Identification of the Genes Involved in Enhanced Fenretinide-Induced Apoptosis by Parthenolide in Human Hepatoma Cells

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Abstract

Fenretinide (N-4-hydroxyphenyl retinamide, 4HPR) is a synthetic anticancer retinoid that is a well-known apoptosis-inducing agent. Recently, we observed that the apoptosis induced by fenretinide could be effectively enhanced in hepatoma cells by a concomitant treatment with parthenolide, which is a known inhibitor of nuclear factor-κB (NF-κB). Furthermore, treatment with fenretinide triggered the activation of NF-κB during apoptosis, which could be substantially inhibited by parthenolide, suggesting that NF-κB inhibition during fenretinide-induced apoptosis has an antiapoptotic effect. This study investigated the molecular mechanism of this apoptotic potentiation by NF-κB inhibition. The genes involved in the enhanced fenretinide-induced apoptosis by parthenolide were identified using the differential display-PCR method and subsequent Northern blot or semiquantitative reverse transcriptase PCR analysis. This study identified 35 apoptosis-related genes including 12 unknown genes that were either up- or down-regulated by parthenolide. Interestingly, one up-regulated gene (HA1A2) was isolated and cloned from the liver cDNA, and was found to be identical to ANKRD1, which is also referred to as the CARP gene. Compared with controls treated with an empty vector or with antisense cDNA, the ectopic expression of ANKRD1 led to reduced colony formation and to enhanced apoptotic cell death in hepatoma cells. These results suggest that ANKRD1 and the other genes, whose expressions were substantially modulated by the parthenolide-mediated inhibition of NF-κB activation, play roles in the enhanced drug-induced apoptosis. In addition, this study suggests that those identified genes may be useful in anticancer strategies against hepatoma. (Cancer Res 2005; 65(7): 2804-14)

Introduction

Fenretinide is a synthetic retinoid with anticancer properties that has been shown to induce apoptosis in a variety of malignant cell lines (1–3). It has been evaluated in humans in several clinical trials and seems to play a role in the chemoprevention of malignancies in humans (4, 5). Furthermore, fenretinide, which is a chemotherapeutic agent, is relatively nontoxic and effective in prostate cancer (6), bladder cancer (7), and a neuroblastoma (8) in preclinical experiments and in early clinical trials. It was previously observed that fenretinide effectively induces apoptosis in hepatoma cells (9, 10), and it was found that this fenretinide-induced apoptosis could be enhanced by the nuclear factor-κB (NF-κB) inhibitor parthenolide. Fenretinide-induced cytotoxicity is retinoic acid receptor (RAR)–independent (11). However, fenretinide has been found to activate the RARs and several RAR-specific antagonists partially inhibited fenretinide-induced apoptosis (2). Therefore, both RAR-independent and RAR-dependent pathways are involved in the fenretinide-mediated apoptosis. Previously, it was observed that a lethal concentration of parthenolide could induce apoptosis, which is associated with oxidative stress via glutathione depletion (12). In contrast, sublethal concentrations of parthenolide could potentiate fenretinide-induced apoptosis, which seems to in part correlate with NF-κB inhibition.

NF-κB activation in cancer cells correlates with tumor resistance to the induction of apoptosis. This activation usually requires phosphorylation of IκB at two specific serine residues at the NH2-terminus of IκBα (Ser52/36) or IκBβ (Ser19/23), which triggers the polyubiquitination and subsequent proteasome-dependent degradation of the inhibitors (13, 14). This leaves free NF-κB, which translocates into the nucleus and binds the DNA to activate the transcription of the responsive genes. Thus, inhibition of NF-κB sensitizes cells to death by various stimuli including tumor necrosis factor-α and cancer therapy drugs (15–17). Recently, proteasome inhibitors have been used to block NF-κB nuclear localization via the inhibition of IκBα degradation and the processing of the p105 NF-κB precursor (18–20). Other molecules have been shown to inhibit NF-κB, including a peptide that blocks the interaction of IκB kinase-γ with the catalytic core of IκB kinase, the nonsteroidal anti-inflammatory drug sulindac, cyclopentenone prostaglandins, arsenic trioxide, thalidomide, and a variety of antioxidants, natural products such as parthenolide and resveratrol, and pharmacologically developed synthetic small-molecule inhibitors of the IκB kinase complex (20–22). Interestingly, The RARγ-selective agonists (MX3350-1 and CD2325) and the retinoid antagonist, MX781, inhibit IκB kinase via a retinoid receptor-independent pathway (23). During most types of chemotherapy, the inhibition of NF-κB activation promotes cell death. Several agents, including arsenic trioxide (24) and thalidomide (25), could inhibit the NF-κB function and are currently in clinical use as primary cancer therapeutics, whereas others are undergoing clinical development. Therefore, the role of NF-κB in anticaspoptotic signaling has also raised hopes that inhibitors will enhance the efficacy of conventional chemotherapeutic drugs as a new adjuvant approach for cancer treatment.

The aim of this study was to identify the gene(s) involved in the enhancement of fenretinide-induced apoptosis by parthenolide in

Note: J-H. Park and L. Liu contributed equally to this work.

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order to elucidate the molecular and cellular mechanism(s) by which parthenolide potentiates fenretinide-induced apoptosis. Using the differential display (DD)-PCR method, the apoptosis-related genes, which are differentially expressed between vehicle-treated control cells and cells treated with fenretinide, were identified and subsequently classified as being either up-regulated or down-regulated genes according to the alteration of their mRNA expression level by parthenolide. Among the genes identified, it was first confirmed that ANKR D1, which is also known as CAR P, was transcriptionally up-regulated by parthenolide and its up-regulation might be involved in the enhancement of drug-induced apoptosis. Furthermore, the other genes identified may also prove to be valuable in dissecting the role of NF-κB inhibition in drug-induced apoptosis and in determining the applicability of NF-κB inhibitors as an adjuvant approach to cancer therapy.

