Toxicology in Vitro 26 (2012) 142–149

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

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

Cell growth inhibition and apoptotic effect of the rexinoid 6-OH-11-O-hydroxyphenantrene on human osteosarcoma and mesenchymal stem cells

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ARTICLE INFO

Article history:
Received 17 May 2011
Accepted 12 October 2011
Available online 25 October 2011

Keywords:
Osteosarcoma cells
Mesenchymal stem cells
Retinoids
Cell growth
Apoptosis

ABSTRACT

Natural derivatives of vitamin A, including all-*trans*-retinoic acid (ATRA), commonly known as retinoids, currently produce favorable results in the treatment of many types of tumors. The rexinoid 6-OH-11-O-hydroxyphenantrene (IIF) is a synthetic derivative of ATRA. Previous *in vitro* and *in vivo* studies demonstrated that IIF is able to induce growth inhibition of various cancer cells and is a potent apoptosis-inducing agent with clinical potential. Osteosarcoma (OS) is the most common type of bone cancer, characterized by a rising aggressiveness. Recent evidences suggest that mesenchymal stem cells (MSC) may favour tumor growth and progression. Thus, it is important to investigate whether a compound with potential anti-tumoral properties such as IIF affects not only tumor cells but also MSC. The current study is an attempt to understand the mode of the potential cytotoxicity of IIF on OS cells and MSC. The response to IIF treatment of osteosarcoma SaOS-2, MG63, and U2OS cells and of bone marrow-derived MSC was the subject of investigation. The results showed that IIF significantly inhibited cell growth in OS cell lines and MSC in both a time- and dose-dependent manner, as evaluated by methylene blue assay. This was also associated with altered cell morphology and an increase in cell death with the involvement of apoptosis as demonstrated by NucleoCounter, Hoechst 33342 staining and FACS analysis. No cell death and apoptosis was found in U2OS cells. Analysis of cells treated with 20 and 40 μM IIF for 24 h by western blot suggests the activation of initiator caspase 9, indicating the involvement of caspases in inducing apoptosis. Furthermore, IIF upregulated the expression of the pro-apoptotic protein Bax and downregulated the anti-apoptotic protein Bcl2. For the first time, our results collectively provide an evidence for cell growth inhibition and activation of apoptosis in human OS cells and MSC by IIF. These results confirm that IIF may be an effective compound for anticancer treatment, including that of OS.

1. Introduction

Retinoids, including all-*trans*-retinoic acid (ATRA), are natural derivatives of vitamin A and currently produce favourable results in the treatment of many types of tumors (Germain et al., 2006). The retinoid signal is mediated in target cells through specific nuclear receptors: the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). Retinoids which are RXR ligands, called rexinoid (Altucci et al., 2007; Kong et al., 2005), are effective in reducing tumor growth, differentiation and apoptosis as ligands of RAR, among which the most employed is ATRA, but the RXR ligands are almost completely devoid of side effects. In fact, two major problems in the clinical use of retinoids are that the doses needed for successful treatment are often toxic, leading to side effects such as "hypervitaminosis A" syndrome (Kong et al., 2005) and drug resistance. On the other hand, the efficacy of rexinoids has been confirmed in several clinical studies for cancer therapy where rexinoids have been shown to have only a mild toxicity as compared to that of the RAR ligands (Blumenschein et al., 2008; Kagechika and Shudo, 2005; Nahoum et al., 2007). These results have paved the way for a wider clinical use of rexinoids.

The rexinoid 6-OH-11-O-hydroxyphenantrene (IIF) is a synthetic derivative of ATRA ((Altucci et al., 2007) pat. WIPO W0 00/

Abbreviations: ATRA, all-*trans*-retinoic acid; IIF, 6-OH-11-O-hydroxyphenantrene; MSC, mesenchymal stem cells; OS, osteosarcoma.

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doi:10.1016/j.tiv.2011.10.009

17143, Bologna, Italy). *In vitro* studies demonstrated that IIF is effective in inducing cell proliferation inhibition and apoptosis in different types of cancer cells, such as colon cancer cells, neuroblastoma, leukaemia cells, breast cancer cells, fibrosarcoma, melanoma, glioblastoma (Bartolini et al., 2003, 2004, 2006, 2008; Orlandi et al., 2003; Papi et al., 2007, 2009a,b). These *in vitro* findings were further substantiated in a murine glioma model *in vivo*, where oral administration of pioglitazone and IIF resulted in significantly reduced tumor volume and proliferation (Papi et al., 2009b).

High grade osteosarcoma (OS) is a malignant tumor of the bone usually arising in the intramedullary cavity of long bones of young adults (ages 30–33). OS is composed of proliferating spindle-shaped cells that directly produce osteoid or immature bone. Standard treatment today combines multiagent chemotherapy and surgery, producing an overall survival rate of 55–70% for patients with high grade OS. However, this percentage represents a plateau with no significant improvement being observed throughout the past 8–10 years (Gatta et al., 2005; Marina et al., 2004; Sakamoto and Iwamoto, 2008). Furthermore, chemotherapy uses drugs to rapidly kill dividing cancer cells as well as healthy dividing cells. Therefore, the need for new curative therapies has motivated the search for new treatments for OS.

