Ascorbic Acid Induces either Differentiation or Apoptosis in MG-63 Osteosarcoma Lineage

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Abstract. *Background/Aim: Osteosarcoma originates from mesenchymal stem cells with impaired bone differentiation. In the present study we investigated the effect of ascorbic acid (AsA) on osteogenic differentiation and apoptosis of the MG-63 osteosarcoma cell line. Materials and Methods: We evaluated the expression of runt-related transcription factor-2 (RUNX2) and secreted phosphoprotein 1 (SPP1) genes by real-time Polymerase Chain Reaction (PCR) and of endogenous bone morphogenetic protein-2 (BMP2) and osteocalcin proteins by immunohistochemistry. We analyzed osteoblast maturation by phosphatase alkaline synthesis and calcium deposition, and apoptosis by (TUNEL) test and Annexin staining. Results: Our results showed that RUNX2 and SPP1 gene expression was increased in cells treated with low concentrations of AsA with respect to untreated cells. At higher concentrations, AsA induced apoptosis of osteosarcoma cells, possibly with the involvement of p21. Conclusion: Our findings support the ability of AsA to induce both differentiation, by affecting the target involved in early and late phases of osteogenic maturation, and apoptosis in poorly-differentiated osteosarcoma cells.*

Osteosarcoma is the most common primary malignant tumour of bone, and chemotherapeutic regimens used in the treatment of osteosarcoma result in significant morbidity, such as cardiac toxicity, infertility, and renal dysfunction (1).

Despite aggressive treatment, more than one-third of patients develop recurrent high-grade osteosarcoma, with metastatic disease, particularly osteolytic bone metastases. Thus, there is a pressing need for the development of new and alternative approaches to the treatment of this disease. Some researchers, have focused on promoting terminal differentiation in order to treat human tumors (2), like the use of *all-trans* retinoic acid receptor in acute promyelocytic leukemia (3).

Ascorbic acid (AsA) is a water-soluble vitamin that acts as a co-factor in many biological reactions (4). The effects of AsA are often attributed to its antioxidative properties, but it is also an important supplement for mesenchymal stem cell (MSC) differentiation towards osteoblast lineage (5), it increases collagen production (6) and it is an essential co-factor for the hydroxylation of proline and lysine residues in collagen (7). AsA increases DNA synthesis, RNA transcription and induces post-translational protein modifications. It promotes cell differentiation at lower concentrations but becomes cytotoxic at higher doses, leading to apoptosis (8).

In vitro and *in vivo* analysis showed that the anti-proliferative activity of AsA is due to the inhibition of expression of genes involved in cell division progression (9). In normal cells, for example, the transition from G_1 phase to S phase requires the activity of two classes of cyclin-dependent kinases (CDKs), namely CDK4/6 and CDK2 (10). Two families of mammalian G1 CDK inhibitors, the CIP/KIP (p21, p27, and p57) and the INK-4 (p15, p16, p18, and p19), have been described (11). Recent studies indicate that vitamin C mediates its anti-proliferative effects on tumor cells by increasing p53, $p21^{waf1/cip1}$ expression and inhibiting CDK2 expression (12). On the other hand, AsA stimulates osteogenic differentiation of MSCs (5) and promotes tumor cell maturation, as observed in some cell lineages, such as HEP-2, KB, bone marrow, melanoma, human osteoblast-like cells (osteosarcoma-derived cells) (8).

It is generally believed that osteogenic sarcoma may originate from MCSs (13). The pluripotent nature of MSCs permits differentiation *in vitro* into several distinct lineages (*e.g.* chondroblastic, fibroblastic, and osteoblastic phenotypes) that account for most of the phenotypes exhibited in osteosarcoma.

MG-63 cells are an osteosarcoma lineage characterized by an atypical bone-forming model since the gene expression profile and the capability to synthesize a correct extracellular

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bone matrix (ECM) are impaired (14). MG-63 cells look like fibroblasts rather than osteoblasts and the loss of osteoblast differentiation is associated with increased proliferation leading to important prognostic significance *in vivo* and impairment of the gene expression profile (14, 15).

The runt-related transcription factor-2 (RUNX2) defines the osteoblastic lineage by mediating the expression of osteoblast-specific genes, and the regulation of its activity is linked to cell proliferation (16). RUNX2 is absent or non-functional in many cell lines of osteosarcoma, and experimental restoration of *RUNX2* gene expression promotes terminal cell differentiation and reduces malignancy (17). RUNX2 is mainly involved in the integration of signals for the induction of bone formation (18), in the regulation of gene expression in osteoblast lineage cells (19), and in mediating the invasive properties of cancer cells which are metastatic to bone (20).

Moreover, several members of the Transforming Growth Factor (TGFβ) superfamily, including the bone morphogenetic proteins (BMP)-2,-4 and -7, have strong osteogenic effects. BMPs in early embryogenesis up-regulate many homeodomain (HD) proteins, which regulate tissue specification, MSC growth, differentiation and apoptosis in skeletal development (21). Many of the bone proteins of the ECM, including collagen, osteopontin, alkaline phosphatase, bone sialoprotein, osteocalcin, together with the transcription factors RUNX2 and osterix, are directly regulated by HD proteins. A recent study showed that BMP2 and BMP5 are down-regulated in adrenocortical carcinoma (22), playing an important role in the regulation of cell growth, apoptosis, and differentiation (23).

Previously research has been performed to investigate the effects of AsA on MG-63 differentiation and proliferation. They showed that AsA increases the expression and activity of alkaline phosphatase (ALP), improves collagen, type I, alpha 1 (COL1A1) production, mainly by post-translational modification (24, 25) and stimulates ECM synthesis (25). However, it is not well-documented if ascorbic acid also has an anti-tumor effect, promoting apoptosis on osteosarcoma, as is described for other cell lineages (9).

In the current study, we hypothesized that by affecting the expression of molecules involved in the early phase of differentiation, a mature osteoblastic phenotype may be obtained from osteoblastic tumoral cells. In particular, we aimed to assess the effect of increasing concentrations of AsA on osteoblastic cell differentiation and cell viability. In addition, cell apoptosis was evaluated since apoptosis and differentiation are correlated events.

