ML-1 myeloid leukemia cells. Logarithmically growing cells were exposed to 20 Gy of γ-radiation and harvested after indicated periods of time. RNA extraction and quantitative dot-blot hybridization were performed as described in "Materials and Methods." The representative results for *KILLER/DR5*, *TRID*, and *TRAIL* were obtained after sequential hybridization of the same blot. *B*, comparison between the kinetics of IR-induced *KILLER/DR5* mRNA accumulation and IR-induced apoptosis. *Data points*, percentage response to IR against IR-induced apoptotic index, which was determined by directly counting the number of apoptotic nuclei over total number of nuclei counted. The percentage *KILLER/DR5* response was determined by considering the mRNA values at 4 h as 100% response. *C*, representative photomicrographs showing the IR-induced apoptosis in ML-1 cells.

other hand, CrmA, a cow pox serpin, has been shown to block the apoptotic signals that originate both from the TNFR1 (24, 25) and KILLER/DR5 (11–14). Thus, although it is not yet clear whether TNFR1 and KILLER/DR5 engage different or similar upstream adaptor molecules, the signals from both of these receptors, nevertheless, converge to activate similar downstream caspases (11–14). We next sought to investigate whether the *KILLER/DR5* mRNA expression was also affected during or prior to TNF-α-induced cell death. We monitored the TNF-α effect on the expression profile of *KILLER/DR5* gene in five different tumor cell lines with wild-type (MCF-7, A549, and RKO) and mutant (H1299 and HeLa) p53 status. Our results demonstrate that TNF-α treatment enhanced the *KILLER/DR5* mRNA levels in all five cell lines, irrespective of p53 status. Fig. 3*A* shows the representative results of TNF-α modulation of *KILLER/DR5* expression in human cancer cells. Treatment with 20–40 ng of TNF-α resulted in near maximal induction of *KILLER/DR5* expression by approximately 4 h (Fig. 3*A*). TNF-α only minimally enhanced the TRID expression in H1299 cells and had no effect in MCF-7 cells; TNF-α also did not alter the expression of *DR4* and *TRAIL* in these cell lines (data not shown).

TNF-α, at higher concentrations, has been shown to induce apoptosis in MCF-7 cells (24). We noted in this study that MCF-7, H1299, and A549 cells exhibited some degree of apoptosis in response to prolonged (72 h) treatment with TNF-α. Because maximal induction of TNF-α-mediated *KILLER/DR5* mRNA accumulation occurred by approximately 4–8 h (long before the morphological features of TNF-α-induced apoptosis became evident), it is reasonable to suggest that the TNF-α-induction of *KILLER/DR5* is a primary effect and not the consequence of TNF-α-induced apoptosis. TNF-α could regulate the expression of *KILLER/DR5* at transcriptional and/or posttranscriptional level. To gain insight into the potential mechanism of TNF-α regulation of *KILLER/DR5* expression, we used protein synthesis inhibitor CHX and transcription inhibitor ActD. Fig. 3*B* illustrates that 8-h treatment with CHX alone did not affect *KILLER/DR5* mRNA levels but significantly enhanced TNF-α induction of *KILLER/DR5* mRNA expression. These results demonstrate that new protein synthesis is not required during TNF-α-mediated increases in *KILLER/DR5* mRNA levels. Unlike CHX, ActD blocked TNF-α-mediated increases in *KILLER/DR5* mRNA levels (Fig. 3*B*), suggesting that TNF-α enhancement of *KILLER/DR5* expression appears transcriptional. TNF-α did not alter the stability of *KILLER/DR5* message because the half-life of *KILLER/DR5* mRNA in the absence and presence of TNF-α was essentially similar, approximately 4 h (Fig. 3*C*). These results, thus, strongly support the contention that TNF-α appears to enhance the rate of *KILLER/DR5* gene transcription.

