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Resveratrol inhibits proliferation and promotes apoptosis of osteosarcoma cells

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ABSTRACT

The phytoalexin resveratrol has been described to have chemopreventive and chemotherapeutic effects in several tumor models while its effects on osteosarcoma have not been extensively studied. Additionally, resveratrol is a potent activator of the Sirt1/Sir2 (silent information regulator 2) family of NAD-dependent deacetylases which plays a role in calorie restriction-mediated tumor suppression. In the present study, we evaluated the effect of resveratrol on growth and apoptosis in four osteosarcoma cell lines (HOS, Saos-2, U-2 OS and MG-63) and a normal human osteoblast cell line (NHOst). We found that Sirt1 protein was relatively higher expressed in the tumor cells than normal osteoblasts. Consistently, resveratrol induced apoptosis in a dose-dependent fashion in the osteosarcoma cells but had minor effect on normal osteoblasts. Also, a similar effect could be elicited by another Sirt1 activator, isonicotinamide. In addition, the pro-apoptotic effect of resveratrol could be enhanced by nutrition restriction elicited by L-asparaginase. We postulate that these effects by resveratrol are mediated via Sirt1 but further studies are needed to confirm or refute this theory.

1. Introduction

Despite significant therapeutic improvements over the past several decades osteosarcoma still has a poor prognosis in about 50% of patients (Lamoureux et al., 2007). Most of the adjuvant chemotherapy regimens cause extensive side effects (Bacci et al., 2000) and multi-drug resistant cases are not uncommon (Ferrari and Palmerini, 2007; Lamoureux et al., 2007).

As a natural phytoalexin, resveratrol is produced by a limited number of plant species such as red grapes and nuts (Athar et al., 2007; Bavaresco, 2003). Proposed benefits of resveratrol on human health include cardioprotection, neuroprotection, as well as cancer suppression (Bradamante et al., 2004; Jang et al., 1997; Sun et al., 2002). The fact that resveratrol can elicit direct cytotoxity to tumor cells without harm on normal tissue indicates a potential for cancer chemotherapy (Athar et al., 2007). Compared with other tumors, data regarding the effects of resveratrol on osteosarcoma are scarce. Additionally, although nearly all stages of carcinogenesis are supposed to be influenced by resveratrol (Delmas et al., 2006) the mechanism(s) through which it exerts such effects remain(s) unclear (Shankar et al., 2007). Resveratrol belongs to the most potent activators of the Sirt1/Sir2 (silent information regulator 2) family of NAD-dependent histone deacetylases (Howitz et al., 2003). With NAD^+ as a cofactor, these decacetylases remove the acetyl group from acetyllysine in histones and non-histone substrates, subsequently causing widespread effects on cell function (Lin et al., 2004). It is known that several beneficial effects of resveratrol are due to activation of Sirt1, including stress resistance and lifespan extension (Howitz et al., 2003; Valenzano et al., 2006). Furthermore, Sirt1 deacetylase was recently found to both suppress tumorigenesis and cancer growth (Firestein et al., 2008). In the present study we evaluated the effects of resveratrol on cell growth and apoptosis in four human osteosarcoma cell lines, HOS, Saos-2, U-2 OS and MG-63 and in the normal human osteoblast cell line, NHOst.

2. Materials and methods

2.1. Reagents

Modified Eagle's Medium alpha (α-MEM), fetal bovine serum (FBS), L-glutamine and gentamicin were purchased from Invitrogen. Resveratrol, isonicotinamide and L-asparaginase were purchased from Sigma-Aldrich.

