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ORIGINAL ARTICLE

Basic Study

Prednisolone inhibits SaOS2 osteosarcoma cell proliferation by activating inducible nitric oxide synthase

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Abstract

AIM: To investigate the effect of prednisolone, a synthetic glucocorticoid used in inflammatory diseases, on the growth of cultured osteosarcoma cells.

METHODS: Two osteosarcoma cell lines with different degree of differentiation were used. SaOS2 show a rather mature phenotype, while U2OS are negative for almost all osteoblastic markers. The cells were exposed to different concentrations of prednisolone (1-9 μmol/L) with or without antioxidants or the inhibitor of inducible nitric oxide synthase (iNOS) l-N^6-(iminoethyl)-lysine-HCl (L-NIL). Cell growth was assessed by counting viable cells. The production of nitric oxide (NO) was measured in the conditioned media by the Griess method. The production of reactive oxygen species was quantified using 2′-7′-dichlorofluorescein diacetate. Western blot with specific antibodies against NOSs was performed on cell extracts.

RESULTS: Prednisolone inhibited SaOS2 cell growth in a dose dependent manner. No significant effects were observed in U2OS. The inhibition of SaOS2 growth is not due to oxidative stress, because antioxidants do not rescue cell proliferation. Since high concentrations of NO inhibit bone formation, we also measured NO and found it induced in SaOS2, but not in U2OS, exposed to prednisolone, because of the upregulation of iNOS as detected by western blot. Therefore, we treated SaOS2 with prednisolone in the presence or in the absence of L-NIL. L-NIL prevented NO release induced by prednisolone at all the concentrations apart from 9 μmol/L. At the same concentrations, we found that L-NIL rescued SaOS2 growth after exposure to prednisolone. In U2OS cells, prednisolone did not induce NO production nor affected cell growth. All together, these data indicate that a link exists between increased amounts of NO and growth inhibition in response to prednisolone in SaOS2.

CONCLUSION: Prednisolone inhibited SaOS2 proliferation by increasing the release of NO through the upregulation of iNOS, while no effect was exerted on U2OS.

Key words: Osteosarcoma cells; Prednisolone; Nitric oxide; Inducible nitric oxide synthase; Endothelial nitric oxide synthase; Reactive oxygen species

Core tip: Since prednisolone, a widely used synthetic glucocorticoid, inhibits osteoblast proliferation, we evaluated its effects on osteosarcoma cells. In particular, we used two osteoblastic osteosarcoma cell lines with different degree of differentiation, *i.e.*, SaOS2, which have a rather mature phenotype, and U2OS, which are less differentiated. We found that prednisolone inhibited SaOS2 proliferation by increasing the release of nitric oxide (NO) through the upregulation of inducible NO synthase (iNOS). Indeed, pharmacological inhibition with the iNOS inhibitor l-N^6-(iminoethyl)-lysine-HCl restored the normal proliferation rate of the SaOS2. On the contrary, prednisolone did not modulate NO production nor cell growth in U2OS.

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INTRODUCTION

Osteosarcomas are aggressive primary malignant tumors of the bone characterized by the deposition of immature bone by the neoplastic cells which most likely arise from mesenchymal stem cells. Osteosarcomas mostly affect teenagers and frequently metastasize. Nowadays, systemic multidrug chemotherapy and surgery are successful in 60%-70% of patients. Therefore, novel approaches are foreseen.

Since glucocorticoids participate to the regulation of survival, differentiation, and proliferation of many cell types, including osteoblasts and bone mesenchymal stem cells[1-3], we asked whether glucocorticoids might control the growth of osteosarcoma cells. Glucocorticoids act by binding their cognate receptor which functions as a hormone-regulated transcription factor. In addition, glucocorticoids interact with transcription factors such as AP1 and nuclear factor kappa B (NF-κB) and inhibit their activity. They can also modulate some intracellular signalling pathways, one of which is the MAP kinase cascade. Because of their effects on cell cycle progression and apoptosis[4], they are also used in the treatment of lymphoid malignancy and of some solid cancers[5,6].