Materials and Methods

Cell lines and reagents. The hepatoma cell lines, Hep 3B, SK-HEP-1, Hep G2, and Huh7, were obtained from the American Tissue Culture Collection (Rockville, MD). The fenretinide was kindly supplied by the Johnson Pharmaceutical Research Institute (Spring House, PA). It was initially dissolved in DMSO at 10^{-1} mol/L, and diluted in absolute ethanol at a concentration of 10^{-2} mol/L and then stored in aliquots at −20°C for a maximum of 2 weeks. The parthenolide was obtained from Calbiochem (San Diego, CA).

Cell culture and detection of apoptotic cells. The cells were cultured in DMEM supplemented with 10% fetal bovine serum in air containing 5% CO2. The percentage of apoptotic cells was determined using a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay using the DeadEnd Colorimetric Apoptosis Detection System kit (Promega, Corp., Madison, WI) according to the manufacturer’s protocol. Briefly, Hep 3B and SK-HEP-1 cells were cultured on slides and treated for 3 days with various fenretinide concentrations (as noted in the figure legends). The cells were fixed in 10% buffered formalin for 1 hour at 4°C in a total volume of 10 μL. The reactions were quenched by incubation at 75°C for 10 minutes. Two microliters of each reaction mixture was PCR-amplified with DyNAzyme II DNA Polymerase (Finnzyme, Espoo, Finland) in 1 μL of 10× PCR buffer (50 mmol/L KCl, 1 mM MgCl2, 10 mM Tris-HCl, pH 8.5, 0.01%, w/v, gelatin, and 0.0005%, w/v, Tween 20). The products were subsequently analyzed by agarose gel electrophoresis.

Table 1. Gene-specific PCR primer sets for the genes in low abundance

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<th>Annealing Temperature (°C)</th>
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The terminal deoxynucleotidyl transferase enzyme mixture was added and then processed for the in situ detection of apoptotic cells. The quantitative measurements of apoptotic cells and the cell cycle distribution were done using a modified technique described elsewhere (10).

Electrophoretic mobility shift assay. The nuclei from Hep 3B cells treated with the vehicle or with fenretinide were prepared according to the modified procedure described by Dignam et al. (26). The nuclear proteins were quantified using a bicinchoninic acid protein assay kit (Sigma, St. Louis, MO), and were used to carry out electrophoretic mobility shift assay. The oligonucleotide probes for NF-κB containing the IgG chain-binding site (5’-CCGTTAACAGGCGGTTCCGGAG-3’; ref. 27) were used to measure the activation of the transcription factors (including NF-κB). Two complementary strands of the oligonucleotides were annealed and labeled with [α-32P]dCTP using a random primer labeling kit (Rediprim II, Amersham Biosciences, United Kingdom). The nuclear extracts (5 μg) were reacted with 2 to 5 ng of the radiolabeled NF-κB (50,000-100,000 cpm/ng). The reactions were done in the presence of 10 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 1 mmol/L DTT, and 4% glycerol (final volume, 25 μL) at room temperature for 30 minutes. The reaction products were subjected to 4% PAGE in 0.5× Tris-borate EDTA buffer [50 mmol/L Tris-HCl (pH 8.5), 50 mmol/L borate, and 1 mmol/L EDTA]. The gels were dried under vacuum for 1 hour. The DNA-binding activity for NF-κB was detected by autoradiography. For the supershift analysis, the nuclear extracts were incubated with the rabbit polyclonal rel-antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) 30 minutes prior to electrophoretic mobility shift assay reaction.

Differential display-PCR. The modified DD-PCR was done using a GenHunter RNAimage kit (GeneHunter Corp, Nashville, TN), based on an improved method described previously (28). Four micrograms of the total RNA from the treated cells or from the untreated cells were reverse-transcribed with 200 units of SuperScript II RT enzyme (Invitrogen, Carlsbad, CA) in the presence of 1 μmol/L one-base anchored oligo(dT) primers for 1 hour at 42°C in a total volume of 10 μL. The reactions were quenched by incubation at 75°C for 10 minutes. Two microliters of each reaction mixture was PCR-amplified with DyNAzyme II DNA Polymerase (Finnzyme, Espoo, Finland) in 1 μL of 10× PCR buffer (50 mmol/L KCl, 1 mM MgCl2, 10 mM Tris-HCl, pH 8.5, 0.01%, w/v, gelatin, and 0.0005%, w/v, Tween 20). The products were subsequently analyzed by agarose gel electrophoresis. The modified DD-PCR was done using a GenHunter RNAimage kit (GeneHunter Corp, Nashville, TN), based on an improved method described previously (28). Four micrograms of the total RNA from the treated cells or from the untreated cells were reverse-transcribed with 200 units of SuperScript II RT enzyme (Invitrogen, Carlsbad, CA) in the presence of 1 μmol/L one-base anchored oligo(dT) primers for 1 hour at 42°C in a total volume of 10 μL. The reactions were quenched by incubation at 75°C for 10 minutes. Two microliters of each reaction mixture was PCR-amplified with DyNAzyme II DNA Polymerase (Finnzyme, Espoo, Finland) in 1 μL of 10× PCR buffer (50 mmol/L KCl, 1 mM MgCl2, 10 mM Tris-HCl, pH 8.5, 0.01%, w/v, gelatin, and 0.0005%, w/v, Tween 20). The products were subsequently analyzed by agarose gel electrophoresis.
amplified cDNAs were cloned into the pGEM-T vector using the TA cloning system (Promega) and the sequenced cDNAs were analyzed via the BLAST program for matches in the Genbank database and were compared with each other via FASTA analysis, as described previously (29).