Materials and Methods

Cells and AsA treatment. Osteosarcoma MG-63 human osteoblast-like cells were obtained from the American Type Culture Collection (Rockville, MD, USA).

AsA was purchased from StemCell Technologies Inc. (Vancouver, BC, Canada) The powder was dissolved in sterile water at a final concentration of 50 mM. Stock solutions were aliquoted and kept at −20˚C for long-term storage and protected from exposure to light and air to prevent oxidation.

Cells were cultured under a humidified atmosphere with 5% CO_2 and passaged in Dulbecco's Modified Eagle's medium (DMEM)/F12 media containing 10% fetal bovine serum (FBS) supplemented with antibiotics (1% penicillin and streptomicyn) and glutamine (1% glutamine). Cells were then harvested using trypsin, washed and counted on a microscope using a Burker haemocytometer and plated again in DMEM/F12 containing 5% FBS, 1% penicillin/ streptomycin and 1% glutamine. Once 80% confluence was reached, AsA was added and medium containing AsA was changed every three days.

For the induction and analysis of cell differentiation, cells were treated with AsA concentrations of 62.5, 125 and 250 μM for a time ranging from 24 h until 10 days, while AsA was used at concentrations ranging from 0 to 1,000 μM for 3 and 24 h for evaluation of apoptosis.

Cell viability. Cell viability was evaluated by a colorimetric assay based on the reduction of the tetrazolium salt sodium 3I-[1-phenylamino-carbonyl-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) (XTT) by mitochondrial dehydrogenase of viable cells to a formazan dye (Cell proliferation kit II – XTT Roche, Milano Italy).

XTT test was performed after 3 and 24 h of AsA exposure. Briefly, 100 μl XTT labelling mixture was added to each well and the cells were incubated at 37˚C under a humidified atmosphere with 5% CO_2 for 4 h. The spectrophotometric absorbance of the samples was measured every hour (four measurements) using a microtitre plate (ELISA) reader at a wavelength of 450 nm. We measured the absorbance every hour in order to obtain the linear region of the XTT assay curve as in this region, the greatest sensitivity is observed.

Total RNA extraction and reverse transcription. After treatment, cells were scraped, and pellets were collected by centrifugation at 1000 ×*g* for 10 min at 4˚C for RNA extraction. Total RNA was extracted from each pellet using the RNeasy minikit (Qiagen, Milano Italy) with DNAse I treatment.

First-strand cDNA was generated using the High-Capacity cDNA Archive Kit, with random hexamers, (Applied Biosystems PE, Foster City, CA, USA) according to the manufacturer's protocol. The cDNA product was aliquoted in equal volumes and stored at −80˚C.

Real time PCR. PCR was performed in a total volume of 20 μl containing 1× Premix Ex Taq™ (2×), 1× Rox Reference Dye (50×) and 20 ng of cDNA. Probe sets for each gene (RUNX2, Hs00231692_m1; SPP1, Hs00167093_m1, B2M, Hs999999_m1; *P21*, Hs00355782_m1; Applied Biosystems) were obtained from Assay-on-Demand Gene Expression Products (Applied Biosystems). Real time RT-PCR reactions were carried out in a two-tube system and in multiplex.

The amplification conditions included 30 s. at 95˚C (initial denaturation), followed by 50 cycles at 95˚C for 5 s (denaturation) and at 60˚C for 31 s (annealing/extension). Thermocycling and signal detection were performed with ABI Prism 7000 Sequence

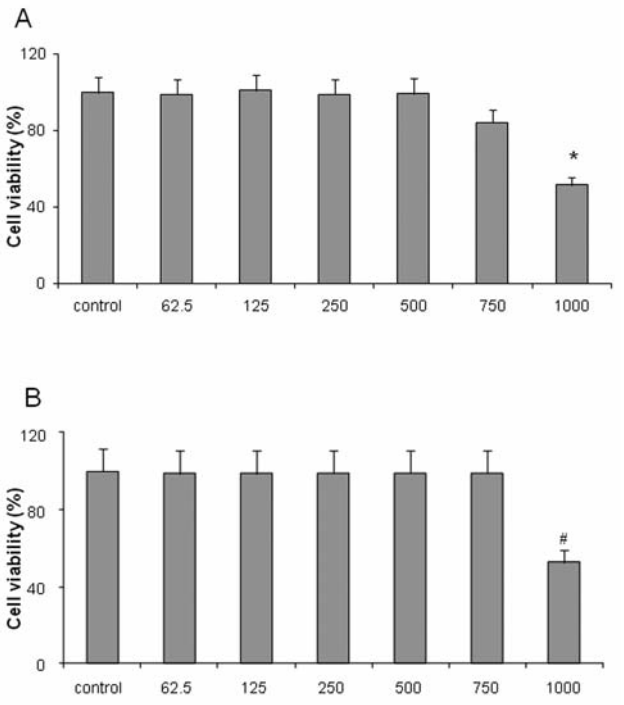

Figure 1. *Cell viability. Cell viability was always >95% when Ascorbic acid (AsA) treatment was used at concentrations ranging from 0 to 500 μM, both after 3 h (A) and 24 h (B) of treatment. On the contrary, a reduction of cell viability was observed when AsA was used at concentrations of 750 μM and 1,000 μM both after 3 h and 24 h of treatment (*p<0.001; #p=0.002).*

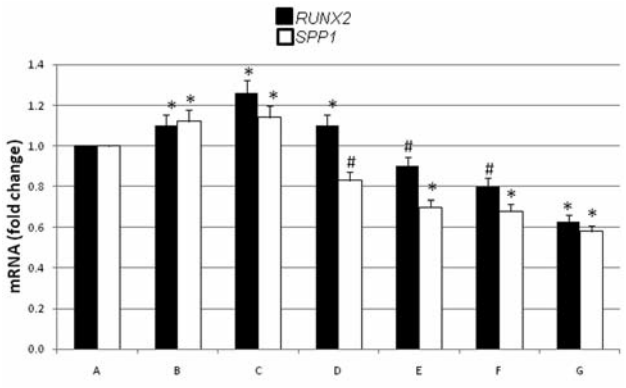

Figure 2. *Runt-related transcription factor-2 (RUNX2) and secreted phosphoprotein-1 (SPP1) gene expression. Ascorbic acid (AsA) treatment increased RUNX2 expression in MG63 cell line and the highest effect was observed at 125 μM (A) (p<0.001). AsA also increased SPP1 gene expression at concentrations of 62.5 μM and 125 μM (B); on the contrary, treatment induced down-regulation of SPP1 when AsA was used at a concentration of 250 μM (B) (*p<0.001; #p=0.002). A: Control; B: 62.5 μM of AsA; C:125 μM od AsA; D:250 μM of AsA; E: 500 μM of AsA; F: 750 μM of AsA; G: 1000 μM of AsA.*

Detector (Applied Biosystems). Signals were detected according to the manufacturer's instructions (26). ΔΔcT values were then calculated with respect to the control.

