Fenretinide up-regulates DR5/TRAIL-R2 expression via the induction of the transcription factor CHOP and combined treatment with fenretinide and TRAIL induces synergistic apoptosis in colon cancer cell lines

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Abstract. Fenretinide (N-[4-Hydroxyphenyl]retinamide; 4HPR) is a semisynthetic retinoid that induces apoptosis in a variety of malignancies. Fenretinide has been examined in clinical trials as a cancer chemopreventive and chemotherapeutic agent. Oxidative stress induced by fenretinide has been shown to mediate apoptosis through a mitochondrial pathway by the induction of a transcription factor CCAAT/enhancer binding protein homologous protein (CHOP) and Bak. In this study, we report that fenretinide induces death receptor 5 (DR5)/TRAIL-R2 up-regulation via the induction of the transcription factor CHOP in colon cancer cell lines. Fenretinide induced DR5 expression at protein and mRNA levels. Furthermore, fenretinide increased DR5 promoter activity and the enhanced activity decreased by mutation of the CHOP binding site. CHOP was also up-regulated by fenretinide at the promoter level. We also showed that combined treatment with fenretinide and TRAIL induced synergistic apoptosis in colon cancer cell lines. The synergistic apoptosis was markedly blocked by DR5/Fc chimeric protein. Fenretinide and TRAIL cooperatively activated caspase-3, -8, -10 and -9 and cleavage of Bid and PARP, and this activation was also blocked in the presence of DR5/Fc chimeric protein. These results indicate that fenretinide-induced apoptosis is sensitized by TRAIL. Therefore, combined treatment with fenretinide and TRAIL might be a promising model for the treatment of colorectal cancer.

Introduction

Colorectal cancer is the fourth most common and ranks as the second leading cause of cancer death in the United States (1). Despite improvements in therapy against advanced colorectal cancer, the overall impact of chemotherapies remains limited and it is hard to prevent recurrences; hence, newer and more effective therapeutic regimens are clearly needed.

Fenretinide (N-[4-Hydroxyphenyl]retinamide; 4HPR) is a semisynthetic retinoid that induces apoptosis in cell lines derived from a variety of malignancies (2). The apoptotic pathway triggered by fenretinide remains unsettled. Both retinoic acid receptor (RAR)-independent and RAR-dependent effects of fenretinide-induced apoptosis have been described (3-6). In the RAR-independent pathway, the upstream signaling events induced by fenretinide include an increase in intracellular levels of ceramide, which is subsequently metabolized to GD3 (4,7,8). GD3 triggers the activation of reactive oxygen species (ROS) leading to the induction of 12-lipoxygenase (12-Lox) (8). The oxidative stress mediates apoptosis via the induction of the transcription factor CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) and the Bcl-2-family member protein Bak (8-10). Bak, which may be directly regulated by CHOP, induces the release of cytochrome-c from mitochondria. Cytochrome-c may activate caspase-9, which in turn activates caspase-3 to initiate the downstream processes of apoptosis (9,11). Fenretinide may exert greater therapeutic activity, because in contrast to other classical retinoic acids, it is able to induce apoptosis rather than differentiation and shows synergistic responses with chemotherapeutic drugs, such as cisplatin, carboplatin or etoposide, in different cell types (12,13). On the basis of these results, fenretinide has been examined in clinical trials as a cancer chemotherapeutic agent.
chemopreventive agent in a variety of malignancies, including a Phase III study (14).

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL/Apo2L) induces apoptosis selectively in cancer cells in vitro and in vivo, and has little or no toxicity to normal cells (15,16). Therefore, TRAIL is one of the most promising agents for cancer therapeutics and was recently examined in a Phase I study (17). However, some tumor types exhibit resistance to TRAIL (18).

Death receptor 5 (DR5; also called TRAIL-R2, or KILLER) is a member of the tumor necrosis factor receptor family. DR5 mediates TRAIL-induced apoptosis through the formation of a death-inducing signaling complex and caspase activation (19-22). Silencing of DR5 expression causes tumor cell growth and resistance against anti-tumor agents (23). Therefore, DR5 is considered to be an attractive candidate for use in cancer therapy.