2.2. Cell cultures

Normal human osteoblast cell line NHOst was obtained from Cambrex. Human osteosarcoma cell lines HOS, Saos-2, U-2 OS and MG-63 were obtained from American Type Culture Collection. All cells were cultured in α-MEM medium supplemented with 10% FBS, 1 mM L-glutamine and 100 μg/ml gentamicin at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. SDS-PAGE and Western blot

Cells were lysed in lysis buffer [10 mM HEPES, pH 7.4, 400 mM NaCl, 5% glycerol, 1.5 mM $MgCl_2$, 0.1 mM EGTA, Complete Mini EDTA-free protease cocktail (Roche Applied Science), 1 mM sodium

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vanadate, 20 mM sodium fluoride, 1 mM PMSF and 1 mM DTT]. Protein extracts were mixed with Laemmli buffer and boiled for 5 min before loading onto SDS-PAGE gels. Proteins were transferred to PVDF membranes by electroblotting and blocked in 5% milk. The membranes were probed with anti-Sirt1 (1:2000) and anti-β-actin (1:5000) antibodies (Gene Tex) diluted in blocking solution followed by horseradish peroxidase conjugated secondary antibody. Proteins were detected with enhanced chemiluminescence detection system (Amersham Biosciences), followed by exposure to X-ray films.

2.4. Cell growth assay

Cell growth was assessed by using the WST-1 assay (Roche Molecular Biochemicals) in 96-well format according to the recommendations of the manufacturer. Briefly, 2000 cells were seeded per well in 96-well flat-bottomed plates in 100 μl α-MEM cell culture medium and incubated for 24 h. The cells were then treated with different concentrations of resveratrol (0–100 μM). Medium was changed after three days when cells were cultured for seven days. Cell growth was analyzed on day 3 and day 7 of culture. Before measurement one-tenth volume of WST-1 reagent was added to each well and cells were incubated at 37 °C for 1 h. The absorbance of the sample was measured against the background (culture medium plus WST-1 reagent), at 450/650 nm. In this assay the OD at 450 nm is proportional to the total number of living cells. At least 4 wells were used for each concentration of the tested reagent and values are expressed as means ± S.E.M.

2.5. Flow cytometric analysis of apoptosis

One hundred thousand cells per well were seeded in 6-well plates in medium containing 10% FBS. After 24 h, the medium was removed and the cells were treated in serum-reduced medium (0.5% FBS) with vehicle or different concentrations of resveratrol (0–50 μM in DMSO), isonicotinamide (0–50 mM in water) or L-asparaginase (0–1 U/ml in medium) for three days. During harvest, adherent cells were digested with trypsin and combined with floating cells in the medium. All cells were centrifuged and washed with PBS. Twenty thousand cells were suspended in 100 μl apoptosis binding buffer, and then stained with Annexin-FITC and/or propium iodide using an Annexin V kit (Caltag Laboratories, Burlingame, CA, USA). Positive controls for apoptosis were obtained by adding 100 nM staurosporine on control cells 4 h before staining with only Annexin-FITC. Positive controls for necrosis were obtained by fixing control cells in −20 °C 70% ethanol for 20 min before staining with only propium iodide. At least 10,000 cells were analyzed by flow cytometry using a FACScan-equipment and CellQuest software (Becton Dickson Co., Franklin Lakes, NJ, USA). The cells binding Annexin V only were recorded as early apoptotic cells, cells binding propium iodide only were recorded as necrotic cells and cells binding both Annexin V and propium iodide were recorded as late apoptotic cells. Both early and late apoptotic cells were recorded as apoptotic cells. The number of cells in each group was then divided by the total number of cells analyzed. Values are expressed as means ± S.E.M.

3. Results

3.1. Resveratrol inhibits cell growth and induces apoptosis of human osteosarcoma cells but has minor effects on human normal osteoblasts

Resveratrol, at increasing concentrations (0–100 μM), was added to cultures of different cell lines, HOS, U-2 OS, Saos-2, MG-63 (osteosarcoma) and NHOst (normal osteoblast) cells for up to 7 days. As shown in Fig. 1, at both tested time points, day 3 and

