Augmentation of Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL)-induced Apoptosis by the Synthetic Retinoid 6-[3-(1-Adamantyl)-4-hydroxyphenyl]-2-naphthalene Carboxylic Acid (CD437) through Up-Regulation of TRAIL Receptors in Human Lung Cancer Cells

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis via the death receptors DR4 and DR5 in different transformed cells in vitro and exhibits potent antitumor activity in vivo with minor side effects. The synthetic retinoid CD437 is a potent inducer of apoptosis in cancer cells through increased levels of death receptors. We demonstrate that treatment of human lung cancer cells with a combination of suboptimal concentrations of CD437 and TRAIL enhanced induction of apoptosis in tumor cell lines with wild-type p53 but not in normal lung epithelial cells. CD437 up-regulated DR4 and DR5 expression. The CD437 and TRAIL combination enhanced activation of caspase-3, caspase-7, caspase-8, and caspase-9 and the subsequent cleavage of poly(ADP-ribose) polymerase and DNA fragmentation factor 45. Caspase inhibitors blocked the induction of apoptosis by this combination. Moreover, this combination induced Bid cleavage and increased cytochrome c release from mitochondria. These results suggest that the mechanism of enhanced apoptosis by this combination involves p53-dependent increase of death receptors by CD437, activation of these receptors by TRAIL, enhanced Bid cleavage, release of cytochrome c, and activation of caspase-3, caspase-7, caspase-8, and caspase-9. These findings suggest a novel strategy for the prevention and treatment of human lung cancer with the CD437 and TRAIL combination.

INTRODUCTION

TRAIL (also called APO-2 L), a type II membrane protein belonging to the tumor necrosis factor death ligand cytokine family, induces apoptosis in a wide variety of transformed cells. However, unlike other members of this family, TRAIL does not seem to be cytotoxic to normal cells in vitro (1–3). Histological analysis of TRAIL-treated tumors revealed an increase in apoptotic cells and confirmed the ability of TRAIL to induce apoptosis in vivo in an animal model without toxicity toward normal tissue (3, 4). Thus, TRAIL is different from the death ligands tumor necrosis factor and Fas ligand which, in addition to inducing apoptosis in cancer cells, cause an inflammatory response and liver damage, respectively, when administered systemically (5). Therefore, TRAIL is considered to be a tumor-selective, apoptosis-inducing cytokine and a promising new candidate for cancer prevention and treatment (5–7).

TRAIL induces apoptosis by interacting with two death domain-containing death receptors DR4 (also known as TRAIL-R1) and DR5 (also called TRAIL-R2, TRICK2, and KILLER/DR5; Refs. 5 and 8). TRAIL signaling involves recruitment of caspase-8 or caspase-10 via Fas-associated death domain or an unknown adaptor protein and activation of the caspase cascade, resulting in apoptotic cell death (5, 8). TRAIL can also bind to three decoy receptors, DcR1 (TRAIL-R3 or TRID), DcR2 (TRAIL-R4 or TRUNDD), and OPG, that contain either no cytoplasmic death domain or truncated death domain and can compete with DR4 and DR5 for ligand binding, thereby acting as an antagonist (5, 8). TRAIL and its receptors DR4 and DR5 are expressed widely in both normal and malignant cells, whereas DcR1 and DcR2 are expressed preferentially in many normal tissues but in only a few transformed cells (5, 8). Therefore, it was suggested that the low toxicity of TRAIL toward normal tissues is attributable to the expression of decoy receptors in normal tissues, which can protect normal cells from induction of apoptosis by TRAIL (5, 8).