To normalize mRNA expression for sample-to-sample differences in RNA input, quality and reverse transcriptase efficiency, we amplified the housekeeping gene β_2M. Endogenous control gene was abundant and remained constant proportionally to total RNA among the samples.

Immunocytochemistry. Cells were grown on slides with different AsA concentrations for six days under a humidified atmosphere with 5% CO_2 at 37˚C. Cultured cells were then fixed and permeabilized with cold acetone and stored at –20˚C. For immunocytochemistry, cells were firstly rinsed two times in PBS and permeabilized with washing solution (Triton ×100 0.1% in PBS). Endogenous peroxidases were neutralized by incubation in 0.3% H_2O_2 in distilled water.

Primary antibodies (mouse anti-human BMP-2, mouse anti-human osteocalcin and goat anti-human RUNX-2; Santa Cruz Biotechnology, Heidelberg, Germany) were diluted 1:100 in washing solution 1% bovine serum albumin (BSA), and the slides were incubated overnight at 4˚C in a humidified chamber. The next day, each chamber was incubated for 1 h at room temperature with horseradish peroxidase (HRP) conjugated secondary antibodies (goat anti-mouse and donkey anti-goat; Santa Cruz Biotechnology) at a dilution of 1:100. Marked cells were revelead using 3,3'-diaminobenzidine (DAB) solution according to the supplier's protocol (DAKO, Milan, Italy). Finally, cells were counter-stained with hematoxylin.

To express data in a semi-quantitative way, the levels of protein in six cell-containing slides and three different fields were measured at a magnification of ×40. The level for each of the proteins evaluated was calculated as the percentage of positive cells with respect to the total number of haematoxylin-stained nuclei .

ALP staining. The Alkaline Phosphatase staining was performed with Alkaline Phosphatase kit N˚ 85 purchased from Sigma-Aldrich, by following the manufacturer's instructions.

Cells were plated in 2×10^4 in a glass chamber. Once they reached 80% confluence, cells were treated with AsA and the medium was removed every two days until the tenth day.

At the end of each treatment period, cultures were washed twice with PBS and ALP Staining solution was used. After incubation, the staining solution was removed, the cultures were washed with distilled water to remove excessive colour and samples were evaluated by optical microscopy. Finally, the slides were counterstained with buffered Neutral Red Solution.

Alizarin red assay. To evaluate calcium deposition, 2×10^4 cells were plated in a glass chamber and treated with AsA for six days, replenishing the medium every three days. The cells were then fixed with 70% ethanol, rinsed three times with deionized water, treated 15 min with 40 mM Alizarin red S at pH 4.1, and then gently washed with 1× phosphate-buffered saline for 15 min.

Apoptosis. Apoptosis was analyzed in paraformaldehyde-embedded cells in a glass chamber by terminal deoxynucleotidyl transferase (TdT) technique for the detection of positive cells by specific staining (TUNEL). The procedure used was that reported in the manufacturer's instructions (ApoTag Fluorescin *In Situ* Apoptosis Detection Kit, S7110, Millipore Corporation, Billerica, MA, USA).