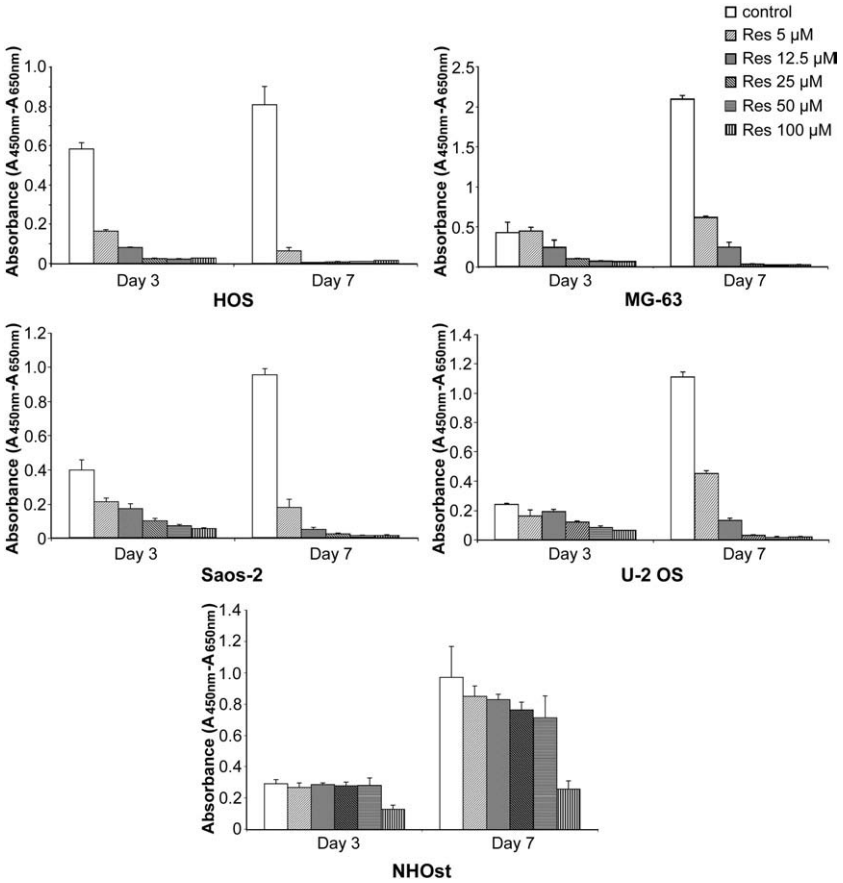

Fig. 1. Resveratrol inhibits cell growth of osteosarcoma cells. Different concentrations of resveratrol (0, 5, 12.5, 25, 50 and 100 μM) were added to the osteosarcoma cell lines, HOS, Saos-2, U-2 OS, MG-63, and the normal human osteoblast (NHOst) cell line. The cells were cultured up to 7 days. WST assay was used to measure cell proliferation. Each data point represents the mean ± S.E.M. of 4 samples. The experiment was repeated 3 times.