TRAIL-induced apoptosis was suggested to be independent of p53 (5, 9). Likewise, p53-independent induction of DR5 expression was observed (10–12). However, TRAIL signaling may be related to p53 because DR5 can be regulated by p53 (10, 13–15) and can be increased by p53-elevating DNA-damaging agents in tight association with induction of apoptosis (10, 13, 16, 17). Thus, it is plausible to assume that agents, which up-regulate the expression of death receptors DR4, DR5, or both, will augment TRAIL-induced apoptosis. Indeed, chemotherapeutic agents such as DOX, 5-Fu, VP-16, and CPT-11 show synergy in apoptosis induction when combined with TRAIL in certain types of cancer cell both in vitro and in vivo (18–20). The molecular mechanism underlying the enhanced efficacy of these combinations is not well understood.

The synthetic retinoid CD437 is a potent inducer of apoptosis in a variety of cancer cell types including human breast cancer (21), melanoma cells (22), cervical cancer (23), leukemia (24), and NSCLC (25–27) cells. In previous studies, we have demonstrated that CD437 induces apoptosis in human NSCLC cells through both p53-dependent and p53-independent pathways (16, 28). CD437 increased the level of p53 protein and subsequently induced the expression of p53-regulated genes such as Bax, p21 (WAF1/CIP1), and DR5, which eventually triggered apoptosis through induction of cytochrome c release from mitochondria and caspase-3 activation (16, 28).

Because CD437 induces DR5 expression in a p53-dependent manner in human NSCLC cells (16), we hypothesized that CD437 may augment TRAIL-induced apoptosis in human NSCLC cells, particu-
larly those with wild-type p53. To test this hypothesis, we examined the effects of CD437 and TRAIL combination on apoptosis induction in human NSCLC cell lines. This combination exhibited more than additive induction of apoptosis, which was p53 dependent.

**MATERIALS AND METHODS**

**Reagents.** CD437 was provided by Dr. B. Shroot (Galderma R&D, Sophia Antipolis, France). It was dissolved in DMSO at a concentration of 10 mM and stored in the dark at ~80°C under N2 atmosphere. Stock solutions were diluted to the desired final concentrations with growth medium just before use. Soluble recombinant human TRAIL was purchased from Biomol (Plymouth Meeting, PA). rhDr2:Fc and rhOpg:Fc were purchased from Alexius Biochemicals (San Diego, CA). The caspase inhibitors Z-VAD-FMK, Z-DEVd-FMK, Z-IEHD-FMK, and Z-LEHD and fluorogenic caspase substrates Ac-Asp-Glu-Val-Asp-AFC, Ac-Ile-Glu-Thr-Asp-AFC, and Ac-Leu-Glu-His-Asp-AFC were purchased from Enzyme System Products (Livermore, CA). DOX, CDDP, VP-16, and 5-FU were purchased from Sigma Chemical Co. (St. Louis, MO).

**Cell Lines and Cell Culture.** Human NSCLC cell lines H460, A549, and H1944, which possess wild-type p53, and NSCLC cell lines H596, H157, and H1792, which express mutant or no p53 (29), were either obtained from Dr. A. Gazdar (University of Texas Southwestern Medical Center, Dallas, TX) or purchased from the American Type Cell Culture (Rockville, MD). H460 cell lines transfected with either Neo or HPV-16 E6 as described previously (16) was obtained from Dr. W. S. El-Deiry (University of Pennsylvania School of Medicine, Philadelphia, PA). These cells were grown in monolayer culture in a 1:1 (v/v) mixture of DMEM and Ham’s F-12 medium supplemented with 5% fetal bovine serum and antibiotics at 37°C in a humidified atmosphere consisting of 5% CO2 and 95% air. In addition, we also used normal human NHBE and SAEC cells, which were purchased from Clonetics (San Diego, CA). These cells were grown in BEGM BulletKit (Clonetics) at 37°C in a humidified atmosphere consisting of 5% CO2 and 95% air.