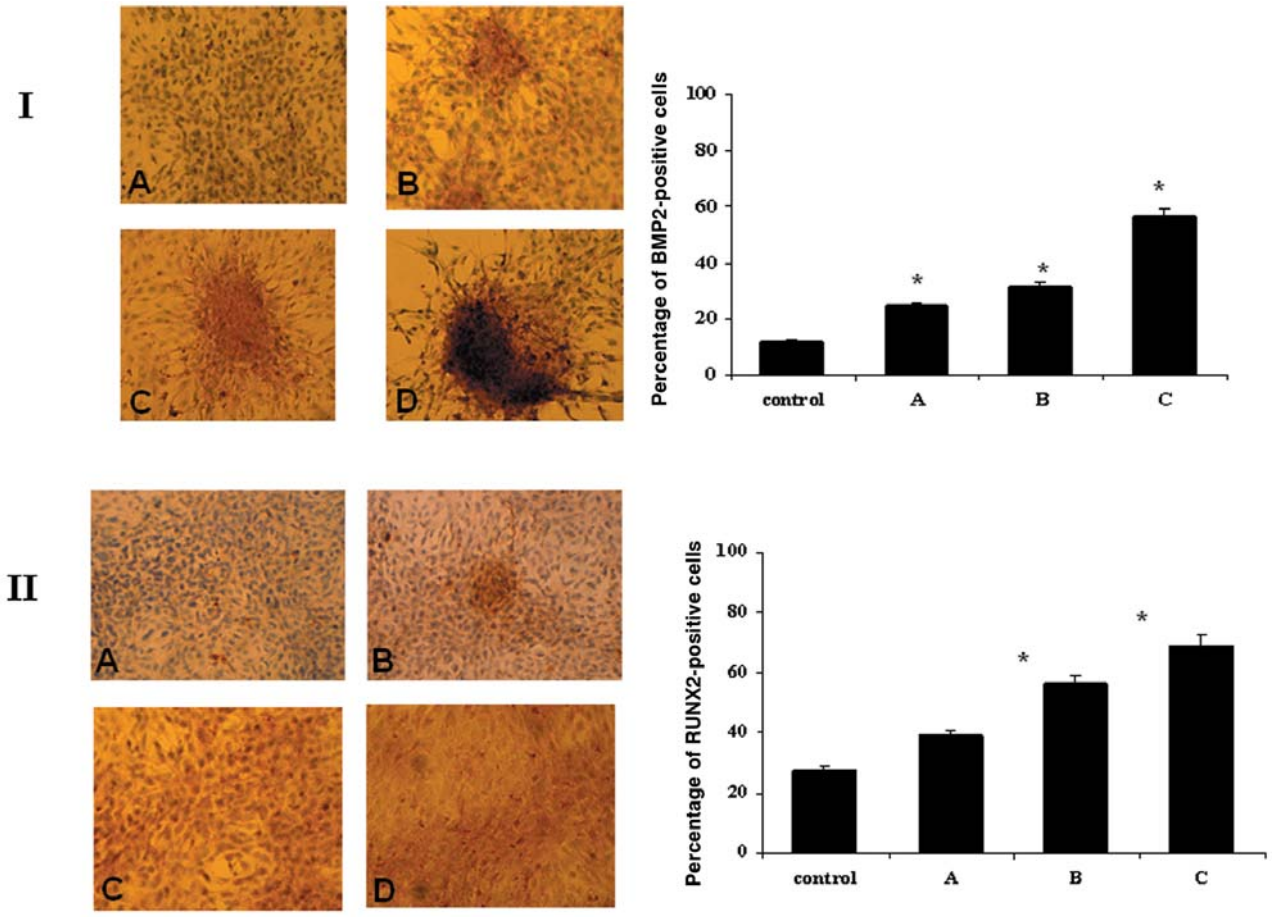

Figure 3. *Bone morphogenetic protein-2 (BMP-2) and runt-related transcription factor-2 (RUNX2) immunocytochemistry. After six days of treatment, the number of cells expressing BMP2 protein (I), and RUNX2 (II) valuated by immunocytochemistry, was higher in treated cells than in control cells at all concentrations (*p<0.05) (magnification ×20). A: Control; B: 62.5 μM of AsA; C:125 μM od AsA; D:250 μM of AsA.*

Apoptotic nuclei were identified by the green fluorescence under microscopy by incubating the glass slides with 25 μl (10%) of equilibration buffer, 10 μl of working strength enzyme (70% reaction buffer and 30% TdT enzyme), 50 μl of working strength stop/wash buffer (1 ml stop/wash buffer and 34 ml dH20), 50 μl working strength fluorescein antibody solution. Finally, total nuclei were counterstained with dihydrochloride (DAPI, Millipore Corporation, Billerica, MA, USA).

The number of apoptotic cells was expressed as the TUNEL index was calculated as the number of TUNEL-positive nuclei divided by the total number of nuclei (evaluated by DAPI) multiplied by 100.

Four different fields for each sample with about 80-100 total cells were measured.

Annexin staining. The exposure of phospholipid phosphatidylserine on the plasma membrane of apoptotic cells was detected using the annexinV-fluorescein isothiocyanate (FITC)/propidium iodide (PI) detection kit (Bender Med System, Vienna, Austria), according to the manufacturer's recommended protocol. Briefly, cells were treated with AsA for 3 or 24 h. After incubation, cells were trypsinized and suspended in 1 ml (PBS). For each cell suspension, 250×10^3 cells were centrifuged and re-suspended in 500 μl of 1× binding buffer. According to the protocol, cells were stained with annexinV-FITC for 10 min. Propidium iodide (PI) was added just before the analysis with the FACSCanto™ flow cytometer (BD Biosciences, San Jose, CA, USA). At least 10000 events were acquired. Samples were analyzed by FlowJo software (TreeStar, Ashland, Oregon, USA). Viable cells were defined as annexinV-negative and PI-negative; early apoptotic cells were defined as annexinV-positive and PI-negative; late apoptotic/necrotic cells were defined as annexin V-positive and PI-positive.

Statistical analysis. Results are expressed as mean±S.E. The Wilcoxon test was used for nonparametric data. For analysis of treatment responses, multiple measurement ANOVA followed by Bonferroni as *post-hoc* analysis was performed. A probability value of less than 0.05 was considered statistically significant. Spearman correlation coefficient and regression curve estimations were performed when indicated. Analyses were applied to experiments carried out at least three times. Statistical analyses were performed using SPSS for Windows, version 16.0 (SPSS Inc, Chicago, IL, USA).