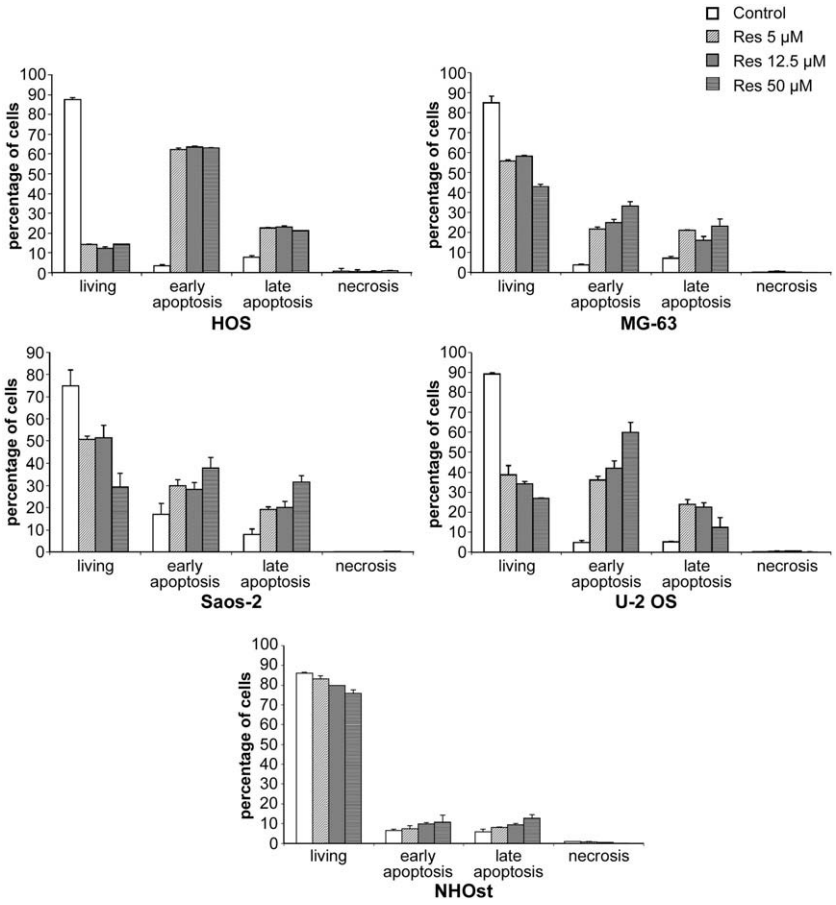

Fig. 2. Resveratrol induces apoptosis of osteosarcoma cells. Different concentrations of resveratrol (0, 5, 12.5 and 50 μM) were added to the osteosarcoma cell lines, HOS, Saos-2, U-2 OS, MG-63 and the normal human osteoblast (NHOst) cell line. The cells were cultured for 3 days after which apoptosis was measured by flow cytometry using Annexin V kit. Each data point represents the mean ± S.E.M. of 3 samples. The experiment was repeated twice.

day 7, resveratrol inhibited cell growth of all four osteosarcoma cell lines dose dependently, an effect already seen at the lowest concentration tested, 5 μM. For the normal osteoblast (NHOst) cells an apoptotic effect could only be seen on at the highest concentration, 100 μM. The inhibitory effect was further confirmed by apoptosis analysis (Fig. 2). This showed that resveratrol, at all concentrations, elicited significant apoptosis on day 3 in all four osteosarcoma cell lines, while it elicited only a moderate effect on normal osteoblasts.

3.2. Sirt1 protein is expressed at higher levels in osteosarcoma cells than in normal osteoblasts

As Sirt1 has been described to be one cellular target for resveratrol we found it interesting to investigate the presence of Sirt1 in the tested cells. Thus, whole cell extracts from the different cell lines were analyzed by Western blot using a Sirt1 antibody. We found that the expression of Sirt1 protein is much higher in the osteosarcoma cells as compared to normal osteoblasts (Fig. 3).

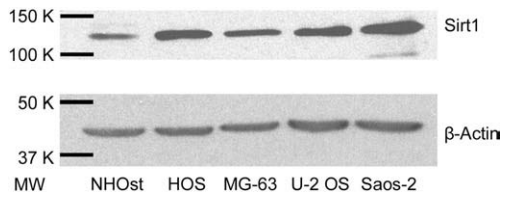

Fig. 3. Western blot identification of Sirt1 protein in osteosarcoma and normal osteoblast cells. Cells were cultured for 3 days after which cell extracts were prepared. Proteins were separated by SDS-PAGE gel electrophoresis and transferred to PVDF membrane. The membranes were probed with anti-Sirt1 and anti-β-actin antibodies followed by horseradish peroxidase conjugated secondary antibody.

3.3. The Sirt1 activator isonicotinamide induces apoptosis of osteosarcoma cells

Isonicotinamide has been described to enhance Sirt1 deacetylase activity by competing with the endogenous Sirt1 inhibitor nicotinamide (Sauve et al., 2005). We found that similar to resveratrol, isonicotinamide dose dependently increased apoptosis in all four osteosarcoma cell lines but elicited almost no effect on normal osteoblasts (Fig. 4).