Bone marrow cavities also contain a subpopulation of stem cells commonly referred to as mesenchymal stem cells (MSC) which can easily replicate in culture and are pluripotent. In particular, MSC can be induced to differentiate along the osteoblastic phenotype. Physiologically, MSC are thought to be responsible for repairing damaged tissue and therefore have been used extensively in regenerative medicine and bone repair (Hall et al., 2007; Pittenger et al., 1999). MSC and OS cells present the common feature to be proliferating-immature-osteocompetent cells located in the medullary cavity of bones. Scientists frequently use OS cells instead of MSC to test *in vitro* biomaterial for tissue engineering purposes (Gandolfi et al., 2008). When MSC are implanted in osteoincompetent mice they do not form tumors (Bernardo et al., 2007; Choumerianou et al., 2008), while OS cells do (Su et al., 2009). Despite these studies, recent evidence suggests that MSC may still favour tumor growth and progression. In fact, MSC have been shown to have immunosuppressive properties (Aggarwal and Pittenger, 2005; Djouad et al., 2003), causing concerns that MSC could prevent tumor cell recognition by the immune system. In addition, MSC have been shown to have proangiogenic properties in that they secrete a broad spectrum of growth factors that promote new vessel formation and maturation (Kinnaird et al., 2004), which could in turn promote the survival of cancer stem cells and protect these cells from drug-induced apoptosis (Roorda et al., 2009). Thus, it is important to investigate whether a compound with potential antitumoral properties affects not only tumor cells but also MSC. Although IIF has been shown to have cytotoxic effects on different types of cancer cell, it remains unknown whether it has such effects on OS cells and MSC. In the present study we investigated whether IIF possesses significant cytotoxic effects against different human OS cell lines and human primary MSC. For the first time, the current study provides evidence for cell growth inhibition and activation of apoptosis in human OS cells and MSC by IIF. These results confirm that IIF may be an effective compound for anticancer treatment including that of OS, which suggests new potential for this drug.

2. Materials and methods

2.1. Reagents and antibodies

6-OH-11-O-hydroxyphenantrene (IIF), kindly provided by Dr. Khodor Ammar (pat. WIPO W0 00/17143, Bologna, Italy), was dissolved in propylene glycol at a concentration of 7800 μM and stored at 4 °C for a maximum of 6 months. Immediately prior to use, the drug was diluted to its final concentrations with the culture medium. The final concentration of propylene glycol in cultures did not exceed 0.1% (v/v). Equivalent quantities of the vehicle were added to control cells.

Cell culture media were from Euroclone (Paignton, UK), fetal bovine serum (FBS) from BioWhittaker (Lonza, Verviers, Belgium), glutamax and TripLe Select from Gibco-Invitrogen Corp. (Paisley, Scotland). MEBCYTO Apoptosis kit, containing AnnexinV-FITC and propidium iodide (PI), was purchased from Medical & Biological Laboratories (Naka-ku Nagaya, Japan).

Anti-caspase-9, anti-PARP and anti-actin were purchased from Sigma–Aldrich Co. (St Louis, MO, USA); anti-Bcl2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-Bax from Calbiochem (San Diego, CA, USA). Hybond TM-C Extra membranes and luminol were from GE Healthcare (Milan, Italy). The rest of the reagents used were from Sigma–Aldrich.

2.2. Human osteosarcoma cell cultures

Human osteosarcoma (OS) cell lines were obtained from LGC Promochem-American Type Culture Collection (Teddington, Middlesex, UK). The following human cell lines were employed in the current study: SaOS-2 (ATCC-HTB-85), MG63 (ATCC-CRL-1427), and U2OS (ATCC-HTB-96). The cells were cultured according to the LGC instructions. Briefly, the culture medium was Dulbecco's modified Eagle's medium (DMEM) for cell lines MG63 and U2OS and McCoy's 5A medium for SaOS-2. The medium was supplemented with 10% FBS and 1% glutamax. The cells were maintained at 37 °C, 95% humidity, and 5% carbon dioxide.

2.3. Isolation and culture of human mesenchymal stem cells

Primary MSC cultures were established from three patients undergoing elective surgery at the Rizzoli Orthopaedic Institute after obtaining informed consent according to a protocol approved by the Ethics Committee. Briefly, a 10-mL bone marrow sample was aspirated from the posterior iliac crest. Mononucleated cells were isolated in a density gradient and resuspended in α-modified minimum essential medium (α-MEM) containing 20% FBS and 1% glutamax. All the nucleated cells were plated in a 150 cm^2 culture flask and incubated in a humidified atmosphere at 37 °C with 5% CO_2. Non-adherent cells were discarded after 2 days and adherent cells were cultured for further expansion. When cultured dishes became near confluent (70–80%), cells were detached by mild trypsinization (TripLe Select) and reseeded onto new plates at 1/3 density for continued passage. Media were changed every 3–4 days.

2.4. Cell treatment

Cells were cultured in multi-well culture plates (Corning Costar, NY, USA) with different numbers of wells depending on the experiment at a confluence of about 50% (corresponding to 25,000/cm^2 for MSC, 36,000/cm^2 for SaOS-2 and MG63, and 73,000/cm^2 for U2OS). After 24 h, the medium was removed and cells were incubated with increasing concentrations of IIF (10, 20, 40, and 80 μM) for 24, 48, and 72 h. After incubation, cell morphology was observed with a inverted phase-contrast microscope (Nikon Eclipse TE2000, Tokyo, Japan) and images were acquired.

2.5. Cell growth assay

Cell growth was measured by methylene blue assay in 96-well culture plates (Oliver et al., 1989). Briefly, cells were fixed by adding 100 μl of 10% formol saline to each well for 30 min. Cells were then stained with 100 μl of filtered 1% (w/v) methylene blue in