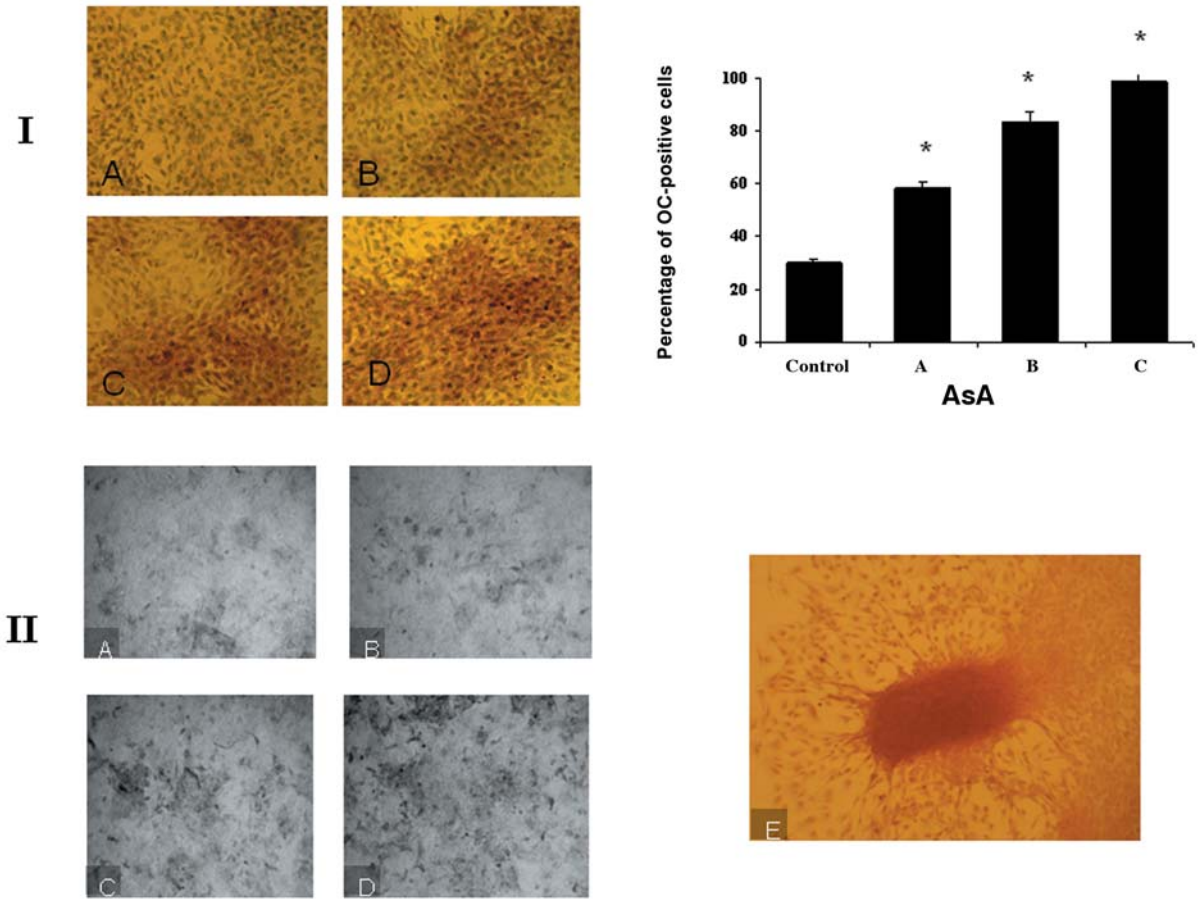

Figure 4. *Osteoblastic maturation. The number of positive cells, evaluated by immunocytochemistry, for the late marker osteocalcin (Panel I) of osteogenic differentiation were increased after 6 days of treatment with 62.5 μM (B), 125 μM (C) and 250 μM (D) of Ascorbic acid (AsA) p<0.05 magnification ×20. ALP expression after 8 days of treatment was increased in cells treated with 62.5 μM (B), 125 μM (C) and 250 μM (D) of AsA in a dose-dependent manner, magnification ×10 (Panel II). In cells treated with 125 μM of AsA (E) some ossification nuclei, evaluated by alizarin red staining, were observed after six days of treatment, magnification ×10 (Panel II).*

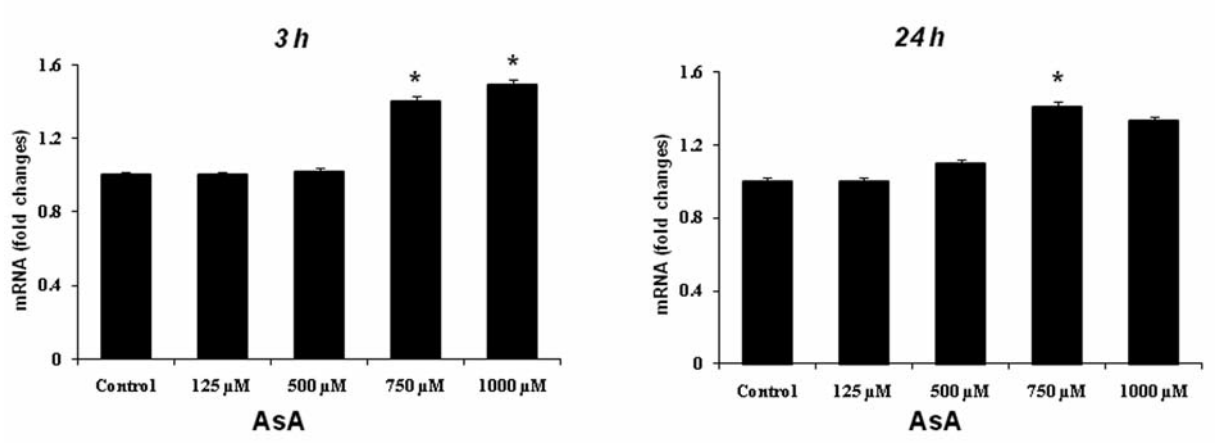

Figure 5. *p21 gene expression. p21 gene expression increased after 3 h of treatment and the mRNA expression became significantly greater than that of the control at the higher concentration of Ascorbic acid (AsA) (p<0.001). After 24 h, the increase was also significant at 500 μM. (*p<0.001).*