Northern blot analysis. The cells were cultured in DMEM with 10% fetal bovine serum until they reached 60% confluence and were then treated with 10 μmol/L fenretinide in either the presence or absence of 4 μmol/L parthenolide for 72 hours. The total RNAs were extracted from the treated and from untreated cells using a phenol and guandine thiocyanate solution (Tri Reagent, Molecular Research Center, Inc., Cincinnati, OH). Northern blot analysis was described as described previously (10). The blots were stripped and rehybridized with the cDNA of the 18S ribosomal protein gene as a loading control.

Semi-quantitative reverse transcriptase-PCR. Ten micrograms of the total RNA were incubated with DNase I and were then reverse-transcribed with random decamers (Ambion, Austin, TX) using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Grand Island, NY). One microliter of the reverse transcriptase product was amplified for 30 or 35 cycles with primer pairs specific for the genes studied. Semi-quantitative PCR assays were designed to compare the reverse transcriptase (RT)-PCR products of the 18S ribosomal protein gene using QuantumRNA Universal 18S Standard (Ambion). The PCR products were analyzed by electrophoresis on 1.2% agarose gels. Table 1 shows the sequences of primers used.

Cloning of the ANKRD1 gene by 5’-rapid amplification of cDNA ends. A 117 bp cDNA of the 3’ untranslated region (UTR) of C-193 was isolated (30). A sense primer (5’-AGAAGTTTGCTGTGTTGCGCT3’) was synthesized based on the 3’ UTR sequence of C-193. 5’-Rapid amplification of cDNA ends was done using the sense primer according to the protocol supplied with the Marathon cDNA Amplification kit of the liver (Clontech, Palo Alto, CA). Based on the sequence of the 5’-rapid amplification of cDNA ends fragment, an antisense primer (5’-AGAAGTTTGCTGTGTTGCGCT3’) was synthesized, and 5’-rapid amplification of cDNA ends was done to obtain the full-length cDNA. The rapid amplification of cDNA ends product was cloned into the TA vector (Promega), and the entire cDNA was sequenced. Nucleotide and predicted amino acid sequence searches were done using the BLAST program.

Plasmids and transfections. The ANKRD1 eukaryotic expression vector was constructed as follows. The human ANKRD1 gene was PCR-amplified with a forward primer containing a KpnI restriction enzyme site (5’-GGGGTACCGACCTACTGATG-3’) and a reverse primer containing XbaI site (5’-CCCTCCGAGGCCCTAGAATTG-3’) from the open reading frame cDNA cloned in the pGEM7 vector and was then cloned into pDNA3.1(+)XbaI (Invitrogen) at the XbaI restriction enzyme site. For the antisense expression vector, the human ANKRD1 gene was PCR-amplified with a forward primer containing a XbaI restriction enzyme site (5’-GCTCTAGACCCCTGAGGACCTT-3’) and a reverse primer containing KpnI site (5’-GGGGTACCGACCTACTGATG-3’), and was cloned into pDNA3 at the XbaI/XhoI restriction enzyme site. For the expression of the GFP-ANKRD1 fusion gene, the human ANKRD1 gene was PCR-amplified with a forward primer containing a KpnI restriction enzyme site (5’-GGGGTACCGACCTACTGATG-3’) and a reverse SP6 promoter primer and was cloned into pFLAG-CMV2 at the KpnI/BamHI site in-frame in the sense orientation or at the SalI/KpnI site in the antisense orientation. Transfection of the ANKRD1 gene into Hep 3B cells was done using an expression plasmid vector encoding the human ANKRD1 cDNA in the sense orientation, in the antisense orientation, or the control empty vector. One day after transfection using lipofectin (Life Technologies) according to the manufacturer’s protocol, the cells were seeded in 10 cm Petri dishes and incubated in the presence of 600 μg/mL G418 for 3 weeks. Finally, the individual colonies were isolated using cloning rings, and then expanded in the same concentration of G418 and assayed for expression of the transfected gene using Northern and Western analysis.

Colony generation assay. Hep 3B and SK-HEP-1 cells was transfected with ANKRD1 expression vectors. One day after transfection, the cells were seeded in 10 cm Petri dishes and incubated in the presence of 600 μg/mL G418 for 4 weeks. The colonies were visualized by washing the plates twice with PBS and incubating them for 1 hour in the fixed (10% [v/v] methanol, 10% [v/v] acetic acid). After staining for 30 minutes with the fixative containing 0.5% (v/v) crystal violet, the plates were washed thrice with a fixation buffer twice with water and then dried in air.

Immunofluorescence. The cells were grown on glass coverslips, fixed with 4% paraformaldehyde, permeabilized in PBS containing 0.2% Triton X-100, and blocked with 1% bovine serum albumin. The cells were incubated with a rabbit polyclonal antibody to ANKRD1 overnight at 4°C, washed, and incubated with TRITC-conjugated swine anti-rabbit immunoglobulin. After a final washing, the cells were stained for 15 minutes with 1 μg/mL Hoechst 33258 in order to visualize the nuclei, and were mounted with 50% glycerol in PBS at 4°C. The cells were examined using laser scanning microscopy (LCM 510, Carl Zeiss, Jena, Germany). The polyclonal antibody to ANKRD1 was affinity purified from the serum of immunized rabbits with the recombinant glutathione S-transferase-ANKRD1 protein produced in E. coli.