3.4. The apoptotic effect of resveratrol on osteosarcoma cells is enhanced by L-asparaginase

Nutritional stresses such as calorie restriction in vivo or glucose and serum deprivation in vitro have all been shown to enhance Sirt1 activity either through an increase of Sirt1 expression (Cohen et al., 2004; Nemoto et al., 2004; Rogina et al., 2002) or depriving cells of the endogenous Sirt1 inhibitor, nicotinamide. We thus found it interesting to investigate if a situation involving nutritional stress could enhance the effect of resveratrol on osteosarcoma cells. The enzyme L-asparaginase is used for the treatment of acute lymphoblastic leukemia through depriving L-asparagine in the tumor surrounding environment. The supposed mechanism is that in comparison with normal tissue tumor

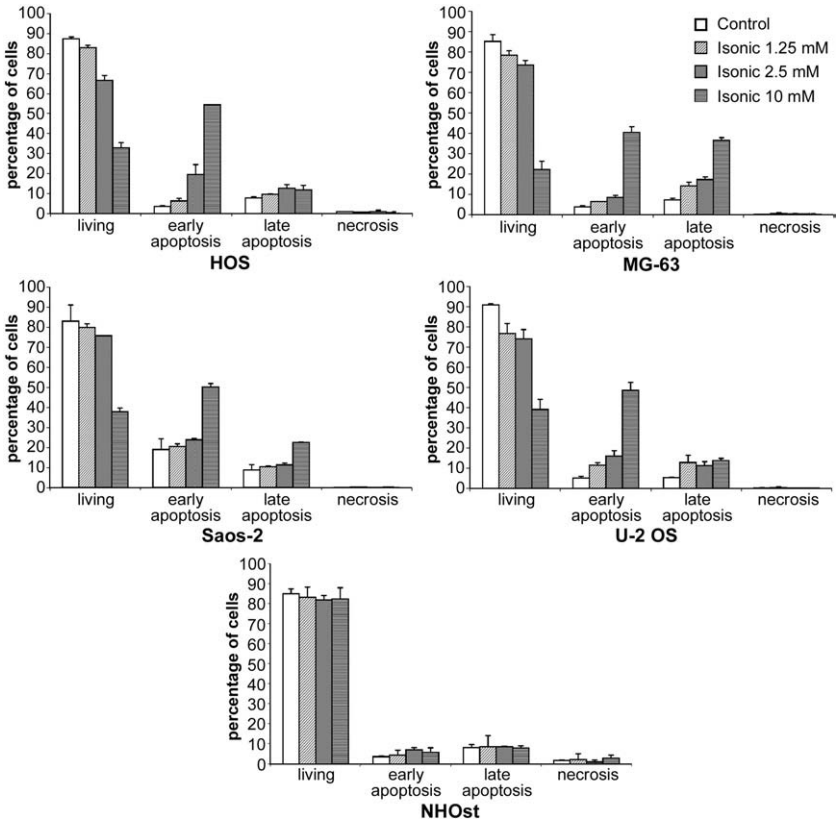

Fig. 4. Isonicotinamide induces apoptosis of osteosarcoma cells. Different concentrations of isonicotinamide (0, 1.25, 2.5 and 10 mM) were added to the osteosarcoma cell lines, HOS, Saos-2, U-2 OS, MG-63 and the normal human osteoblast (NHOst) cell line. The cells were cultured for 3 days after which apoptosis was measured by flow cytometry using Annexin V kit. Each data point represents the mean ± S.E.M. of 3 samples. The experiment was repeated twice.

cells are usually deficient of asparagine synthetase and have to rely on an external source of L-asparagine to keep up with their rapid growth (Narta et al., 2007; Verma et al., 2007). As indicated in Fig. 5, the osteosarcoma and normal osteoblastic cells, and as a comparison, the lymphoblastic leukemia Jurkat cells were analyzed for apoptosis after incubation with different concentrations of L-asparaginase for 3 days. We found that these cell lines demonstrated different responses to L-asparaginase treatment. However, as indicated in Fig. 6, regarding all the osteosarcoma cell lines, the presence of 0.125 U/ml L-asparaginase could apparently enhance the pro-apoptotic effects of 1 μM resveratrol. One possible explanation for these synergic effects could be that the treatment of L-asparaginase increases Sirt1 expression. However, our Western blot analysis showed no apparent difference in Sirt1 protein levels before and after 3 days of L-asparaginase treatment (data not shown).