In this study, we report, for the first time, that fenretinide up-regulates DR5 expression via the induction of the transcription factor CHOP, and combined treatment with fenretinide and TRAIL induces synergistic apoptosis in colon cancer cells.

Materials and methods

Reagents. Fenretinide and soluble recombinant human TRAIL/Apo2L were purchased from Toronto Research Chemicals (North York, Canada) and PeproTech (London, UK), respectively. Recombinant human DR5 (TRAIL-R2)/Fc chimera protein, and the caspase inhibitors zVAD-fmk, zDEVD-fmk, zETD-fmk, zLEHD-fmk, zAEVD-fmk, were purchased from R&D Systems (Minneapolis, MN).

Cell culture. Human colon cancer, SW480, SW620, HCT116 and HT29 cells were maintained in DMEM with 10% fetal bovine serum, 4 mmol/l L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. DLD-1 and HCT15 cells were maintained in DMEM with 10% bovine serum, 4 mmol/l L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. DLD-1 and HCT15 cells were incubated at 37˚C in a humidified atmosphere containing 5% CO2.

RNase protection assay and Northern blot analysis. Northern blot analysis was performed as previously described (24) using a full-length DR5 cDNA as a probe. The RNase protection assay was also performed as previously described (24).

Western blot analysis. Western blot analysis was performed as described previously (24). Rabbit polyclonal anti-GADD153 (CHOP) (1:200) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-DR5 (1:250) antibody (Cayman Chemical, Ann Arbor, MI), mouse monoclonal anti-Bid (1:1,000), anti-caspase-8 (1:1,000), anti-caspase-9 (1:1,000), or anti-caspase-10 (1:1,000) antibodies (MBL, Nagoya, Japan), mouse monoclonal anti-procaspase-3 (1:10) antibody (Immunotech, Marseille, France), rabbit monoclonal cleaved caspase-3 (1:1,000) antibody (Cell Signaling Technology, Beverly, MA) or mouse monoclonal anti-ß-actin antibody (Sigma Chemical Co., St. Louis, MO) were used as the primary antibodies. The signal was developed with the enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech, Piscataway, NJ).

Plasmid preparation. pDR5PF and its deletion mutants, such as pDR5/BamHI, pDR5/-1188, pDR5/-605, pDR5/-347, pDR5/-318, pDR5/-301, pDR5/-252 and pDR5/-198, were described previously (25,26). pDR5/mtCHOP, which has a mutation in the CHOP binding site at -272/-269, was generated with a site-directed mutagenesis kit (Stratagene, La Jolla, CA) using the following synthesized oligonucleotides: sense, 5’-CGCTTGCGGAGGAGGTAGTTGACGAGAC-3’; and antisense, 5’-AGAGTCTCGTAACTACCTCTCCGCC-3’. The reporter plasmid CHOP 3K, which contains the CHOP promoter, was described previously (27).

Transfection and luciferase assay. Colon cancer cells (1x10⁵ cells) were seeded in 6-well plates, and were transfected with a series of DR5 reporter plasmids and a vacant vector plasmid (1.0 μg) using the DEAE-dextran method (CellPhect, Amersham Pharmacia Biotech). Twenty-four hours after the transfection, luciferase activities were measured and normalized with the protein concentrations. Luciferase assays were carried out in triplicate, and the experiments were repeated several times. Data were analyzed using Student’s t-test, and the differences between solvent DMSO and fenretinide treatment were regarded as significant when P<0.05.

Detection of apoptosis. Colon cancer cells were treated with or without fenretinide (15 or 20 μM). Twenty-four hours after the treatment, cells were treated with or without TRAIL (10-100 ng/ml) for twelve hours, and then harvested. Cells were fixed with 0.1% Triton X-100 and treated with RNase A (Sigma). The nuclei were stained with propidium iodide (Sigma). DNA fragmentation was quantified as the percentage of cells with hypodiploid DNA (sub-G1). The DNA contents were measured using a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson, Franklin Lakes, NJ). For all assays, 10,000 events were counted. Detection of apoptosis was carried out in triplicate.