Cell lysis and immunoblotting. The cells were lysed in a buffer containing 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% Triton X-100, 5 mmol/L EDTA, 5 mmol/L ethylene glycol-bis(β-aminoethyl ether) N,N,N’,N’-tetraacetic acid, 10 mmol/L NaF, 1 mmol/L Na3VO4, 1 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, 1 mmol/L pepstatin, and 1 μg/mL aprotinin. Thirty micrograms of each cell lysate was separated by SDS-PAGE and was subjected to Western blot analysis as described previously (12).

Figure 1. Parthenolide enhances the fenretinide-induced apoptosis in hepatoma cells. A, apoptotic cell death was determined using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining in Hep 3B and SK-HEP-1 cells treated for 72 hours with 10 or 15 μmol/L fenretinide, respectively, in the presence or absence of 4 μmol/L parthenolide (P), or treated with vehicle alone. Columns, mean; bars, ± SE of quadruplicate experiments; **, P < 0.01; compared with cells treated with fenretinide alone. B, quantitation of the apoptotic fraction by FCM analysis in Hep 3B (top) and SK-HEP-1 (bottom) with 10 μmol/L fenretinide in the presence or absence of 4 μmol/L parthenolide (P) for 72 hours, or treated with vehicle alone. The sub-G1 fraction was estimated by gating hypodiploid cells in the histogram using the LYSIS II program. The cell cycle distribution of the cells was analyzed after the fenretinide treatment using a FACScan. The data was analyzed as a single-variable frequency histogram in an SFIT model. The DNA contents were plotted on the linear abscissa (M1, apoptotic fraction). Each value represents the mean ± SE of triplicate experiments; *, P < 0.05; **, P < 0.01, compared with the control cells treated with either the vehicle or fenretinide alone.
Statistical analysis. All the data was entered into the Microsoft Excel 5.0 and GraphPad Software was used to perform the two-tailed t tests or ANOVA, where appropriate. All the values are expressed as means ± SE. P values < 0.05 are considered significant.

Results

Parthenolide enhances fenretinide-induced apoptotic cell death. We previously observed that fenretinide effectively induced cell death in a concentration-dependent manner after 3 days in culture. Hep 3B and SK-HEP-1 cells, which have a defective and wild-type p53 function, respectively (31), were susceptible to fenretinide-induced cell death. The IC50 of fenretinide for Hep 3B and SK-HEP-1 cells were 12.5 and 17.5 μmol/L, respectively. The cells exposed to 10 or 15 μmol/L fenretinide alone or in combination with 4 μmol/L parthenolide for 3 days were positive for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining. Fenretinide effectively induced apoptosis in up to 35% to 40% of the hepatoma cells (Fig. 1A). Parthenolide alone (at 4 μmol/L) did not significantly increase the level of apoptotic cell death compared with vehicle controls. However, parthenolide elicited a 2-fold increase in apoptotic cell death induced by fenretinide, suggesting that a sublethal dose of parthenolide potentiates the fenretinide-induced apoptotic cell death. Similarly, flow cytometric analysis (FCM) revealed that parthenolide significantly enhanced fenretinide-induced apoptosis 2-fold in Hep 3B (71.6% versus 35.6%; P < 0.01) and SK-HEP-1 (31.6% versus 62.5%; P < 0.01; Fig. 1B). In addition, the cells treated with a sublethal dose of parthenolide were arrested in the G1-S stage of the cell cycle, which was accompanied by a significantly lower number of cells in the G2-M stage. Moreover, the G1-S arrested cells did not elicit an apoptotic response.

Parthenolide effectively inhibits NF-κB activation during fenretinide-induced apoptosis. Parthenolide is a well-known natural product that can inhibit NF-κB (32). Therefore, this study examined whether or not NF-κB is activated during fenretinide-induced apoptosis in Hep 3B cells. A statistically significant increase in the level of NF-κB activation in the Hep 3B cells began 10 minutes after the treatment with fenretinide and reached a peak around 12 hours after treatment. However, there was an increase and decrease in the level of NF-κB activation in SK-HEP-1 cells 10 and 20 minutes after treatment, respectively. A substantial increase of the level of NF-κB activation in SK-HEP-1 cells began again after 30 minutes of treatment and peaked 12 hours after treatment (Fig. 2A). This different time course NF-κB activation in Hep 3B cells seems to be caused by the lower level of constitutive NF-κB activation, whereas there is a higher level of constitutive NF-κB activation following the early phase NF-κB inactivation by fenretinide in SK-HEP-1 cells. The NF-κB transcription activator is composed of a homodimeric or heterodimeric complex of...
NF-κB/Rel family proteins. In order to assess the protein composition of the NF-κB/Rel complex in the DNA binding reaction, supershift experiments were done using the specific antibodies against several NF-κB/Rel family members, including p65, p50, c-Rel, p50, and RelB. A supershift assay with the p65-antibody was observed in the Hep 3B cells treated with fenretinide (Fig. 2B). Thus, fenretinide seems to activate NF-κB mainly as a p65 homodimer. Next, the effect of parthenolide on the NF-κB activation was examined. Parthenolide effectively inhibited the NF-κB activation induced by fenretinide at the indicated times (Fig. 2C). Therefore, it was concluded that the fenretinide-induced activation of NF-κB is antiapoptotic and that the inhibition of NF-κB by parthenolide sensitizes the cells to apoptotic cell death by fenretinide. In order to support the hypothesis that NF-κB inhibition sensitizes the cells to apoptotic cell execution by fenretinide, a proteasome inhibitor (MG132) that functions to reduce the degradation of IκB and to prevent activation of the NF-κB pathway was used (18, 33). Nonlethal doses of MG132 (0.2 μmol/L) enhanced the efficacy of fenretinide in inducing apoptotic cell death almost 2-fold similarly to parthenolide (Fig. 2D). Furthermore, MG132 also inhibited NF-κB activation by 50% (Fig. 2D).