**Northern Blot Analysis.** Preparation of total cellular RNA and the Northern blotting were described previously (30). Thirty µg of total RNA were loaded in each lane. Human Killer/DR5 cDNA was obtained from Dr. W. S. El-Deiry. Human Bax cDNA was provided by Dr. S. J. Korshmeyer (Washington University School of Medicine, Saint Louis, MO). Human DR4, Dr2, and Dd2 cDNAs were purchased from Alexis Biochemicals. Human glyceraldehyde-3-phosphate dehydrogenase cDNA was purchased from Ambion, Inc. (Austin, TX).

**Western Blot Analysis.** Preparation of whole-cell lysates and the Western blotting were described previously (28). Fifty µg of protein were loaded in each lane. Mouse monoclonal anti-Bcl-2 (100), rabbit polyclonal anti-Bcl-XL (S-18), and goat polyclonal anti-Bid (C-20) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal anti-Bax antibody, mouse monoclonal anti-caspase-3 (clone 19), mouse monoclonal anti-caspase-7 (clone B94-1), mouse monoclonal anti-cytochrome c (clone 7H8.2C12), and mouse monoclonal anti-caspase-9 (clone B40) antibodies were purchased from Pharmingen (San Diego, CA). Mouse monoclonal anti-caspase-8 (clone S57F), rabbit polyclonal anti-caspase-10, and rabbit polyclonal anti-DR5 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal PARP antibody (VICT 5) and rabbit polyclonal β-actin antibody were purchased from Roche Molecular Biochemicals (Indianapolis, IN) and Sigma, respectively.

**DNA Fragmentation Assay.** Cells were plated in 96-well cell culture plates or 10-cm-diameter dishes 1 day before treatment. After 24 h treatment, DNA fragmentation was evaluated by examination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) using a Cell Death Detection ELISA kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. In addition, DNA fragments with 3’-hydroxyl ends were quantitated using an APO-DIRECT TUNEL kit (Phoenix Flow Systems, Inc., San Diego, CA) following the manufacturer’s protocol.

**Measurement of Cytochrome c Release.** Cells were plated onto 10-cm-diameter dishes 1 day before treatment. After the cells were exposed to CD437 for the indicated time, both floating and attached cells were harvested, and cytosolic extracts were prepared as described previously (16).

**Measurement of Caspase Activity.** Cells were plated onto 10-cm-diameter dishes 1 day before treatment. After the cells were exposed to CD437 for different times, both floating and attached cells were harvested by trypsinization and counted. Caspase activity was measured as described by Deveraux et al. (31). The fluorogenic substrate Ac-Asp-Glu-Val-Asp-AFC (for caspase-3), Ac-Ile-Glu-Thr-Asp-AFC (for caspase-8), and Ac-Leu-Glu-His-Asp-AFC (for caspase-9) were used, respectively.

**RESULTS**

**Augmented Induction of Apoptosis by CD437 and TRAIL Combination in H460 NSCLC Cells.** Because we have shown previously that CD437 induced DR5 expression in H460 cells (16, 28), we first examined the effect of combination of CD437 with TRAIL on apoptosis induction in these cells. As shown in Fig. 1, A and B, this combination caused additive or more than additive induction of apoptosis evidenced by the ELISA method. The lowest concentrations of CD437 and TRAIL required for this effect were 0.1 µM and 5 ng/ml, respectively. This finding was confirmed by the TUNEL-flow cytometric apoptosis assay (Fig. 1C). At a concentration of 0.2 µM, CD437 enhanced TRAIL-induced apoptosis, whereas several chemotherapeutics agents including CDDP, VP-16, 5-FU, and DOX used at the same concentration did not enhance induction of apoptosis when combined with 10 ng/ml TRAIL in the H460 cells (Fig. 1D).