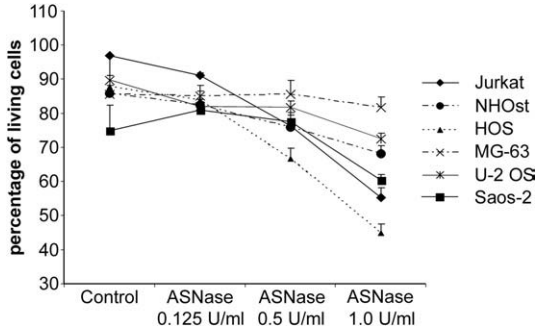

Fig. 5. The effects of L-asparaginase on apoptosis of osteosarcoma cells. Different concentrations of L-asparaginase (0.125, 0.5 and 1 U/ml) were added to the osteosarcoma cell lines, HOS, Saos-2, U-2 OS, MG-63 and the normal human osteoblast (NHOst) cell line, as well as to lymphoblastic leukaemia Jurkat cells. The cells were cultured for 3 days after which apoptosis was measured by flow cytometry using Annexin V kit. Each data point represents the mean ± S.E.M. of 3 samples. The experiment was repeated twice.

4. Discussion

Osteosarcomas are characterized by complex chromosomal abnormalities including a high degree of aneuploidy, gene amplification, and multiple unbalanced chromosomal rearrangements. Such genomic aberrations often vary between cases (Sandberg and Bridge, 2003). Therefore, cells from different origins are needed for in vitro evaluation of therapeutic effects of a particular agent. In this study, four commonly used osteosarcoma cell lines, HOS, Saos-2, U-2 OS and MG-63, were studied for the cytotoxic effects of resveratrol. All these cell lines demonstrate typical characters of osteosarcoma such as different degrees of chromosomal ploidy (Lim et al., 2005), genomic and phenotypic instability (Hausser and Brenner, 2005; Sihn et al., 2005) and expression of immature osteoblastic markers (Kuroda et al., 2003; Mayr-Wohlfart et al., 2001; Son et al., 2007). In addition, many previous reports indicate that similar to the clinical osteosarcoma cases these cell lines demonstrate variable responses to conventional chemotherapeutic agents. For example, cisplatin exerted cytotoxic effects on HOS and MG-63 cells with an ED_{50} of around 9.0 and 12.2 μM, respectively, while it elicited no cytotoxicity to U-2 OS cells at a concentration as high as 20 μM (Robson et al., 2002). Doxorubicin could induce significant cell death on Saos-2 cells, whereas such an effect was not observed for HOS and U-2 OS cells (Gomes et al., 2006). The sensitivity to methotrexate is even more complicated with inconsistent results having been reported for each individual cell line (Gomes et al., 2006; Li et al., 1995; Orosco et al., 2007; Seki et al., 2000). Our results demonstrate that unlike the above mentioned chemotherapeutic drugs, resveratrol exerted a potent pro-apoptotic effect on all tested osteosarcoma cell lines while it had only a