**Differential display analysis of genes expressed in apoptotic Hep 3B cells.** DD-PCR was used to compare the profiles of gene expression between the untreated Hep 3B cells and Hep 3B cells treated with fenretinide. The expression of the cDNAs was analyzed on 6% denaturing polyacrylamide gels using 240 different combinations of three one-base anchored oligo-dT primers and 80 arbitrary 13-mers (Fig. 3). In total, 240 PCRs were done and each reaction displayed 70 bands. Therefore, approximately 16,000 different cDNAs were screened in this study. Approximately 93 differentially displaying cDNA patterns between the control cells and the cells treated with fenretinide were obtained, and those cDNA clones were then cloned into the TA vector. The cDNAs sequenced were analyzed via the BLAST program for matches in the Genbank database and compared with each other via FASTA analysis.

**Identification of genes regulated by parthenolide.** Using the DD-PCR method, 35 apoptosis-related genes that had been modulated were identified, which included 12 unknown genes. All the cDNA clones were subjected to Northern blotting using the total RNA isolated from the control cells and from the cells treated with either fenretinide alone or in combination with parthenolide. The expression level of the genes in low abundance was analyzed semiquantitatively by RT-PCR using the gene-specific primers (Table 1), and was compared with 18S rRNA as an internal control. The genes that showed decreased or increased expression between the cells treated with fenretinide alone and those treated in combination with parthenolide were classified as being either up-regulated or down-regulated genes (Table 2). Parthenolide up-regulated SNTB1, ALB, PSMD2, RPS23, DMNT, GADD153, SERPIND1, ARPP-19, ND2, ANKRD1, and two unknown genes >1.5-fold, and down-regulated Gas5, TF, ND3, CYR61, KTN1, and five unknown genes by >50%. Among the genes listed in Table 2, GADD153, NACA, TF, and CYR61 were reported to correlate with NF-κB activation (34–37).

**ANKRD1 overexpression during drug-induced apoptosis.** Among the genes differentially expressed during apoptosis, this study was interested in a gene fragment containing 117 bp whose mRNA was preferentially overexpressed in 4-HPR-induced
apoptotic cells and whose expression level was steadily enhanced by at least 1.5-fold by parthenolide. The sequence of this cDNA fragment was found to be a part of the cardiac ankyrin repeat protein gene. Based on the 3'-UTR sequence, the full length of this cDNA was identified by rapid amplification of cDNA ends PCR and found to be C-193 (accession X83703). This gene was previously designated as a cytokine-inducible nuclear protein gene, which is an immediate early response gene induced by the inflammatory

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<tr>
<td>8</td>
<td>HA52C2</td>
<td>Serine (or cysteine) proteinase inhibitor, clade D (heparin cofactor), member 1 (SERPIND1)</td>
<td>0.41</td>
<td>1.69</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>9</td>
<td>HC15C2</td>
<td>3 BAC RP11-543D10</td>
<td>2.5</td>
<td>1.60</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>10</td>
<td>HC2C1</td>
<td>Cyclic AMP phosphoprotein, 19 kDa (ABPP-19)</td>
<td>0.86</td>
<td>1.58</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>11</td>
<td>HG34C1</td>
<td>Mitochondrion NADH dehydrogenase subunit 2 (ND2)</td>
<td>0.08</td>
<td>1.55</td>
<td>Northern</td>
</tr>
<tr>
<td>12</td>
<td>HA1A2</td>
<td>Ankyrin repeat domain 1 (ANKRD1)</td>
<td>1.41</td>
<td>1.5</td>
<td>Northern</td>
</tr>
<tr>
<td>13</td>
<td>HC15C1</td>
<td>Actin related protein 2/3 complex, subunit 1A (41 kDa; ARPC1A)</td>
<td>0.48</td>
<td>1.35</td>
<td>Northern</td>
</tr>
<tr>
<td>14</td>
<td>HC75C2</td>
<td>α-Fetoprotein (AFP)</td>
<td>0.24</td>
<td>1.28</td>
<td>Northern</td>
</tr>
<tr>
<td>15</td>
<td>HC4C1</td>
<td>Clone RP5-863C7 on chromosome 20p12.3-13. contains the CSNK2A1 gene for casein kinase-2α1 polypeptide</td>
<td>0.33</td>
<td>1.24</td>
<td>Northern</td>
</tr>
<tr>
<td>16</td>
<td>HG33C1</td>
<td>Chromosome 5 clone CTC-273M15</td>
<td>1.52</td>
<td>1.17</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>17</td>
<td>HC55A1</td>
<td>RIKEN cDNA 2700047N05 gene, clone MGC:23968, IMAGE:4698187, mRNA</td>
<td>0.45</td>
<td>1.09</td>
<td>Northern</td>
</tr>
<tr>
<td>18</td>
<td>HC2A1</td>
<td>Nascent-polypeptide-associated complex α polypeptide (NACA)</td>
<td>1.62</td>
<td>1.09</td>
<td>Northern</td>
</tr>
</tbody>
</table>