**CD437 Augments TRAIL-induced Apoptosis by Up-Regulating the Expression of Death Receptors DR4 and DR5.** Previously, we reported that 1 µM CD437 can increase expression of DR5 in certain NSCLC cell lines (16). To understand the mechanism underlying the interaction between suboptimal CD437 concentration and TRAIL, we next analyzed whether 0.2 µM CD437 up-regulates the expression of DR4 and DR5 in H460 cells. As shown in Fig. 2A, CD437 at 0.2 µM indeed induced the expression of both death receptors. TRAIL itself neither induced nor enhanced the effect of CD437 on the expression of these genes. DcR1 and DcR2 mRNAs were neither detected in H460 cells nor induced by CD437 or CD437 plus TRAIL (data not shown). CD437 did not change Bax expression (data not shown). Soluble rhOpg:Fc or rhDr2:Fc abolished the augmented induction of apoptosis by the CD437 and TRAIL combination in H460 cells (Fig. 2B). Taken together, these results indicate that enhanced induction of apoptosis by combination of CD437 and TRAIL is attributable to the up-regulation of DR4 and DR5 by CD437.

**The Effects of Combination of CD437 and TRAIL on Apoptosis Induction in Human NSCLC Cells Is Dependent on p53 Status.** Previously, we demonstrated that CD437 induced DR5 expression in a p53-dependent fashion in human NSCLC cell lines (16). The combination of CD437 and TRAIL caused enhanced induction of apoptosis in several NSCLC cell lines with wild-type p53 but not in NSCLC cell lines with mutant p53 (Fig. 3A), even when the concentration of CD437 was increased to 0.5 µM in the cell lines with mutant p53 (data not show). Furthermore, the augmented induction of apoptosis by the CD437 and TRAIL combination, which was observed in H460 cells transfected with Neo control gene, was not observed in H460 cells transfected with HPV-16 E6 gene, in which p53 protein has been degraded (Fig. 3B). These results further support the conclusion that the interaction between CD437 and TRAIL in apoptosis induction in human NSCLC cells is dependent on p53 status.

**CD437 Does Not Augment TRAIL-induced Apoptosis in Normal Human Lung Epithelial Cells.** The finding that CD437 augments TRAIL-induced apoptosis in human NSCLC cells in a p53-dependent manner raised the possibility that the combination of these agents may cause enhanced induction of apoptosis in normal human lung cells, which express wild-type p53. To address this question, we compared and contrasted the effects of CD437 and TRAIL combination on apoptosis in normal human lung epithelial cells and H460 NSCLC cells. As shown in Fig. 3C, each of the agents induced
induction of apoptosis in H460 cells but not in the two types of normal human lung epithelial cells (i.e., NHBE and SAEC). Furthermore, the combination of CD437 and TRAIL induced more than additive effects on H460 but not on normal cells, indicating that the combination targets lung cancer cells selectively.

Involvement of Caspases in Augmented Induction of Apoptosis by CD437 and TRAIL Combination. Caspase activation is known to be a critical event in signaling death receptor-mediated apoptotic pathway (5). To determine which caspases are involved in apoptosis induced by the combination of CD437 and TRAIL, we analyzed their effects on the pattern of caspase activation in H460 cells. CD437 and TRAIL alone did not activate or only slightly activated the initiator caspases caspase-8 and caspase-9. However, the combination of CD437 and TRAIL resulted in activation of both caspases, as evidenced by their auto-cleavage (activation; Fig. 4A). The enhanced activation of these two caspases by the combination was also reflected by measuring caspase-8 and especially caspase-9 activity (Fig. 4B). The combination of CD437 and TRAIL did not activate caspase-10 because no cleavage was detected. A similar analysis of effector caspases revealed that the combination of CD437 and TRAIL resulted in enhanced cleavage of both caspase-3 and caspase-7 and the subsequent cleavage of their substrates PARP and DFF45 (Fig. 4C). Caspase-3 activity was also enhanced in cells treated with the combination relative to each agent alone (Fig. 4D). The enhanced induction of apoptosis by the combination of CD437 and TRAIL could be blocked by the pan caspase inhibitor Z-VAD-FMK, the caspase-3 inhibitor Z-DEVD-FMK, the caspase-8 inhibitor Z-IETD, and the caspase-9 inhibitor Z-LEHD-FMK, respectively (Fig. 5). Taken together, these results demonstrate that initiator caspases caspase-8 and caspase-9 and effector caspases caspase-3 and caspase-7 are involved in mediating the augmented induction of apoptosis by the CD437 and TRAIL combination.