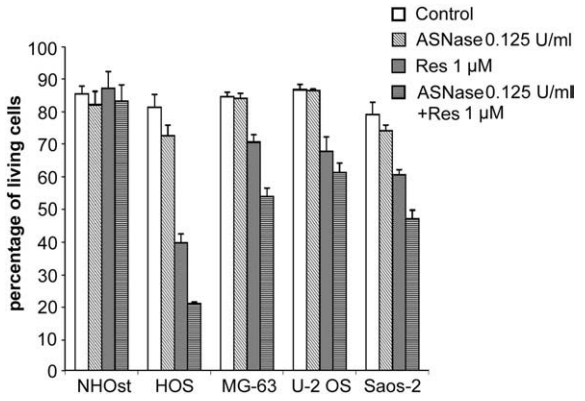

Fig. 6. The effects of L-asparaginase and resveratrol, separate or in combination, on apoptosis of osteosarcoma cells. The osteosarcoma cell lines, HOS, Saos-2, U-2 OS, MG-63 and the normal human osteoblast (NHOst) cell line were treated with 0.125 U/ml L-asparaginase, 1 μM resveratrol or the combination of both. The cells were cultured for 3 days after which apoptosis was measured by flow cytometry using Annexin V kit. Each data point represents the mean ± S.E.M. of 3 samples. The experiment was repeated twice.

moderate effect on normal osteoblasts. Thus, resveratrol could be a potential agent to complement the arsenal used in the treatment of osteosarcoma.

Resveratrol is one of the most potent Sirt1 activators. Through binding to a yet not well defined binding site it induces a conformational change in Sirt1, which lowers K_m for both the acetylated substrate and NAD, resulting in an increased enzymatic activity (Howitz et al., 2003). Many of the Sirt1 substrates are transcription factors and key regulators described to take part in cancer development, such as the tumor suppressor gene p53, the nuclear factor-κB (NF-κB), the DNA repair factor Ku70 and the forkhead transcription factors (FoxOs) (Saunders and Verdin, 2007). However, the relationship between Sirt1 activity and tumorigenesis is still open to debate (Saunders and Verdin, 2007). On one hand, Sirt1 was found to be highly expressed in several types of tumors (Huffman et al., 2007) and may be responsible for the development of chemotherapy resistance (Chu et al., 2005). On the other hand, Sirt1 is an important mediator in calorie restriction-associated tumor prevention (Bordone and Guarente, 2005; Patel et al., 2004). Effects such as suppressing intestinal tumorigenesis and inhibition of breast cancer have also been recently reported (Firestein et al., 2008; Wang et al., 2008). Our results demonstrate that despite the described chromosomal variability and instability all the tested osteosarcoma cell lines expressed higher level of Sirt1 protein compared with the normal osteoblasts. Furthermore, resveratrol and isonicotinamide, another known Sirt1 activator, could induce apoptosis in the osteosarcoma cells in a similar pattern. The fact that both Sirt1 activators induced apoptosis is an indication that activation of Sirt1 initiated apoptosis in the osteosarcoma cells.

The Sirt1 inhibitor nicotinamide is an endogenous by-product of NAD-dependent deacetylation (Jackson et al., 2003; Sauve and Schramm, 2003). It is supposed that over 95% of Sirt1 activity is inhibited in mammalian cells due to a high nuclear concentration of nicotinamide (Bitterman et al., 2002; Sauve et al., 2005; Sauve and Schramm, 2003). Furthermore, nicotinamide-mediated Sirt1 inhibition could be relieved by in vivo deamination of nicotinamide by Pnc1, a nicotinamidase (Ghislain et al., 2002). The expression and enzymatic activity of Pnc1 have been shown to increase dramatically in response to nutritional stresses such as reduction of glucose or amino acid (Anderson et al., 2003; Gallo et al., 2004). Additionally, the mechanism for nicotinamide-regulated Sirt1 deacetylation is different from that used by resveratrol. Therefore we speculate that a combination of the two mechanistically distinct pathways may synergistically enhance deacetylase activity of Sirt1 in vivo. Our results show that the pro-apoptotic effect of reseveratrol on osteosarcoma cells could be significantly enhanced under nutritional stress caused by L-asparaginase. This indicates a potential way to enhance the anti-osteosarcoma effects of resveratrol although more experimental data are needed to support such a mechanism.

In summary, our study provides important information regarding resveratrol's effect on osteosarcoma cells. The results presented here suggest that resveratrol might be useful in the treatment of osteosarcoma. More studies are needed, for example the cellular mechanism of action of Sirt1 in resveratrol-mediated apoptosis demands further investigation and, furthermore, the resveratrol-induced apoptosis of osteosarcoma cells needs to be investigated in appropriate in vivo models.

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