CrmA has been shown to block TNF-α-induced apoptosis, but it does not block TNF-α activation of NFκB and Jun NH_2-terminal kinase activation (24, 26). To further investigate the relationship between TNF-α enhancement of *KILLER/DR5* and TNF-α-induced apoptosis, we used cells stably transfected with CrmA. As shown in Fig. 3*D*, CrmA did not affect TNF-α enhancement of *KILLER/DR5* mRNA expression in two different CrmA transfected cell lines. Similar results were obtained in CrmA-transfected H1299 cells (data not shown). Cumulatively, these results further substantiate that TNF-α-induction of KILLER/DR5 expression is a primary effect and not the consequence of TNF-α-induced apoptosis.

In this study, we have demonstrated that IR predominately enhanced the *KILLER/DR5* expression in p53 wild-type cells, the majority of which also undergo IR-induced apoptosis. Introduction of exogenous wild-type *p53* transgene into p53-negative cells also stimulates the expression of endogenous *KILLER/DR5* (10). The p53-mediated increase in the endogenous *KILLER/DR5* expression precedes p53-induced apoptosis.[3] Thus, IR-induced apoptosis may occur, in part, via p53-mediated increases in the KILLER/DR5 expression

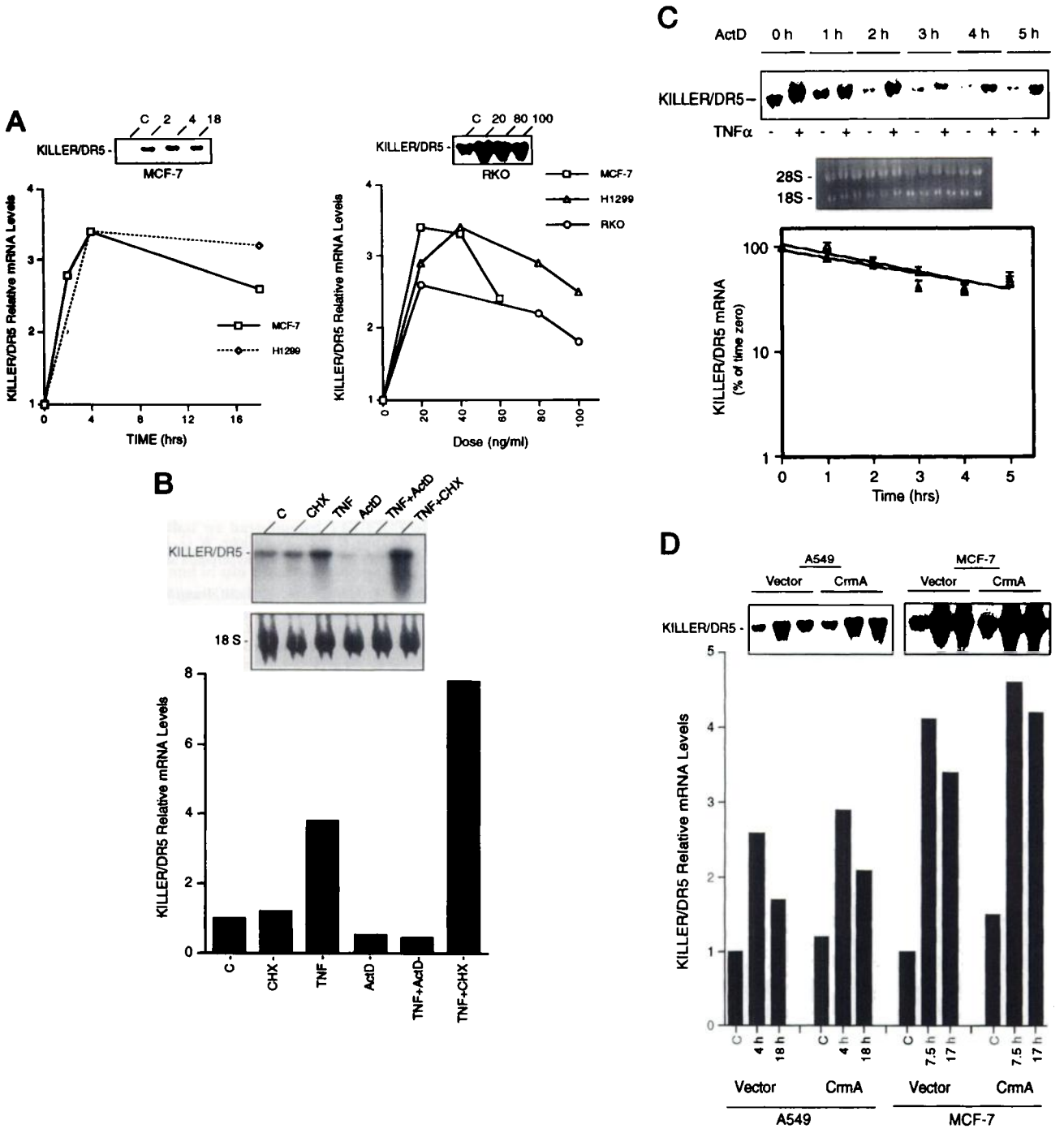

Fig. 3. *A*, dose response and time course of TNF-α regulation of *KILLER/DR5* mRNA expression in human cancer cells. p53 wild-type (MCF-7, RKO, and A549) and p53-null (H1299) cells were treated with TNF-α (20 ng/ml for MCF-7 and A549 and 40 ng/ml for H1299) for indicated periods of time or with indicated doses of TNF-α for 4 h. Representative Northern blots show TNF-α effects on *KILLER/DR5* mRNA levels in MCF-7 and RKO cells. *B*, CHX and ActD effect on TNF-α regulation of *KILLER/DR5* expression in MCF-7 cells. Logarithmically growing cells were either untreated or treated with 20 ng/ml TNF-α in the presence or absence of CHX (10 μg/ml) or ActD (4 μg/ml) for approximately 8 h. RNA extraction and Northern blot hybridizations were performed as described in "Materials and Methods." *C*, *KILLER/DR5* mRNA half-life in untreated and TNF-α-treated MCF-7 cells. Cells were either left untreated or treated