Small interfering RNAs. The CHOP and LacZ small interfering RNA (siRNA) sequences were previously described (26) (synthesized by Proligo, Kyoto, Japan). The LacZ siRNA was used as an siRNA control. In brief, 1 day prior to the transfection, SW620 cells were seeded without antibiotics at a density of 30-40%. CHOP and LacZ siRNAs (20 nmol/l) were transfected into cells using a modified Oligofectamine protocol (Invitrogen, Carlsbad, CA), in which the volume of Oligofectamine was reduced to one-third of the recommended volume to limit toxic effects. Twenty hours after transfection, cells were treated with fenretinide (20 μM) for 24 h and/or with TRAIL (25 ng/ml) for an additional 12 h, and then harvested.

Results

Effects of fenretinide on the death receptor-related gene in SW480 cells. To date, the role of the mitochondrial pathway in fenretinide-induced apoptosis has been well elucidated. However, the effect of fenretinide has not been examined in
the death-receptor pathway. Therefore, we investigated the effects of fenretinide on the expression of death receptor-related genes using an RNase protection assay. As shown in Fig. 1A, DR5 mRNA was significantly up-regulated by fenretinide in SW480 cells. Lane 1, 1/50 probes not treated with RNase; lane 2, RNase-protected probes following hybridization with yeast tRNA; lanes 3 and 4, total RNA from SW480 cells treated with 20 μM fenretinide or solvent DMSO for 24 h. The housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein L32 are shown as loading controls. B and C, SW480 cells were treated with the indicated concentrations of fenretinide for 24 h (B) and with 20 μM of fenretinide for the indicated periods (C). Western blotting was performed as described in Materials and methods. β-actin was used as a loading control. Arrows show DR5 protein bands. D, Western blotting was performed in a variety of colon cancer cell lines as described in B. Arrows show DR5 protein bands. E, SW480 cells were treated with the indicated concentrations of fenretinide for 24 h. Northern blotting was performed as described in Materials and methods. Ethidium bromide-stained 28S and 18S ribosomal RNA are shown as controls. UT, treated with medium only; CT, treatment with 0.1% DMSO. F, Colon cancer cells were treated with 20 μM of fenretinide for 24 h. Northern blotting was performed as described in E.

**Fenretinide induces DR5 mRNA and protein in colon cancer cell lines.** To confirm DR5 up-regulation by fenretinide, we carried out Western and Northern blotting. As shown in Fig. 1B and C, we found that fenretinide increased the DR5 protein expression in a dose- and time-dependent manner in SW480 human colon cancer cells. In other colon cancer cell lines, the DR5 protein expression was also increased by fenretinide in a dose-dependent manner (Fig. 1D). We also found that fenretinide increased DR5 mRNA expression in a dose-dependent manner in colon cancer cell lines (Fig. 1E and F).