Enhanced Induction of Cytochrome c Release from Mitochondria by CD437 and TRAIL Combination. Because the CD437 and TRAIL combination caused enhanced activity of caspase-9, it was plausible to assume that cytochrome c release from mitochondria would also be increased because it is involved in caspase-9 activation (32). As shown in Fig. 6, treatment of H460 cells with the CD437 and TRAIL combination resulted in enhanced cytochrome c release compared with CD437 or TRAIL alone, which only weakly induced cytochrome c release.

Effects of the Combination of CD437 and TRAIL on the Expression of Bcl-2 Family Genes in H460 Cells. Because Bcl-2 family genes are known to play important roles in regulating apoptosis (33), we also analyzed the effects of the combination of CD437 and TRAIL on the expression of Bcl-2 family genes. The expression of Bcl-2, Bax, and Bcl-XL in H460 cells was not altered by CD437 or TRAIL or by their combination. In contrast, the level of Bid protein was decreased in cells treated with the CD437 and TRAIL combination (Fig. 7). Because Bid can be activated through cleavage by caspase-8 during apoptosis (34, 35), it is possible that the decrease in the level of Bid in the cells treated with CD437 and TRAIL combination is attributable to its cleavage by caspase-8.

DISCUSSION

In this study, we have demonstrated that the synthetic retinoid CD437 in combination with the death ligand TRAIL caused enhanced induction of apoptosis in certain human NSCLC cell lines at concentrations that each agent alone is ineffective or only poorly effective. Because at these concentrations, CD437 could still induce the expression of the death receptors DR4 and DR5 and addition of soluble rhDr2R2:Fc and rhOPG:Fc to the cells, which are supposed to compete with DR4 and DR5 for binding TRAIL, suppressed the augmented induction of apoptosis by the CD437 and TRAIL combination, we conclude that CD437 augmented TRAIL-induced apoptosis by up-regulating DR4 and DR5 expression. The concentrations of CD437 and TRAIL, which augmented apoptosis induction, were 0.2 μM and 10 ng/ml, respectively. At such low concentration, CD437 was more effective in inducing apoptosis in human NSCLC cells than cytotoxic chemotherapeutic agents such as DOX, CDDP, VP-16, and 5-FU when combined with 10 ng/ml TRAIL. DOX, 5-FU, or VP-16 have...
been reported to enhance TRAIL-induced apoptosis in human breast cancer cells (18, 19). However, the concentrations for either chemotherapeutic agents or TRAIL were much higher than used here (5 μM, 30 mM, and 100 μM for DOX, 5-FU, and VP-16, respectively, and 200–25,000 ng/ml for TRAIL; Refs. 18 and 19).

TRAIL-induced apoptosis has been reported to be independent of p53 (5, 9). Likewise, the sensitization of human breast cancer cells to TRAIL-induced apoptosis by chemotherapeutic agents such as DOX and VP-16 were independent of p53 status (18, 19). However, the TRAIL receptor DR5 is regulated by p53 (10, 13–15). We found that augmentation of TRAIL-induced apoptosis by CD437 in human NSCLC cells was dependent on wild-type p53 as evidenced by the findings that the effect was observed only in human NSCLC cell lines carrying wild-type p53 and that targeting degradation of wild-type p53 protein by HPV-16 E6 abolished the interaction between CD437 and TRAIL on apoptosis induction in H460 cells. These results were consistent with our previous study that indicated that CD437 induces DR5 expression in a p53-dependent manner in human NSCLC cells (16). A recent study also showed a p53-dependent augmentation of TRAIL-induced apoptosis by radiation through up-regulation of DR5 in human breast cancer cells (36). It should be pointed out that the role of p53 in augmentation of TRAIL-induced apoptosis by CD437 might be dependent on cell types. Recently, we found that CD437 augmented TRAIL-induced apoptosis independently of p53 status in human prostate cancer cells (12).