In this study, we evaluate the effect of prednisolone, a synthetic glucocorticoid widely used to treat inflammatory diseases, on cultured osteosarcoma cells. It is well known that cultured neoplastic cells have been the basis of cancer biology and the chase to identify drug treatments[7]. Two human osteosarcoma cell lines are particularly intriguing, *i.e.*, SaOS2 and U2OS, which are among the first generated cell lines used for anticancer research[8]. U2OS were derived from a moderately differentiated sarcoma of a 15-year-old girl, and SaOS2 from an osteogenic sarcoma of an 11-year-old girl. SaOS2 are relatively resistant to drugs because of the mutation of major oncosuppressors, *i.e.*, p53 and Rb[9], which are functional in U2OS. While SaOS2 show a mature phenotype, U2OS are negative for almost all osteoblastic markers but positive for cartilage markers like collagen Ⅱ, Ⅸ and Ⅹ and for type Ⅳ collagen, which is only expressed in very early differentiation stages but not by mature osteoblasts. These two cell lines were selected for this study because of their different degree of differentiation and gene expression.

MATERIALS AND METHODS

Cell culture

SaOS2 and U2OS (American Type Culture Collection) were cultured in DMEM containing 10% fetal bovine serum. Proliferation assays were performed on cells at low density (7000/cm^2) with different concentrations of prednisolone. After trypsinization and staining with trypan blue solution (0.4%), the viable cells were counted. In some experiments cells were exposed to apocynin (10 μg/mL), trolox (40 μmol/L), or l-N^6-(iminoethyl)-lysine-HCl (L-NIL) (100 μmol/L), a selective inhibitor of inducible nitric oxide synthase (iNOS).

Reactive oxygen species evaluation

Intracellular oxidative stress was quantified using 2′-7′-dichlorofluorescein diacetate (DCFH). Cells were seeded into black bottomed 96 plates (Greiner Bio-One) and 24 h later exposed for 30 min to different concentrations of prednisolone dissolved in a 20 μmol/L DCFH solution. The rate of intracellular oxidative stress was evaluated by monitoring the emission at 529 nm of the DCFH dye using Promega Glomax Multi Detection System[10]. Data are shown as the mean of three independent experiments in triplicate ± SD. H_2O_2 (50 μmol/L) was used as a positive control.

NOS activity

NOS activity was measured in the conditioned media by the Griess method as described[11]. Data are shown as the mean of four independent experiments in triplicate ± SD.

Western blot analysis

Western blot was performed using anti-iNOS, total endothelial nitric oxide synthase (eNOS) and p-eNOS^{Ser1177} antibodies (Cell Signalling Technology) followed by