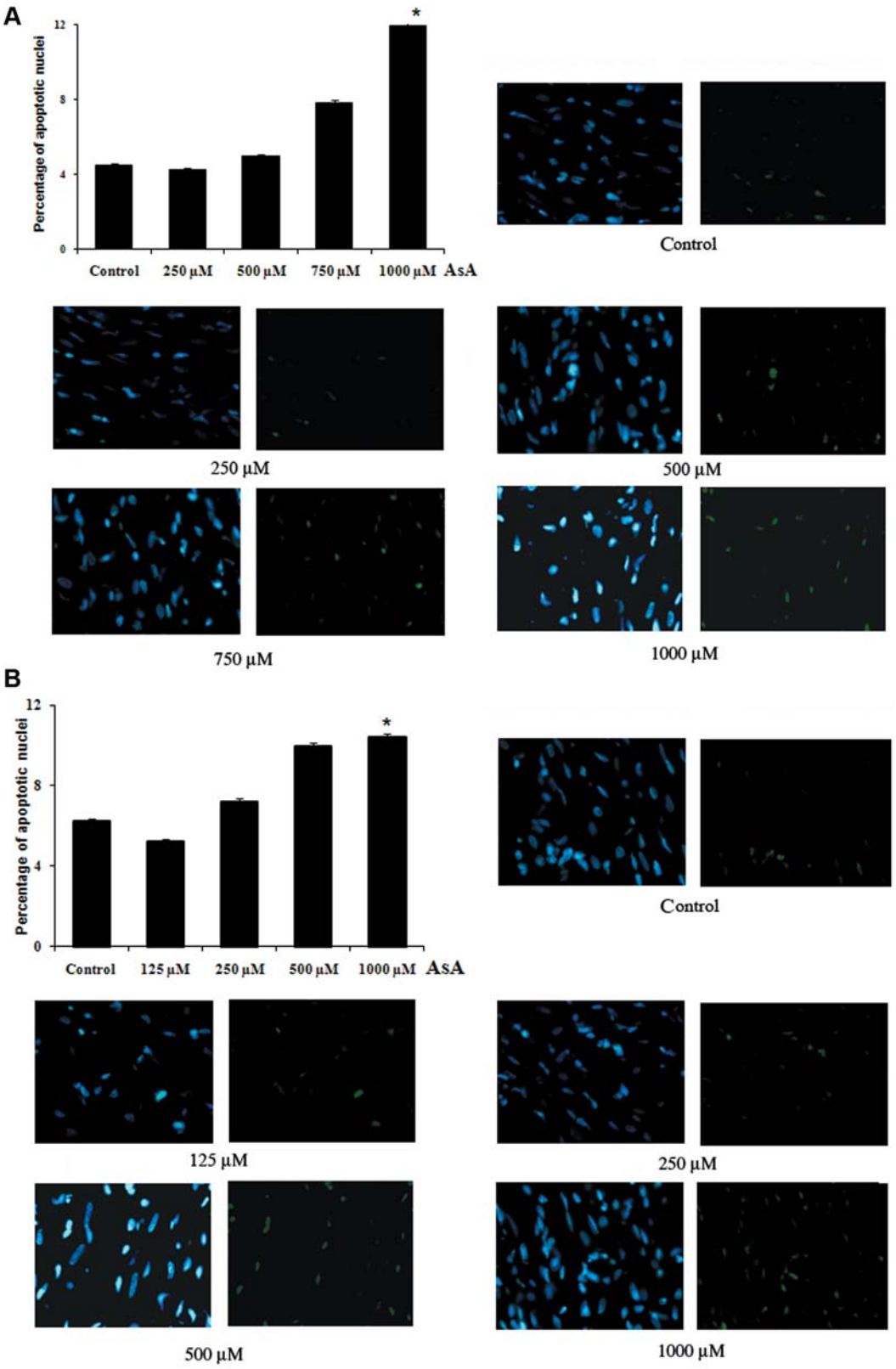

Figure 6. *Apoptotic nuclei evaluation. The number of apoptotic cells, evaluated by TUNEL method, increased in a dose-dependent manner, especially after treatment with 750 and 1,000 µM, and became significantly higher than control at 1,000 µM both after 3 h (A) and 24 h (B) (*p<0.01).*

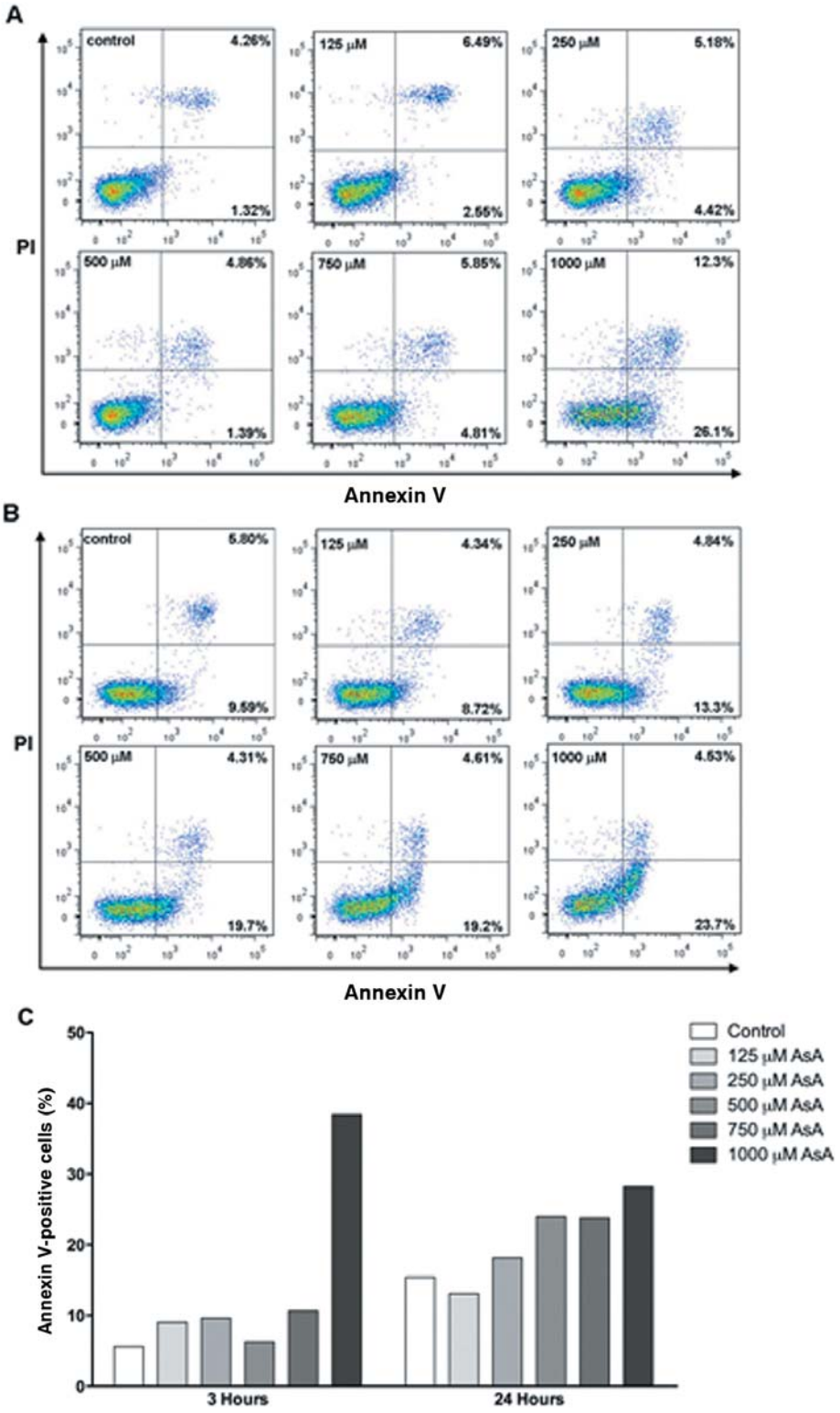

Figure 7. *Annexin staining. Flow cytometric analysis of annexin/PI-stained MG63 cells treated for 3 h (A) and 24 h (B) with different concentrations of Ascorbic acid (AsA) (0-1,000 μM). Early apoptotic cells were defined as annexinV-positive and PI-negative; late apoptotic/necrotic cells were defined as annexin V-positive and PI-positive. Analysis of MG63 cells treated with AsA for 3 and 24 h and stained with annexin V and PI showed a dose-dependent increase of annexinV-positive cells. Data represent two independent experiments.*

