Biochemical Pharmacology 79 (2010) 948–954

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Induction and intracellular localization of Nur77 dictate fenretinide-induced apoptosis of human liver cancer cells

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ARTICLE INFO

Article history:
Received 22 September 2009
Accepted 2 November 2009

Keywords:
Fenretinide
Nur77
Apoptosis
ROS
Nuclear receptor
Retinoids

ABSTRACT

Fenretinide, a synthetic retinoid, is known to induce apoptosis in various cancer cells. However, the mechanism by which fenretinide induces apoptosis remains unclear. The current study examines the mechanisms of fenretinide-induced apoptosis in human hepatoma cells. The induction of Nur77 and the cytoplasmic distribution of Nur77 induced by fenretinide were positively correlated with the apoptotic effect of fenretinide in HCC cells. The sensitivity of Huh-7 cells was related to Nur77 translocation and targeting mitochondria, whereas the mechanism of resistance for HepG2 cells seemed due to Nur77 accumulating in the nucleus. The intracellular location of Nur77 was also associated with the differential capability of fenretinide-induced ROS generation in these two cell lines. In addition, the knockdown of Nur77 expression by siRNA greatly reduced fenretinide-induced apoptosis and cleaved caspase 3 in Huh-7 cells. Therefore, our findings demonstrate that fenretinide-induced apoptosis of HCC cells is Nur77 dependent and that the intracellular localization of Nur77 dictates the sensitivity of the HCC cells to fenretinide-induced apoptosis.

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1. Introduction

Retinoids, natural and synthetic derivatives of vitamin A, have a profound effect on cellular homeostasis including growth, differentiation, and apoptosis [1,2]. Clinically, retinoids have displayed therapeutic activity against a number of proliferative diseases. Experimental and clinical studies have demonstrated that retinoids can inhibit or reverse the carcinogenic process in certain organs, including premalignant and malignant lesions in the oral cavity, head and neck, breast, skin, and liver [3].

Hepatocellular carcinoma (HCC), the primary liver malignancy, is the fifth most common neoplasm in the world and the third most common cause of cancer-related mortality [4]. HCC is highly resistant to available chemotherapy, thus resulting in a 5-year relative survival rate of less than 7% [5]. Therefore, discovery of new and effective therapies against HCC is greatly needed.

Fenretinide (N-(4-hydroxyphenyl) retinamide; 4HPR) is a structure analogue of all-trans retinoic acid that was first synthesized by R.W. Johnson Pharmaceuticals in the late 1960s. Many laboratory and clinical studies have demonstrated that fenretinide may hold great potential in cancer chemoprevention and therapy. Data from *in vitro* models demonstrate that fenretinide not only inhibited cell proliferation, but also induced apoptosis in human cancer cell types derived from a variety of tumors including head and neck, lung, melanoma, prostate, bladder carcinoma, neuroblastoma, and leukemia [6–13]. Furthermore, fenretinide is effective against carcinogenesis of the breast, prostate, pancreas, and skin in animal models [14–16]. In clinical trials, fenretinide slowed the progression of prostate cancer in men and protected against the development of ovarian cancer and a second breast malignancy in premenopausal women [17]. Therefore, fenretinide offers great promise as a therapeutic agent in cancer treatment and prevention.

The different signaling pathways involved in fenretinide-induced apoptosis in cancer cells including reactive oxygen species (ROS) generation, ceramide and ganglioside GD3 and the intrinsic or mitochondrial-mediated pathways seem to play a central role in cancer cells elimination [17]. The most commonly observed property of fenretinide-induced apoptosis in cancer cells is its inhibition by antioxidants such as vitamin C, vitamin E, and N-acetylcysteine, and pyrrolidine dithiocarbamate, thus suggesting an essential role of ROS and oxidative stress in fenretinide's cytotoxicity [18–20].

Nur77 (NR4A1, TR3, NGFI-B) belongs to nuclear receptor superfamily NR4A subfamily. Nur77 is one of the orphan nuclear receptors with no identified physiological ligands. Nur77 is highly

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0006-2952/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.bcp.2009.11.004

expressed in various tissues including liver [21]. Nur77 was initially classified as an immediate-early response gene as it can be rapidly induced by growth factors, phorbol esters, calcium ionophores and other stimuli acting via cyclic AMP-dependent synthesis pathways [22]. Most importantly, a number of studies have indicated that Nur77 plays an important role in chemotherapeutic agent-induced apoptosis. One retinoid-related compound, 6-(3-(1-adamantyl)-4-hydroxyphenyl)-2-naphthalenecarboxylic acid, also known as AHPN/CD437, was shown to trigger Nur77 nuclear export and mitochondria targeting, which is the key mechanism responsible for CD437-induced apoptosis of cancer cells [23,24]. It is unknown whether Nur77 plays a role in fenretinide-induced apoptosis.

In the present study, we provide direct evidence that Nur77 is involved in mediating the apoptotic effect of fenretinide in HCC cells. Furthermore, our findings establish the distinct modes of action of Nur77 between the sensitive and resistant cells in response to fenretinide. Our data show the intracellular localization of Nur77 determines the susceptibility of HCC cells to the apoptotic effect of fenretinide.