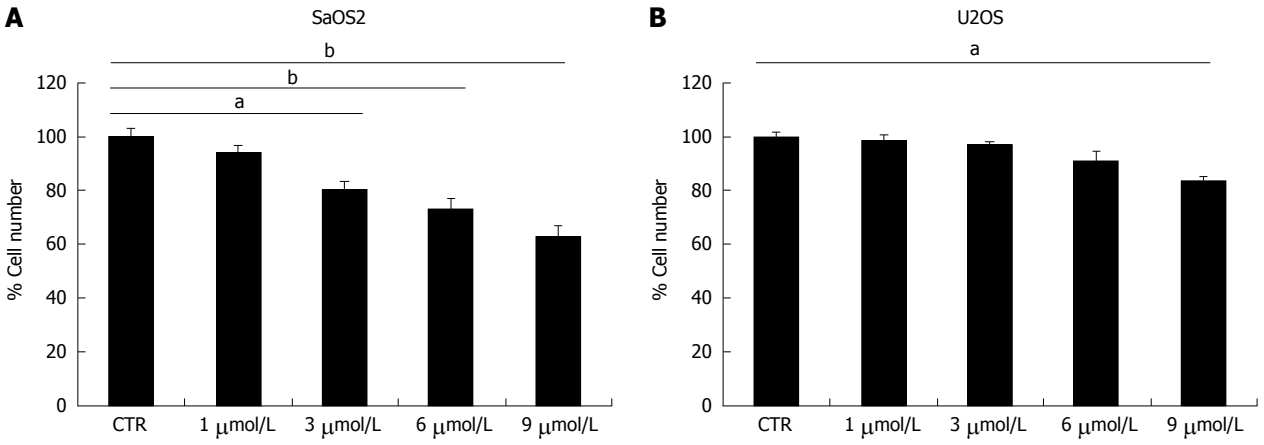

Figure 1 Prednisolone inhibits the growth of SaOS2 but not of U2OS. A: Viable SaOS2 cells were counted after 4 d in the presence of different concentrations of prednisolone ($^{a}P < 0.05$; $^{b}P < 0.01$); B: Viable U2OS cells were treated as above and counted after 96 h ($^{a}P < 0.05$). Results are shown as the mean of three separate experiments ± SD. CTR: Control.

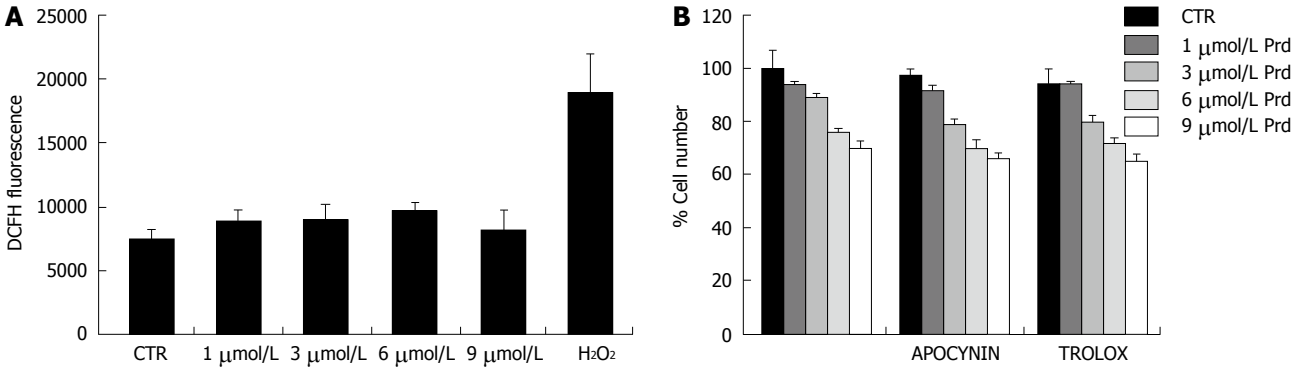

Figure 2 Prednisolone does not induce the formation of reactive oxygen species and antioxidants do not prevent growth inhibition. A: SaOS2 cells were treated with various concentrations of prednisolone. H_2O_2 was used as positive control. Reactive oxygen species generation was measured. Data are shown as the mean of three separate experiments ± SD. *P* value was calculated *vs* untreated cells and found not significant; B: SaOS2 cells were treated with apocynin (10 μg/mL) or trolox (40 μmol/L) in the presence of prednisolone (Prd). Viable cells were counted after 96 h. Results are shown as the mean of three separate experiments ± SD. CTR: Control.

incubation with secondary antibodies labelled with horseradish peroxidase (GE Healthcare). Anti-actin antibodies (Sigma-Aldrich) were used to show that equal amounts of proteins were loaded per lane. The SuperSignal chemiluminescence kit (Thermo Fisher Scientific) was utilized to detect immunoreactive proteins. Densitometry was performed using ImageJ software and results are shown as the mean ± SD of three separate experiments. A representative blot is shown.