The combination of CD437 and TRAIL failed to induce apoptosis in two normal human lung epithelial cells derived either from bronchus (NHBE) or from small airway (SAEC) in short-term culture, despite their presumed wild-type p53 status. This finding is important from a clinical point of view because it implies that the CD437 and TRAIL combination may selectively kill cancers while sparing normal cells. Although the mechanism of this selectivity is not known, it could be attributable to differences in expression levels of decoy receptors on cancer cells versus normal cells (12).
receptors or different regulation of death receptors by CD437 in lung cancer cells and normal lung epithelial cells.

Caspases play important roles in apoptosis triggered by various proapoptotic signals (37, 38). Ligation of death ligands (e.g., Fas ligand) with their receptors activates the caspase cascade, leading to apoptosis through adaptor molecules (5, 8). In general, activation of the caspase cascade requires both initiator caspases, such as caspase-8, caspase-9, and caspase-10, and effector caspases such as caspase-3 and caspase-7. Caspase-3 and caspase-7 cleave several key substrates such as PARP and DFF45, leading to apoptosis (37, 38). It is well known that both caspase-8 and caspase-9 can activate caspase-3 and caspase-7 in response to different death signals (37, 38). Caspase-8 is thought to mediate apoptosis signaled by death receptors such as Fas, whereas caspase-9 is thought to mediate apoptosis triggered by signals such as chemotherapeutic agents (37–39). However, possible cross-talk between caspase-8- and caspase-9-mediated apoptotic pathways may exist. Recent studies indicate that caspase-10, and that caspase-8 and caspase-9 inhibitors blocked apoptosis by this combination. These results indicate that caspase-8 and caspase-9, rather than caspase-10, are the initiator caspases involved in the caspase cascade leading to apoptosis by the combination of CD437 and TRAIL.

It is well known that both caspase-8 and caspase-9 can activate caspase-3 and caspase-7 in response to different death signals (37, 38). Caspase-8 is thought to mediate apoptosis signaled by death receptors such as Fas, whereas caspase-9 is thought to mediate apoptosis triggered by signals such as chemotherapeutic agents (37–39). However, possible cross-talk between caspase-8- and caspase-9-mediated apoptotic pathways may exist. Recent studies indicate that caspase-8 and caspase-9, rather than caspase-10, are the initiator caspases involved in the caspase cascade leading to apoptosis by the combination of CD437 and TRAIL.

Fig. 4. Augmented activation of initiator caspases caspase-8 and caspase-9 (A and B) and effector caspases caspase-3 and caspase-7 (C and D) and the cleavage of PARP and DFF45 (C) by CD437 and TRAIL combination in H460 cells. After 14 h treatment with 0.2 μM CD437 or 10 ng/ml TRAIL or CD437 plus TRAIL, both floating and attached cells were harvested, and whole-cell protein lysates were prepared for Western blot analysis (A and C) and caspase activity assay (B and D) as described in “Materials and Methods.” Columns, means of triplicate determinations; bars, SD. Casp, caspase.
TRAIL not only activated caspase-9 but also induced cytochrome c.

Bid, a Bcl-2 interacting protein that is activated (truncated) by caspase-8, can bridge these two pathways by mediating death receptor-triggered cytochrome c release from mitochondria.

In summary, we found that CD437 can augment TRAIL-induced apoptosis by CD437 through p53-dependent up-regulation of death receptors DR4 and DR5 in human NSCLC cells. Moreover, we provide evidence indicating the involvement of caspase-8, caspase-9, caspase-7, and caspase-3 in this effect. Our results also suggest a potential strategy of using CD437 in combination with TRAIL for prevention or treatment of certain lung cancers.

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