**Exogeneous TRAIL enhances fenretinide-induced apoptosis in colon cancer cell lines.** We demonstrated that fenretinide specifically and drastically induced DR5 expression. Therefore, we hypothesized that exogenous TRAIL, a ligand for DR5, might enhance fenretinide-induced apoptosis due to its ability to increase DR5 expression. We examined the effect of combined treatment with fenretinide and TRAIL on apoptosis by measuring the sub-G1 population. As a single agent, fenretinide weakly induced apoptosis, whereas TRAIL scarcely induced apoptosis, even in high concentrations. However, combined treatment with fenretinide and TRAIL markedly induced apoptosis (Fig. 2).
DR5/Fc chimera protein and caspase inhibitors block apoptosis enhanced by the combination of fenretinide and TRAIL. We estimated that TRAIL and its receptors participated in the apoptosis mediated by combined treatment with fenretinide and TRAIL. In order to confirm the interaction between TRAIL and its receptor, we employed human recombinant DR5/Fc chimera protein to block TRAIL from interaction with its receptor. As shown in Fig. 3A, human recombinant DR5/Fc chimera protein efficiently blocked apoptosis. This result indicates that apoptosis caused by the combined treatment of fenretinide and TRAIL is mediated through its receptors. We also demonstrated that caspase-3, -8, -10, -9 inhibitors and zVAD-fmk pancaspase inhibitor efficiently blocked the apoptosis induced by treatment with fenretinide and TRAIL (Fig. 3B). These observations were concurrent with previous reports on the role of caspase in TRAIL-mediated apoptosis (28-30). Next, we carried out Western blot analysis to confirm the effect of combined treatment with fenretinide and TRAIL on the activation of caspases. As shown in Fig. 3C, fenretinide and TRAIL synergistically induced the activation of caspase-3, -8, -10, -9 and the cleavage of BH3 interacting domain death agonist (Bid) and poly[ADP-ribose] polymerase (PARP). Bid is cleaved downstream of TRAIL and subsequently mediates between the death receptor and mitochondrial pathways (30). PARP is a substrate of caspases and a marker of apoptosis (31). Moreover, the DR5/Fc chimera and the zVAD-fmk pancaspase inhibitor efficiently blocked the activation of these caspases and the cleavage of Bid and PARP (Fig. 3C). These results indicate that the apoptosis mediated by combined treatment with fenretinide and TRAIL occurs via both the death receptor pathway and the mitochondrial pathway in a caspase-dependent manner.

CHOP is involved in the induction of DR5 expression by fenretinide in colon cancer cell lines. To investigate the mechanism of the DR5 up-regulation by fenretinide, we carried out a luciferase assay, using a series of DR5 reporter plasmids. At first, we found that the promoter activity of
pDR5PF, a luciferase reporter plasmid containing a 2.5-kbp fragment of the DR5 promoter region, was increased by fenretinide in SW480 cells. SW480 cells were transiently transfected with the reporter plasmids. Twenty-four hours after transfection, the cells were treated with or without 20 μM of fenretinide for 24 h, and cell lysates were harvested for luciferase assay, as described in Materials and methods. Columns, means of triplicate experiments; bars, SD; *P<0.05. B and C, mutation of the CHOP-binding site attenuates the DR5 promoter activity enhanced by fenretinide in SW480 cells (B) and other colon cancer cell lines (C). The luciferase assay was performed as described in A.

**Figure 4.** CHOP is associated with the fenretinide-mediated induction of DR5. A, induction of the DR5 promoter activity by fenretinide in SW480 cells. SW480 cells were transiently transfected with the reporter plasmids. Twenty-four hours after transfection, the cells were treated with or without 20 μM of fenretinide for 24 h, and cell lysates were harvested for luciferase assay, as described in Materials and methods. Columns, means of triplicate experiments; bars, SD; *P<0.05. B and C, mutation of the CHOP-binding site attenuates the DR5 promoter activity enhanced by fenretinide in SW480 cells (B) and other colon cancer cell lines (C). The luciferase assay was performed as described in A.

CHOP is increased by fenretinide treatment in colon cancer cell lines. The result of luciferase assays shows that CHOP is involved in the fenretinide-mediated induction of DR5. Indeed, fenretinide induced CHOP protein expression in a dose- and time-dependent manner in SW480 cells (Fig. 5A). As shown in Fig. 5B, combined treatment with fenretinide and TRAIL induced CHOP protein, while this was not blocked by zVAD-fmk pancaspase inhibitor and DR5/Fc chimera protein. In other colon cancer cell lines, fenretinide induced CHOP protein expression in a dose-dependent manner (Fig. 5C). We also found that fenretinide increased CHOP mRNA expression in a dose-dependent manner in SW480 cells (Fig. 5D) and other colon cancer cell lines (Fig. 5E). Moreover, fenretinide also enhanced CHOP promoter activity (Fig. 5F). These results indicate that CHOP is also induced by fenretinide at a transcriptional level through the promoter.