Statistical analysis

Statistical significance was determined using the student's *t* test and set at *P* values less than 0.05. In the figures $^{a}P < 0.05$; $^{b}P < 0.01$; $^{d}P < 0.001$.

RESULTS

Prednisolone inhibits SaOS2 cell proliferation

SaOS2 were cultured in media containing different concentrations of prednisolone and counted after 4 d. Figure 1A shows that prednisolone inhibits SaOS2 cell growth in a dose dependent manner. No effect is observed in cells treated with 1 μmol/L prednisolone, while growth inhibition is significant with 3, 6, and 9 μmol/L. Similar results were obtained when the MTT assay was used (data not shown). Under the same experimental conditions U2OS were less sensitive to prednisolone than SaOS2 since a modest growth inhibition was observed only with 9 μmol/L of prednisolone (Figure 1B).

We focused on SaOS2 to understand the mechanisms underlying the inhibitory effect of prednisolone. Since the detrimental effects of glucocorticoids in osteoblasts are mediated by the induction of oxidative stress[12], we measured intracellular reactive oxygen species (ROS) by DCFH fluorescence in SaOS2. Prednisolone did not significantly affect the basal levels of DCFH-detectable ROS (Figure 2A). Accordingly, antioxidants, *i.e.*, apocynin, a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor, and trolox, a water soluble analog of α-tocopherol, did not prevent growth inhibition by prednisolone (Figure 2B).