with TNF-α (20 ng/ml) for 4 h; ActD (4 μg/ml) was then added, and cells were harvested at 1-h interval from the point at which ActD was added. The decay of *KILLER/DR5* mRNA was determined by quantitative Northern blot hybridizations. Values are percentage values, with the values at time 0 being defined as 100%. △, samples without TNF-α treatment; ▲, TNF-α-treated samples. *Data points*, mean of four determinations (1–3 h) and two determinations (4–5 h); *bars*, SE. The half-life of the *KILLER/DR5* mRNA was determined from the computer-generated curves. *D*, TNF-α regulation of *KILLER/DR5* in mock- or CrmA-transfected A549 and MCF-7 cells. Logarithmically growing mock- or CrmA-transfected A549 and MCF-7 cells were treated with 20 ng/ml TNF-α for indicated periods of time. RNA extraction and Northern blot hybridizations were performed as described in "Materials and Methods." A representative Northern blot for each cell line is shown; RNA integrity and equal loading was determined by ethidium bromide staining of the gel.

and subsequent activation of downstream caspases. Another important finding demonstrated in this study is the lack of a UV irradiation effect on *KILLER/DR5* expression, which is in striking contrast to the UV effect on the other p53-regulated genes. UV irradiation has been shown to enhance the expression of $p21^{WAF1/CIP1}$, *GADD45*, and *MDM2* in the same cell lines (17, 18), in which UV-irradiation did not induce *KILLER/DR5* expression (this study). The absence of UV effect on *KILLER/DR5* mRNA levels demonstrates that the regulation of its expression is insensitive to UV-type DNA damage. UV irradiation is known to induce apoptosis in these cells, yet it did not alter the

KILLER/DR5 mRNA levels. In the p53-negative cells, the IR-induced apoptosis also occurred in the absence of *KILLER/DR5* induction. Taken together, these findings highlight the dissociation of *KILLER/DR5* regulation and apoptosis and indicate that (*a*) the *KILLER/DR5* gene expression is regulated in a cell type- and trigger-specific manner and (*b*) its induction is not a general/secondary cellular response of all types of apoptotic stimuli.