**CHOP siRNA blocks DR5 induction by fenretinide and partially reduces fenretinide-induced apoptosis enhanced by TRAIL in colon cancer cells.** To examine whether the DR5 up-regulation mediated by CHOP relates to the sensitization of fenretinide-induced apoptosis by TRAIL, we employed CHOP siRNA. Transiently transfected CHOP siRNA partly inhibited the apoptosis induced by fenretinide and TRAIL (Fig. 6). The induction of DR5 protein by fenretinide was also partially reduced by CHOP siRNA. These findings together suggest
Figure 5. Fenretinide up-regulates CHOP expression in colon cancer cell lines. A-C, Western blotting was performed as described in Fig.1 and Materials and methods. D and E, Northern blotting was performed as described in Fig. 1 and Materials and methods. F, activation of the CHOP promoter activity by fenretinide in colon cancer cell lines. The luciferase assay was performed as described in Fig. 4 and Materials and methods. Colon cancer cell lines were transiently transfected with the reporter plasmids containing the CHOP promoter and the luciferase gene (CHOP 3K) or control plasmid pGVB2.

Figure 6. Down-regulation of DR5 by CHOP siRNA partially reduces fenretinide-induced apoptosis enhanced by TRAIL in SW620 cells. CHOP siRNA or LacZ control siRNA was introduced into SW620. Twenty hours after the transfection, cells were treated with fenretinide (20 μM) for 24 h and/or TRAIL (25 ng/ml) for 12 h. Apoptosis was determined by flow cytometry as described in Materials and methods. Columns, means of triplicate experiments; bars, SD; *P<0.01. Western blotting was performed after treatment with fenretinide (20 μM) for 24 h. β-actin was used to ensure equal gel loading.

Figure 7. Novel mechanisms of enhanced apoptosis by combination with fenretinide and TRAIL in colon cancer cell lines. TRAIL enhances fenretinide-induced apoptosis due to the amplification in activation of multiple caspases through both mitochondrial and death receptor pathways. Fenretinide induces the transcription factor CHOP and Bak. Bak, which may be directly regulated by CHOP, acts on the mitochondria and subsequently activates caspase-9 and -3 to initiate the downstream processes of apoptosis. In the present study, CHOP induced by fenretinide up-regulates DR5 expression at a transcription level through the promoter region. DR5 mediates the TRAIL-induced apoptosis through caspase-8 or -10 and caspase-3 activation. In addition, Bid subsequently mediates between the death receptor and mitochondrial pathways.
that CHOP is partially involved in the up-regulation of DR5 expression by fenretinide and that the induction of DR5 by fenretinide is at least partly responsible for the apoptosis by the combination of fenretinide and TRAIL.

Discussion

Since fenretinide can induce apoptosis rather than differentiation and shows synergistic responses with chemotherapeutic drugs, in contrast to other classical retinoic acids, it has been examined in clinical trials as a cancer chemopreventive and chemotherapeutic agent (13,14). Current trials are underway to re-examine both the dose and schedule of fenretinide as a single agent administration to achieve the expected plasma levels that induce cytotoxicity in vitro (14,32). Furthermore, to increase the efficiency of cancer therapy, fenretinide has been widely investigated in ongoing trials in combination with other agents, such as paclitaxel, cisplatin or tamoxifen (14,33).

We recognized that fenretinide also induced apoptosis in human colon cancer cell lines. It has been well elucidated for its role in the mitochondrial pathway in fenretinide-induced apoptosis (9). However, death-receptor pathway has mostly not been examined with regard to apoptosis. Therefore, we investigated the effects of fenretinide on the expression of death receptor-related genes, and found that DR5 is especially increased by fenretinide among the death receptor-regulated genes.

DR5 is an apoptosis-inducing receptor for TRAIL (19). Therefore, at first, we attempted to determine whether fenretinide-inducing apoptosis was affected by TRAIL in SW-480 colon cancer cells. Consequently, exogenous TRAIL significantly enhanced fenretinide-induced apoptosis, whereas fenretinide induced weak apoptosis (Fig. 2). TRAIL is a promising agent for cancer therapy; however, many tumors remain resistant to treatment with TRAIL (18). In fact, several colon cancer cell lines were resistant to TRAIL, even in high concentrations. We also found that fenretinide up-regulated DR5 expression, and combined treatment with fenretinide and TRAIL induced synergistic apoptosis in human colon cancer cell lines (Fig. 2). In conclusion, combined treatment with fenretinide and TRAIL may have the potential to overcome the resistances to both agents. To add significance to this study, as the next step, we need to examine whether the combination of TRAIL and fenretinide can effectively kill tumor cells in vivo.