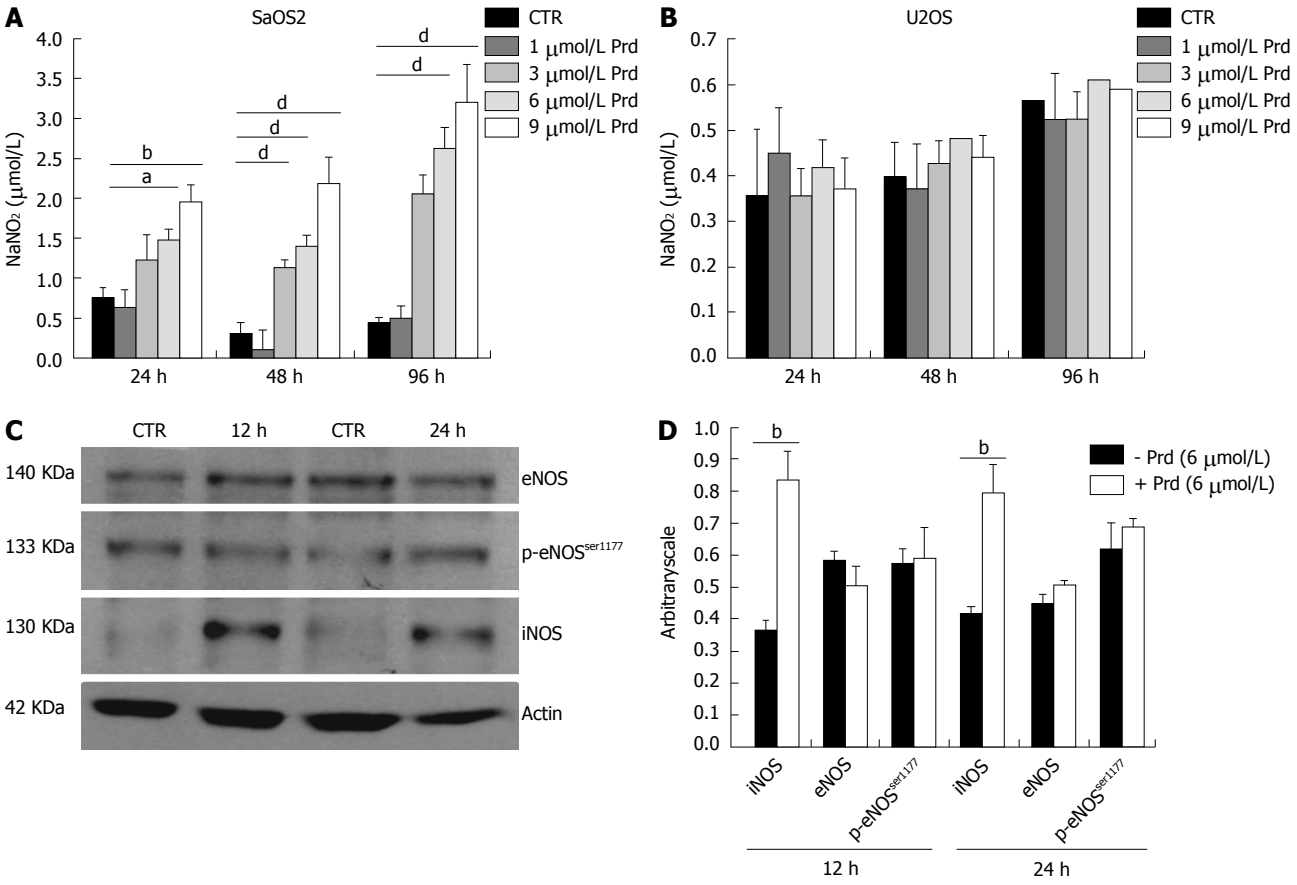

Figure 3 Prednisolone increases the release of nitric oxide in SaOS2. A: SaOS2 were cultured in the presence of different concentrations of prednisolone. Nitric oxide was measured after 24, 48 and 96 h. Results are expressed as the mean ± SD of four different experiments ($^aP < 0.05$, $^bP < 0.01$, $^dP < 0.001$); B: U2OS cells were processed as described in (A). No statistical significance was achieved; C: SaOS2 cells were exposed to prednisolone for 12 and 24 h and then lysed. 80 μg of protein extracts were loaded on SDS-PAGE. Western blots using specific antibodies against iNOS, eNOS, p-eNOS-P-Ser1177 were performed. Actin shows that equal amounts of protein were loaded per lane. The figure shows a representative blot; D: The histogram shows the quantitative evaluation of NOS/actin ratio by densitometry. Results are expressed as the mean ± SD of three separate experiments ($^bP < 0.01$). CTR: Control; iNOS: Inducible nitric oxide synthase; eNOS: Endothelial nitric oxide synthase; Prd: Prednisolone.

Prednisolone induces nitric oxide release in SaOS2

Because of the role of nitric oxide (NO) in bone homeostasis[13], we evaluated whether prednisolone affected NOS activity. After 24, 48 and 96 h of culture in various concentrations of prednisolone, we found that NOS activity was higher in SaOS2 treated with the glucocorticoid as detected by Griess assay (Figure 3A), while no increase of NO was detected in U2OS (Figure 3B). Since iNOS and eNOS were described in cultured osteoblast-like cells from various species[14], we evaluated the amounts of these enzymes by western blot in SaOS2. The phosphorylation of p-eNOS^{Ser1177} was also investigated because it enhances enzyme activity[11]. After 12 and 24 h exposure to prednisolone (6 μmol/L), iNOS was up-regulated (Figure 3C), while the amounts of total eNOS and p-eNOS^{Ser1177} remained almost unvaried.

Inhibition of iNOS activity rescues SaOS2 cell proliferation

To study whether an increased activity of NOS was responsible for SaOS2 growth retardation by prednisolone, the cells were cultured in medium containing various concentrations of prednisolone in the presence or in the absence of the iNOS inhibitor L-NIL (100 μmol/L) for 96 h. Figure 4A shows that L-NIL (100 μmol/L) prevented NO release induced by prednisolone up to 6 μmol/L, but not at 9 μmol/L. We then counted the cells and found that L-NIL prevents prednisolone-dependent growth inhibition up to 6 μmol/L (Figure 4B).