Results

Effects of AsA on cell viability. We first investigated the effects of AsA on MG-63 cell viability. The cell viability obtained by XTT is expressed as a percentage in Figure 1. When the cells were treated with AsA at concentrations ranging from 0 to 250 μM, cell viability was always >95% (Figure 1). However, when higher concentrations were used, a dose-dependent reduction of cell viability (especially at 750 and 1000 μM) was observed. In addition, the decrease became significant with the highest concentration of Asa (1000 μM) both at 3 ($p<0.001$) (Figure 1A) and 24 h ($p=0.002$) (Figure 1B).

Osteoblastic differentiation. To evaluate the role of AsA on osteogenic differentiation, we analyzed the effects of AsA at concentrations ranging from 0 to 1,000 μM on the transcription of *RUNX2* and on the bone related gene *SPP1*, involved in osteoblast differentiation process.

AsA induced a significant increase of *RUNX2* mRNA with respect to the control after 72 h of culture up to concentrations of 250 μM ($p<0.001$). In particular, the effect of AsA at 125 μM led to the highest increment of Runx2 expression, as shown in Figure 2. On the contrary, at all concentrations ranging from 500 to 1,000 μM of AsA, *RUNX2* mRNA expression decreased ($p<0.001$).

SPP1 was also significantly up-regulated after 72 h of treatment with AsA at a concentration of 62.5 and 125 μM ($p<0.001$; Figure 2). On the contrary, we observed significantly reduced expression from 250 up to 1,000 μM ($p<0.001$; Figure 2).

We evaluated the effects of AsA on protein expression of early (BMP2 and RUNX2) and late (osteocalcin) markers of osteoblastic differentiation.

After four days of culture we observed an increase of protein expression of BMP2 and RUNX2 in AsA-treated cells. BMP2 and RUNX2 levels evaluated by immunocytochemistry were higher in treated cells than in controls in a statistically significant manner ($p<0.05$ and $p<0.005$, respectively) at all concentrations of AsA (Figure 3).

Effect of AsA on osteoblast maturation. The evaluation of immunocytochemistry for osteocalcin revealed the number of positive cells to be higher in treated compared to untreated cells after six days of culture and the difference was statistically significant when AsA was used at a concentration of 125 and 250 μM (Figure 4I).

To assess the ability of AsA to induce osteoblastic maturation, the ALP and the presence of calcifications, detected by Alizarin red, were evaluated. We observed a progressive increase of ALP expression from two to ten days of treatment, as suggested by more intense cell staining. AsA treatment did not produce any difference in ALP expression between groups until the eighth day of observation, when a dose-dependent increase was observed (Figure 4II).

MG-63 sarcoma cells are characterized by an impaired mineralization capacity, so we tested calcium deposition after six days of treatment with AsA.

As shown in Figure 4II, we observed the formation of some ossification nuclei after treatment with AsA, especially at a concentration of 125 μM.

p21 gene expression and apoptosis. Since vitamin C mediates its anti-proliferative effects on tumor cells by increasing p21 expression, we evaluated the level of the oncosuppressant gene *p21* in the MG63 cell line treated with AsA at concentrantions ranging from 125 μM to 1,000 μM.

We observed a dose-dependent increase of p21 after 3 h of treatment that became significant at the higher concentration of AsA ($p<0.001$) (Figure 5). After 24 h the increase was already significant at 500 μM and the greater rise of p21 was described with 750 μM ($p<0.001$) (Figure 5).

The number of apoptotic cells increased in a dose-dependent manner, especially after treatment with 750 and 1,000 μM, and became significantly higher than control at 1000 μM ($p<0.01$) either after 3 h and 24 h (Figure 6).

We found a significant direct correlation between *p21* gene expression and TUNEL test both at 3 and 24 h ($p<0.001$, r2 0.84 and $p<0.001$, r2 0.88, respectively).

On the contrary, a highly significant inverse correlation was found between p21 and cell viability ($p<0.001$, r2 0.90 and $p<0.01$, r2 0.61, respectively).

Annexin V staining confirmed the dose-dependent increase of apoptotic cells both at 3 and 24 h of AsA treatment (Figure 7A-B), especially at 1000 μM. Comparing the data, we observed a different trend for apoptosis between 3 and 24 h. After 3 h, the increase of apoptotic (early and late) cells was prevalent at 1,000 μM, whereas after 24 h, apoptosis also increased at lower concentrations, starting from 250 μM (Figure 7C). At higher concentrations apoptosis was detectable early after treatment with AsA, while lower concentrations of AsA required longer exposure to induce apoptosis.

It is interesting to note that at 250 μM of AsA we obtained the reduction of osteopontin and *RUNX2* gene expression and a slight increase of early-phase apoptosis.

Discussion

The clinical management of osteosarcoma is very difficult: the effects associated with chemotherapy reduce the quality of patient's life and the high rate of metastases elevates osteosarcoma-related mortality (27). Thus, there is a pressing need for the development of new approaches to the treatment of osteosarcoma.

Anti-proliferative effects, for example, of the steroid hormone 1,25 dihydroxy-vitamin D have been previously

reported in different cell types, including osteogenic sarcoma (28). In osteoblasts, 1,25 dihydroxy-vitamin D has been shown to inhibit cell-cycle progression at the G_1 phase and to promote cell differentiation, involving *p21*$^{waf1/cip1}$ gene up-regulation (29).

To study alternative treatments, we analysed the therapeutic potential of AsA in human osteosarcoma and its capability to influence on gene expression and on tumour cell differentiation *in vitro* and apoptosis.