2. Materials and methods

2.1. Reagents

All reagents and chemicals used were from Sigma–Aldrich (St. Louis, MO) unless noted otherwise. Fenretinide (10 mM) dissolved in DMSO was stored at −80 °C. MitoSOX™ Red mitochondrial superoxide indicator, Hank's balanced salt solution (HBSS) with calcium and magnesium, TRIzol reagent and Lipofectamine™ RNAiMAX Transfection Reagent were purchased from Invitrogen. (Carlsbad, CA). VECTASHIELD Mounting Medium with DAPI was purchased from Vector Laboratories (Burlingame, CA). Rabbit polyclonal antibodies for Nur77, goat polyclonal cleaved caspase 3, Poly ADP-ribose polymerase (PARP) and goat anti-rabbit IgG-Texas Red were purchased from Santa Cruz (Santa Cruz, CA). Protease and phosphatase inhibitors and In Situ cell Death Detection Kit were purchased from Roche Applied Science (Indianapolis, IN).

2.2. Cell culture and treatment

Huh-7 cells were maintained in Dulbecco's Modification of Eagle's Medium. HepG2 cells were maintained in Minimum Essential Medium (Mediatech, Herndon, VA). The media were supplemented with 10% fetal calf serum (FBS) (Atlanta Biologicals, Lawrenceville, GA). Cells were cultured at 37 °C in 5% CO_2 atmosphere with a relative humidity of 95%. Cells were plated with approximately 1×10^6 cells per T-25 flask or 5×10^4 per well of 24-well plates/4-well chamber slides 12–16 h prior to the treatments and cultured overnight. Cells were incubated with DMSO or fenretinide (10 μM) in serum-free media for indicated time. The final concentration of DMSO in the culture medium was 0.1% in all treatments. Fresh medium containing corresponding compounds was provided every 24 h.

2.3. Apoptosis assay

Apoptosis was evaluated by caspase 3/7 activity and cell survival. Caspase 3/7 activity and cell viability were determined by CellTiter-Glo® Luminescent Cell Viability and Caspase-Glo® 3/7 kit following the protocol supplied in the kit (Promega, Madison, WI).

2.4. Total RNA preparation

Total RNA was extracted with TRIzol reagent according to the manufacturer's instruction. RNA was quantified and assessed for purity on a UV spectrophotometer.

2.5. Reverse transcription and quantitative real-time PCR

Total RNA (1 μg) was reverse-transcribed with oligo (dT) primer and M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) for first strand cDNA synthesis. cDNA corresponding to 32 ng total RNA was used as the template in a 20 μL real-time PCR reaction with the ABI TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and the appropriate primer pair and Taqman probe. The primer pairs and Taqman probes for Nur77 were designed with Primer Express software v2.0 (Nur77 forward primer: AGCATTATGGTGTCCGCACAT; reverse primer: TTGGCGTTTTTCTGCACTGT; probe: TGAGGGCTGCAAGGGCT-TCTTCAA. β-Actin forward primer: CCTGGCACCCAGCACAAT; reverse primer: GCCGATCCACACGGAGTACT; probe: ATCAAGAT-CATTGCTCCTCCTGAGCGC). Real-time PCR was conducted using the ABI Prism 7300 real-time PCR system (Applied Biosystems, Foster City, CA). The quantification analysis for target gene expression was performed using the relative quantification comparative CT method.

2.6. Confocal microscopy

Huh-7 and HepG2 cells were grown in Chamber BD Falcon™ Cultureslides (BD Biosciences, Bedford MA, USA). Following treatment, cells were fixed for 15 min at room temperature with freshly prepared 1% paraformaldehyde (Mallinckrodt Baker Inc., Phillipsburg, NJ) in PBS. After fixation, cells were rinsed with PBS containing 0.2% Triton X100; cells were then incubated with PBS containing 0.2% Triton X100 and 5% normal goat serum (Abcam, Cambridge, MA) for 30 min at room temperature. Primary antibody specific for Nur77 (1:100 dilution) was applied to cells overnight at 4 °C or 1 h at 37 °C in a humidified chamber. After washed with PBS containing 0.2% Triton X100 and 1% normal goat serum, cells were incubated with FITC-conjugated secondary antibody (diluted at 1:400, in PBS containing 0.2% Triton X100 and 1% normal goat serum) for overnight at 4 °C or 1 h at 37 °C. After washing with PBS, cells were mounted with VECTASHIELD Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI) and analyzed under confocal microscope (Nikon, EZ-C1, Japan).

2.7. Subcellular fraction isolation

Trypsinized cells (1×10^7) were collected by centrifugation at 1000 rpm for 5 min. Cell pellets were resuspended with 5.5 mL of cold RSB buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.5, supplemented with protease and phosphatase inhibitors) and incubate on ice for 90 min. Cells were then lysed in a dounce homogenizer and mixed with 4 mL 2.5× MSB buffer (525 mM Mannitol, 175 mM Sucrose, 12.5 mM Tris–HCl, pH 7.5, 2.5 mM EDTA, pH 7.5). The lysate was centrifuged at 1300 × *g* for 5 min at 4 °C for two times and the combined pellets were the nuclear fraction. The supernatant was centrifuged at 17,000 × *g* for 15 min at 4 °C and the subsequent supernatant represented cytosol fraction and the pellets represented crude mitochondria fraction.

2.8. Western blotting and antibodies

Cells from indicated treatments were collected and lysed with lysis buffer (50 mM Tris·Cl pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% (v/v) NP-40 with protease and phosphatase inhibitors). Equal amounts of lysates (50 μg total protein) were electrophoresed on SDS-PAGE and electroblotted onto PVDF membrane (Bio-Rad, Hercules, CA). The membranes were first incubated with PBS supplemented with 0.1% Tween 20 and 5% nonfat dry milk