| B Down-regulated Genes | | | | | |
| 1 | HC26C1 | Kinectin 1 (kinesin receptor; KTNI) | 21.38 | 0.12 | RT-PCR |
| 2 | HA63A1 | Cysteine-rich, angiogenic inducer, 61 (CYR61) | 2.83 | 0.21 | Northern |
| 3 | HG36C2 | Mitochondrion NADH dehydrogenase subunit 3 (ND3) | 0.33 | 0.28 | Northern |
| 4 | HC70A1 | Novel | 2.43 | 0.32 | Northern |
| 5 | HA48C1 | cDNA FLJ21288 fs | 2.28 | 0.38 | Northern |
| 6 | HG22C1 | Transferrin (TF) | 0.50 | 0.44 | Northern |
| 7 | HA49A2 | BAC clone RP11-329A6 from 2 | 0.48 | 0.45 | Northern |
| 8 | HG29A1 | Novel | 0.40 | 0.47 | Northern |
| 9 | HA47C2 | cDNA 40895 fs | 2.33 | 0.49 | Northern |
| 10 | HC61A1 | RB1-inducible coiled coil 1 (RB1CC1) | 1.62 | 0.51 | Northern |
| 11 | HA46C1 | Uba80 mRNA for ubiquitin | 2.54 | 0.60 | Northern |
| 12 | HA62A2 | Splicing factor 30, survival of motor neuron-related (SFP30) | 1.86 | 0.68 | RT-PCR |
| 13 | HA73C1 | Chromosome 8 map 8p23-p22 clone CTC-306G11 | 5.33 | 0.71 | Northern |
| 14 | HG32C1 | Ribosomal protein L21 (RPL21) | 2.20 | 0.72 | Northern |
| 15 | HG42A1 | Mitochondrial transcription factor 1 | 0.28 | 0.79 | Northern |
| 16 | HG11A1 | Gas5 | 1.53 | 0.83 | Northern |
| 17 | HC75A1 | Novel | 2.14 | 0.95 | Northern |

Table 2. Genes regulated during the fenretinide-mediated apoptosis enhanced by a NF-κB inhibitor parthenolide

* C, vehicle control.
* P, parthenolide.

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cytokines in human umbilical vein endothelial cells (30), and was identical to ANKRD1 (NM_014391). However, this gene had a different nucleotide sequence at its 3’-UTR compared with NM_014391 (Fig. 4A). Fenretinide increased the ANKRD1 mRNA expression level in a time-dependent manner in the Hep 3B cells. However, the level increased until 24 hours after treatment in the SK-HEP-1 cells and then decreased at 48 hours after treatment. The addition of fenretinide accentuated ANKRD1 mRNA expression in Hep 3B and SK-HEP-1 cells. ANKRD1 mRNA induction was more prominent (up to 600%) in the SK-HEP-1 cells than in Hep 3B cells (Fig. 4B). Immunoblot analysis was done in Hep 3B and SK-HEP-1 cells treated with fenretinide either alone or in combination with parthenolide in order to confirm this induction of the ANKRD1 protein (Fig. 4C). As expected, fenretinide increased the ANKRD1 protein levels in Hep 3B cells. However, in the SK-HEP-1 cells, the level increased until 24 hours after treatment and then decreased at 48 hours after treatment. Parthenolide effectively accentuated the fenretinide-induced ANKRD1 protein expression in the Hep 3B and SK-HEP-1 cells.

**Decreased clonogenicity and apoptotic morphology by ectopic ANKRD1 overexpression.** In order to determine the functional role(s) of ANKRD1, colony generation assays were done in Hep 3B and SK-HEP-1 cells overexpressing ANKRD1. The Hep 3B cells were transfected with ANKRD1 under the control of a cytomegalovirus promoter in a sense (ANKRD1/S) or in an antisense orientation (ANKRD1/AS), or with an empty vector (pCDNA3.1/HisA). The SK-HEP-1 cells were transfected with a GFP fusion vector with ANKRD1 in a sense (ANKRD1/S) or in an antisense orientation (ANKRD1/AS), or with an empty vector (pEGFP-C3), all of which contained the genetin resistance gene. The ectopic overexpression of ANKRD1 in these cells was confirmed by either immunoblot analysis or fluorescence microscopy, respectively. The number of ANKRD1/S stable transfectants obtained was always significantly lower (<50%, P < 0.01) than the ANKRD1/AS transfectants or the vector control cells (Fig. 5A), nonetheless the colony sizes of the ANKRD1/S stable transfectants were larger than or equal to the vector control cells. In addition, indirect immunofluorescence staining was done. The Hep 3B cells transiently transfected with the GFP fusion vector containing ANKRD1 in the sense (ANKRD1/S) or antisense orientation (ANKRD1/AS) were later stained with a polyclonal ANKRD1 antibody and Hoechst 33258 and were examined using laser scanning microscopy (Fig. 5B). The fluorescence of the GFP-ANKRD1 fusion protein was mainly localized in the cytoplasm, in the perinuclear region, or in the nuclei of the hepatoma cells, which was consistent with a previous report of cardiomyocytes (30, 38). The cells transfected with ANKRD1/AS showed a lower immunoreactivity with ANKRD1. In contrast, the cells transfected with GFP-ANKRD1 revealed a perfect overlap of GFP expression with a strong immunoreactivity for ANKRD1 in the cytoplasm or in the nucleus, suggesting that the polyclonal antibody against ANKRD1 is immunologically functional. Of note, Hep 3B cells ectopically expressing ANKRD1 showed condensed or fragmented nuclear