When we treated MG63 osteosarcoma cells with lower concentrations of AsA, no significant anti-proliferative effect was observed. However, the level of BMP2 protein, evaluated either by western blotting or immunocytochemistry, increased in treated samples. BMPs are members of the TGF-β superfamily, they are important regulators of osteoblastic differentiation of both immature osteoblasts and less committed cells (30-32). Our data showed that AsA increased BMP2 levels in MG63 cells and the increase of this protein is obviously beneficial to MG63 differentiation (33).

BMP2 has been shown to have different effects on cancer cells. It was found to stimulate the growth of pancreatic carcinoma, lung carcinoma and prostate cancer cells (34-36), yet on the contrary, it inhibits the growth of tumor cells of myeloma (37), breast (38) gastric (39), colon (40) and prostate (41) cancer. In addition, Wang and co-workers demonstrated that BMP2 inhibited the tumorigenicity of osteosarcoma cells (42). These findings suggest that the role played by BMP2 depends on the kind of tissue and related context.

Gene expression analysis showed up-regulation of *RUNX2* mRNA in treated *versus* untreated cells and, since *RUNX2* is a master gene involved in the early phase of differentiation, our findings support the ability of AsA to stimulate osteogenic lineage. The same trend was also observed for mRNA of *SPP1* that was up-regulated in a dose dependent manner in cells treated with AsA at concentrations ranging from 0 to 125 μM.

AsA treatment increased osteocalcin-positive cells and ALP activity in a dose-dependent manner, indicating that it supports the later stage of osteoblast differentiation (43) and the establishment of a normal osteoblast phenotype (44).

To analyze the effect on mineralization, the Alizarin Red Staining (AR-S) method was applied, which is a common method for evaluating deposition of minerals in tissues and cultures. Our results showed that AsA-treated cells were induced to form matrix mineralization. Very interestingly, some random nodule-like accumulations of Alizarin Red Staining were observed in cells treated with AsA, which most likely represent the centre of nodule formation (Figure 4IIE); this effect was not observed at the lower concentration of AsA used.

On the contrary, at a concentration of 250 μM, AsA down-regulated the expression of some bone-specific genes at the transcriptional level. The reduction of mRNA expression of *RUNX2* is in agreement with its role in bone gamma-carboxyglutamate (gla) protein (BGLAP)-positive mature osteoblasts in which RUNX2 expression decreases in the late-phase of differentiation (45). The inhibition of osteopontin, considered an important factor in the neoplastic and metastatic process (46), suggests an anti-metastatic activity, or, at least, a cytostatic effect of AsA at the highest concentrations.

Vitamin C has been already associated with apoptosis of tumor cells in some previous research (47, 30). In our work, cell viability was reduced in a dose-dependent manner: this effect became significant at a concentration of AsA of 1,000 μM and cell death was caused mainly by an increase in apoptosis, as TUNEL test and annexin staining clearly demonstrated.

Apoptosis is a process mediated by multiple factors, and, for example, increased p21 expression causes cell-cycle arrest and suppression of cell growth in response to hormone stimulation or cytotoxic agents. It is well-known that the *p21* gene promoter region has a binding site for activator protein 1 (AP-1), which links cytoplasmic Mitogen-activated protein (MAP) kinase activation to p21 up-regulation (25). AP-1, one of the first identified transcription factors, regulates a wide range of cellular processes, including cell proliferation, death, survival, and differentiation (31).

The dose-dependent increase of apoptosis, detected both by TUNEL test and annexin evaluation, was associated with a significant increase of *p21* gene expression, indicating that apoptosis might be p21-mediated. This finding is in accordance with previous work where the authors demonstrated that apoptosis of MG63 cells might be due to modulation of p21 expression (48). It is known that p21 can induce apoptosis in cells with mutant non-functional *p53* (49), in a p53-independent manner and, considering MG63 cells are p53 null, this mechanism could be responsible for apoptosis in these osteosarcoma cells. In addition, Wu and co-worker, identified the role of p21 pathway in *p53*-deficient osteosarcoma cells (50).

Our results showed that p21 changes were significant at the highest concentrations of AsA, but began to appear much earlier, when cell viability was not yet affected. The p21 changes revealed in advance a visible apoptosis and we could reasonably suppose that this process starts at lower concentrations of AsA (from 250 μM); at this concentration, cell viability was not impaired neither after 3 h nor after 24 h, but a cytostatic effect was probably induced. It is interesting to note that SPP1 expression starts to decrease at this concentration, suggesting RUNX2-independent regulation of this gene. Osteopontin regulation involves multiple pathways, including AP-1, Myc, v-Src, RUNX/CBF, TGF-β/BMPs/small mothers against decapentaplegic homolog (SMAD)/HOX, and wingless-type (WNT)/β-catenin/ adenomatous polyposis coli (APC)/(glycogen synthase kinase) GSK– 3β/transcription

factor (TCF)-4 and it is not only involved in osteogenic differentiation, but also in prevention of apoptosis (51). The finding that SPP1 is down-regulated on exposure of cells to 250 μM of AsA yet enforces the concept that cytostatic effects start at this concentration.

This could be considered as a sort of pre-conditioning action and this effect could be interestingly used, for example, in a combined treatment with common chemotherapeutic agents.

Our results support the ability of AsA to influence tumor growth and differentiation in an *in vitro* model of osteosarcoma cell line by affecting target genes involved both in osteogenic differentiation and in cell apoptosis. These data are very interesting as proliferation, together with differentiation and apoptosis, are important processes for which balance is crucial in order to avoid neoplastic transformation. *In vitro* and *in vivo* experiments demonstrated that treatment with AsA controlled cell proliferation, lowered tumor progression and prolonged survival of xenografted mice (9).

According to our data AsA seems to have a bi-modal effect, stimulating cell differentiation at lower concentrations and inducing cell apoptosis at higher concentrations. These data certainly need further investigation and could have important and interesting clinical consequences. For example, since AsA is easily available, cheap and well-tolerated, it could be used at high dosages as an adjuvant to the standard chemotherapy in order to reduce cell proliferation and tumour mass. On the contrary, long-term treatment with lower dosages could be considered to prevent/reduce the risk of metastases or local recurrence and to prevent carcinogenesis.

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