TNF-α also stimulated the expression of *KILLER/DR5*; TNF-α regulation of *KILLER/DR5* appears to occur in a p53-independent manner. The pleiotropic effects of TNF-α are mediated via TNFR1 and TNFR2 (26). TNF-α-mediated apoptotic signals are predominantly transduced via TNFR1, whereas the TNF-α engagement of TNFR2 activates anti-apoptotic NFκB (26 and refs. therein). TNF-α is believed to also activate NFκB via TNFR1 (26). TNF-α also induces Jun NH_2-terminal kinase activity (26) and may consequently activate activator protein-1. TNF-α did not alter the stability of *KILLER/DR5* mRNA, and thus, TNF-α effect on *KILLER/DR5* appears transcriptional. Whether TNF-α-induced KILLER/DR mRNA accumulation occurs via NFκB and/or activator protein-1-mediated transcriptional activation is an issue that remains to be investigated. Because TNF-α-induced *KILLER/DR5* mRNA accumulation was noted in MCF-7 cells that have been reported to express only TNFR1 (26), it appears likely that the TNF-α enhancement of *KILLER/DR5* expression occurs via signals relayed from TNFR1. KILLER/DR5, when expressed at higher levels, has been shown to induce apoptosis in the absence of its ligand (11–15). Our demonstration that TNF-α enhanced the mRNA levels of *KILLER/DR5* but had no effect on the mRNA levels of its closely related DR, DR4, and their ligand TRAIL are novel findings. These results, thus, support the notion that TNF-α may mediate its apoptotic effects by engaging the downstream caspase cascade not only through its own receptor (TNFR1) but also by increasing the levels of the related DR KILLER/DR5. Further studies are needed to test whether TNF-α-mediated increases in the *KILLER/DR5* mRNA levels are coupled with similar increases at the protein levels.

KILLER/DR5 and DR4, the closely related DRs, are generally expressed in same types of cells, bind the same ligand (TRAIL), engage the same downstream caspase cascade, and, therefore, appear functionally redundant. The physiological rationale for the coexistence of functionally redundant KILLER/DR5 and DR4 in the same cell type remains unclear. Our results demonstrate that TNF-α and genotoxic stress selectively regulate *KILLER/DR5* but not *DR4* (*DR4* was regulated by doxorubicin in HCT116 Neo/E6 cells). Hence, the evidence of the differential regulation of *KILLER/DR5* and *DR4* genes in the same cell type in response to apoptotic stimuli may provide some explanation as to why these two DRs with redundant function coexist in a given cell. Because independent overexpression of these DRs results in induction of ligand-independent apoptosis, it can be envisioned that certain apoptotic stimuli may mediate their apoptotic effects, in part, by selectively up-regulating the levels of either of the two molecules.

In conclusion, we have demonstrated that the DR *KILLER/DR5* gene expression is induced by DNA damage, p53, and TNF-α. The regulation of *KILLER/DR5* gene expression is not secondary to DNA damage or p53- or TNF-α-induced apoptosis but may play an active role in mediating the apoptotic effects of these agents. The regulation of *KILLER/DR5* gene expression, however, occurs in a cell type- and trigger-specific manner. On the basis of several lines of evidence, we also conclude that *KILLER/DR5* appears to be a bona fide target of p53. For example, one consistent finding has been that the genotoxic agents IR and doxorubicin increase the expression of p53-regulated genes only in p53 wild-type cells (27). From our results, it is clear that only *KILLER/DR5* was regulated by both of these agents in a manner similar to other p53-regulated genes; none of the other related genes, such as *DR4*, *TRAIL*, or *TRID*, exhibited a similar pattern of regulation.

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