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**Figure 4.** Isolated ankyrin repeat domain 1/cardia ankyrin repeat protein gene (ANKRD1/CARP) cDNA and its up-regulation by the NF-κB inhibitor parthenolide. A, nucleotide and amino acid sequences of the full-length ANKRD1 cDNA derived from liver cDNA. The first rectangle with a dashed line represents the coiled coil region. The four rectangles represent the nucleotide coding for each ankyrin repeat. The different sequences of 3'-UTR between hepatic ANKRD1 and cardiac ANKRD1 are underlined. B, Northern blot analysis of ANKRD1 in Hep 3B (top) and SK-HEP-1 (middle) cells treated either with fenretinide alone or in combination with parthenolide (4HPR + P) at the indicated time intervals, or treated with the vehicle alone (C). The total RNAs were extracted and fractionated by electrophoresis on 1% agarose gels containing formaldehyde, and were then transferred to the membranes. The blot was stripped and subsequently rehybridized with a probe for 18S cDNA as a loading control. The experiments were done at least thrice, and the result of one representative experiment is shown. Relative densitometric plots on the quantification of ANKRD1 mRNA induction (% of control, bottom). C, immunoblot analysis of ANKRD1 expression in Hep 3B (top) and SK-HEP-1 (bottom) cells treated with either fenretinide alone or in combination with parthenolide (4HPR + P) at the indicated time intervals, or treated with the vehicle alone (C). Thirty micrograms of the extracted proteins were separated by 12% SDS-PAGE and were transferred to the membrane. The blots were probed with polyclonal antibodies against ANKRD1 and were then stripped and reprobed with a monoclonal antibody to actin as the loading control. The experiments were done at least thrice, and the result of one representative experiment is shown.
chromatin suggesting that the ectopic expression of ANKRD1 can induce apoptotic cell death. The ectopic expression of ANKRD1 consistently induced an apoptotic cell death in the SK-HEP-1, Hep G2 and HuH7 cells (Fig. 5B, bottom). Alternatively, in order to support the proapoptotic role of ANKRD1, the SK-HEP-1 cells transiently transfected with a sense GFP-ANKRD1 (S), an antisense GFP-ANKRD1 (AS), or an empty vector control (GFP), were analyzed by FCM (Fig. 5C). The apoptotic sub-G1 fraction of the GFP-positive cells was higher in the cells expressing the sense ANKRD1 (S) compared with cells transfected with the empty vector (GFP) 48 hours after transfection (27.5% versus 9.5%; \( P < 0.01 \)), suggested that the ectopic expression of ANKRD1 effectively induces apoptotic cell death.

**Increased drug-susceptibility by ectopic ANKRD1 overexpression.** Finally, Hep 3B cells stably expressing ANKRD1 (ANKRD1/S1 and ANKRD1/S6), of which the expression was confirmed by immunoblot analyses (upper panels), were established and treated with 10 \( \mu \)mol/L fenretinide for 48 hours. Apoptotic cell death was further confirmed using FCM analysis. The apoptotic sub-G1 fraction was higher in the Hep 3B cells stably expressing GFP-ANKRD1 compared with the cells transfected with the empty vector (VC-2 and VC-3; 62.7 ± 5% or 65.1 ± 7.2% versus 8.5 ± 0.8% or 9.3 ± 0.5%; \( P < 0.01 \); Fig. 6). The apoptotic Hep 3B cells stably transfected with an antisense GFP-ANKRD1 was similar to the vector control cells. In contrast, 4 \( \mu \)mol/L parthenolide alone did not increase the level of apoptotic cell death in the Hep 3B cells stably expressing GFP-ANKRD1 compared with the cells transfected with either the empty vector (VC-2 and VC-3) or the antisense ANKRD1 (AS8 and AS13). These results suggest that ANKRD1 plays a proapoptotic role in the enhanced fenretinide-induced cell death. The drug-susceptibility of the cells stably transfected with the antisense ANKRD1 was similar to that of the empty vector control cells.

**Discussion**

A number of sequiterpene lactones, which are cytotoxic against tumor cells, have been reported to be chemotherapeutic drugs. Of these, helenalin induces apoptosis via a CD95 receptor-independent mechanism (39), and has been suggested to be a new drug to overcome chemoresistance. In addition, parthenolide, which is a well known NF-\( \kappa \)B inhibitor that inhibits IκB kinase-\( \beta \) and subsequently suppresses NF-\( \kappa \)B activation (40), has been proposed for combinational chemotherapies because it increases the sensitivity to paclitaxel in breast cancer cells with constitutively active NF-\( \kappa \)B (41), which mediates the prometastatic, proangiogenic, antiapoptotic, and multidrug resistance gene expression (42). In particular, NF-\( \kappa \)B activation is an important mechanism for chemotherapy resistance, and the inhibition of NF-\( \kappa \)B activation significantly enhances the tumor cell response to chemotherapeutic agents (43). This study investigated the effect of parthenolide on the
NF-κB activation pathway during the fenretinide-induced apoptosis as well as the expression of the apoptosis-related genes that were induced by fenretinide. We found that a sublethal dose of parthenolide effectively inhibits the activation of NF-κB, and enhances the apoptotic response to fenretinide in hepatoma cells. A recent report indicated that the constitutive activation of NF-κB inhibits the fenretinide-induced apoptosis via the inhibition of c-fjun NH2-terminal and caspase-3 activation, and that the NF-κB inhibition increases the sensitization to apoptosis in prostate cancer cells (39). Additionally, NF-κB inhibition increases the expression of ANKRD1 protein. The ectopic overexpression of ANKRD1 sensitizes the Hep 3B cells to drug-induced apoptosis. The expression of ANKRD1 protein in the stably transfected Hep 3B cells with a sense GFP-ANKRD1 (S1 and S6) were compared with the antisense GFP-ANKRD1 (AS8 and AS13), or GFP vector control cells (VC2 and VC3; top). Ectopic, ectopic expression of ANKRD1 protein; Endogenous, endogenous expression of ANKRD1 protein. The stable transfectants and vector control cells were treated with either 10 μmol/L fenretinide or 4 μmol/L parthenolide (P) for 48 hours as indicated. Quantification of the apoptotic fractions was done using FACScan. The sub-G0 fraction was estimated by gating the hypodiploid cells in the histogram using a C-30 program. The DNA contents are plotted on the *linear abscissa* (M0, apoptotic fraction). Each value represents the mean ± SE of two independent, triplicate experiments; **, P < 0.01 compared with controls.