We demonstrated here that fenretinide acts as a DR5 inducer. Fenretinide also induces DR5 expression at a protein level. A recent report has indicated that fenretinide induces DR5 in meningioma cell lines (34), but the molecular mechanism underlying DR5 up-regulation has not been investigated. In this study, we showed for the first time that fenretinide induces DR5 up-regulation via the induction of the transcription factor CHOP. CHOP is a growth arrest and DNA damage-inducible transcription factor (35). The expression of CHOP is highly induced in response to a variety of cellular stresses, including nutrient deprivation, oxidative stress and endoplasmic reticulum stress (36-38). We identified that the CHOP-binding site is crucial as the fenretinide-responsive element in the DR5 promoter (Fig. 4). CHOP siRNA at least partly inhibited DR5 expression induced by fenretinide and prevented the apoptosis induced by fenretinide and TRAIL (Fig. 6), suggesting that DR5 up-regulation by CHOP is one of mechanisms required to sensitize colon cancer cells to the apoptosis by fenretinide and TRAIL. A recent report indicated that ceramide cluster DR5 receptors into caveolae as a means to TRAIL sensitization without up-regulating DR5 membrane expression (39). This pathway might also affect the apoptosis induced by the combination of fenretinide and TRAIL, which may explain the reason why the inhibition by CHOP siRNA was partial. We previously demonstrated that tunicamycin and proteosome inhibitor MG132 up-regulate DR5 at a transcriptional level via the induction of the transcription factor CHOP (26,40). These findings together indicate that a variety of stimuli up-regulating CHOP possess the ability of DR5 up-regulation. Moreover, we showed for the first time that CHOP is also up-regulated by fenretinide through the promoter region. However, the responsive elements induced by fenretinide have not yet been elucidated. Thus, further analysis is required to completely reveal the fenretinide-signaling pathway.

A recent report has shown that fenretinide enhances TRAIL-mediated apoptosis in ovarian cancer cells (41). However, they could not detect DR5 up-regulation. The discrepancy between this report and our results may be due to differences of cell types and experimental conditions. Previous reports demonstrated that DR5 expression was highly correlated with sensitivity to TRAIL in Jurkat subclones (42) and that DR5 overexpression by an expression vector could overcome TRAIL resistance in cancer cells (43-45). Therefore, DR5 up-regulation by fenretinide may at least be partially responsible for the sensitization of TRAIL-induced apoptosis.

Our findings provide important possibilities regarding the combined treatment with fenretinide and TRAIL. More than half of malignant tumors possess an inactivating mutation in the p53 tumor-suppressor gene (46,47) and its inactivation contributes to the resistance to chemotherapeutic agents (48). Fenretinide has been reported to kill cancer cells in a p53-independent manner (3). On the other hand, DR5 is a down-stream gene of p53, and p53 transactivates DR5 via a p53-binding site on intron 1 of the DR5 gene (49-51). However, in this study, we show that the CHOP-binding site is responsible for the transactivation of the DR5 promoter by fenretinide in human colon cancer cell lines. In addition, all human colon cancer cell lines except for HCT116 in this study have a mutation in the p53 gene. Therefore, these results indicate that fenretinide up-regulates DR5 in a p53-independent manner. Furthermore, the combined treatment with fenretinide and TRAIL may be useful for the treatment of p53-deficient tumor cells.

We have demonstrated that fenretinide up-regulates DR5 expression via the transcription factor CHOP in human colon cancer cell lines. CHOP plays critical roles in mitochondrial and death-receptor pathways in fenretinide-induced apoptosis (Fig. 7). The results theoretically demonstrate that the combined treatment with fenretinide and TRAIL has the possibility of overcoming the limitation of fenretinide as a single agent. Therefore, combined treatment with fenretinide and TRAIL might be a promising model for the treatment of colorectal cancer.
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References