NF-κB activation pathway during the fenretinide-induced apoptosis as well as the expression of the apoptosis-related genes that were either up- or down-regulated by parthenolide and the functional role(s) of ANKRD1, an up-regulated gene, was determined. The ectopic expression of ANKRD1 led to reduced colony formation and to enhanced apoptotic cell death in hepatoma cells, indicating that the induction of ANKRD1 seems to be correlated with apoptotic cell death in hepatoma cells. Previously, ANKRD1/CARP expression was reported to be restricted to cardiac myocytes, endothelial cells, and vascular smooth muscles cells (45, 46). Its expression level is increased during human heart failure and in animal models of cardiac hypertrophy (47), and is decreased in cardiomyocytes exposed to doxorubicin, which is a cardiotoxic antineoplastic drug (48). ANKRD1 has been suggested to act as a nuclear transcription cofactor that negatively regulates expression of the cardiac gene, and can play a key role in the pathophysiology of heart failure (48). We first observed that ANKRD1 is also expressed in hepatoma cells exposed to the apoptogenic drug fenretinide and that its expression is further enhanced by simultaneous treatment with the NF-κB inhibitor parthenolide. The precise mechanisms whereby ANKRD1 leads to cell apoptosis and whereby the NF-κB directly or indirectly regulates the expression of ANKRD1 needs to be further investigated in hepatoma cells. However, in the heart, the β-adrenoceptor agonist, isoprorenaline, induces hypertrophy and increases the ANKRD1 expression level, whereas the inhibition of calmodulin-dependent protein kinases completely reverses these effects of isoprorenaline. Calmodulin-dependent protein kinase-II, which is activated by stimulation of β-adrenoceptor, is involved in a novel linkage of β1-adrenoceptor stimulation to cardiomyocyte apoptosis (47). Furthermore, ANKRD1 is expressed in the vasculature after a balloon injury and in cultured vascular smooth muscle cells in response to transforming growth factor-β (46). This suggests that ANKRD1 may be associated with apoptotic cell death in cardiomyocytes.

The exposure of mammalian cells to the agents that disrupt the function of the endoplasmic reticulum leads to the activation of the proapoptotic transcription factor, GADD153/CHOP. Paradoxically, several of these agents also induce the antiapoptotic transcription factor, NF-κB. NF-κB inhibits GADD153 activation in breast cancer cells exposed to these agents. Therefore, parthenolide sensitizes breast cancer cells to endoplasmic reticulum stress agents (34). Furthermore, parthenolide and an IκBα super-repressor increase the palmitaxel-induced apoptosis via the inhibition of a distinct NF-κB-regulated cell survival pathway (41). These results also show that NF-κB is activated during fenretinide-induced apoptosis and that its inhibitor, parthenolide, enhances the fenretinide-mediated overexpression of GADD153, as previously described elsewhere (10). Another similar approach to identify the antiapoptotic or survival genes involved in a distinct NF-κB-regulated cell survival pathway was done using a mutated inhibitor IκBα in ovarian and in breast cancer cells (35). GADD153 and NAC4 are reported to be the NF-κB repressed genes that are up-regulated by NF-κB inhibition, which is consistent with our data. A novel protein, the proteolysis-inducing factor, which is isolated from the urine of pancreatic cancer patients, activates both the transcription factors, NF-κB, and signal transducers and activators of transcription-3, which result in the increased production of interleukin-8, interleukin-6, and the C-reactive protein, and the decreased production of transferrin (36). Cyr61 plays an important role in the resistance to chemotherapeutic agent–induced apoptosis in human breast cancer MCF-7 cells via a mechanism involving the activation of...
the integrins/NF-κB/XIAP signaling pathway (37). However, the Cyr61-overexpressing cells had a significantly higher NF-κB activity than the neo control cells, and the blockage of NF-κB activity in Cyr61-expressing cells by transecting them with either a dominant-negative-IκB α or a NF-κB decoy rendered them more susceptible to anti-cancer drug-induced apoptosis. This suggests that NF-κB activation is downstream of Cyr61.

In summary, hepatocellular carcinoma cells are known to be resistant to chemotherapy and radiation during anticancer therapy. However, the molecular mechanism of such drug-resistance and radioresistance remains unclear. Nevertheless, the activation of NF-κB is recognized as an antiapoptotic or survival factor that contributes to chemoresistance and to radioresistance.

Therefore, the role of NF-κB in antiapoptotic signaling raises new hopes that novel inhibitors that can enhance the efficacy of conventional chemotherapeutic drugs will be developed.

This study examined the possibility of enhanced chemotherapeutic efficacy by inhibiting NF-κB activation in human cancer cells. Furthermore, this study identified the potential target genes that are modulated by the inhibition of NF-κB activation. Such adjuvant treatment with NF-κB inhibition and functional analysis of these target genes might contribute to advanced strategies for the effective chemotherapy of human cancer cells.

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