DISCUSSION

High levels of glucocorticoids impact on the generation and lifespan of osteoblasts[15]. In humans, prednisolone, even at low doses[16], causes significant bone loss and increases the risk of fractures through a direct action mainly on osteoblasts and osteocytes[17]. Because of the inhibitory effect of prednisolone on osteoblast proliferation and viability, we asked whether prednisolone might inhibit also osteosarcoma cell proliferation. Indeed, the outcome of antineoplastic therapies in osteosarcoma is not satisfactory and the quest for novel treatments continues. Here, we investigated the effects of prednisolone on two human osteoblastic osteosarcoma cell lines that reveal a different degree of differentiation, *i.e.*, SaOS2 and U2OS[8]. We found that SaOS2 are

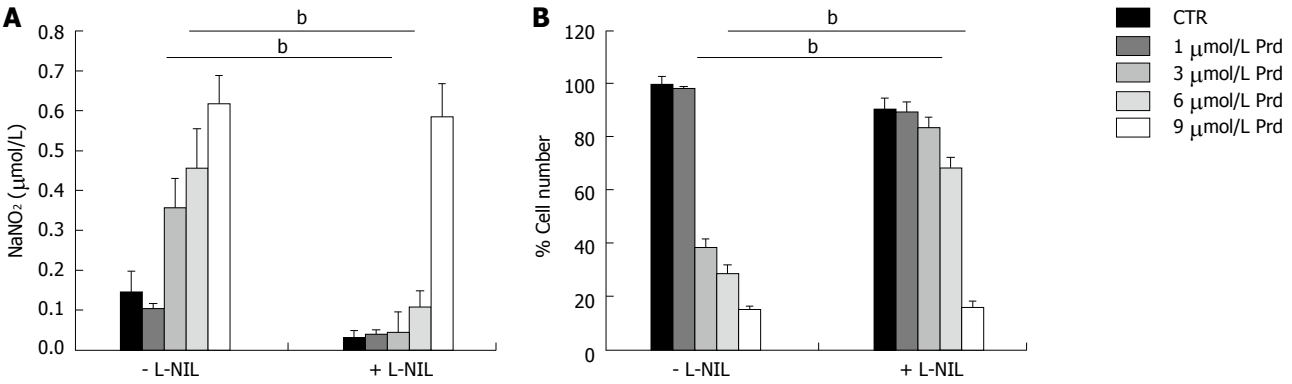

Figure 4 Pharmacological inhibition of inducible nitric oxide synthase rescues SaOS2 cell growth. A: SaOS2 were treated with prednisolone in the presence or in the absence of L-NIL (100 μmol/L). Nitric oxide was measured as described ($^bP < 0.01$); B: Viable SaOS2 cells were counted after 4 d in the presence of different concentrations of prednisolone with or without L-NIL ($^bP < 0.01$). CTR: Control; L-NIL: l-N6-(iminoethyl)-lysine-HCl.

growth inhibited by prednisolone while U2OS are not. We therefore investigated the mechanisms underlying prednisolone inhibition of SaOS2 cell growth, which also means to understand why U2OS are far less sensitive to the drug. Glucocorticoids are known to alter redox balance. Indeed, the administration of prednisolone to mice increased ROS production in the bone and dexamethasone had similar effects on osteoblastic cells *in vitro*[12]. Moreover, prednisolone enhanced the formation of superoxide by augmenting NADPH oxidase activity in pulmonary endothelial cells[18]. We found no significant induction of ROS production in prednisolone-treated SaOS2. In agreement with this result, two anti-oxidants with different mechanisms of action have no effect in preventing SaOS2 cell growth inhibition by prednisolone.

Also NO has a role in bone homeostasis. Low NO levels stimulate, while high concentrations inhibit bone formation. It is eNOS, constitutively expressed in the bone, that is implicated in maintaining the basal levels of NO[19]. Accordingly, eNOS$^{-/-}$ mice show defective bone formation and are osteopenic[11]. Also iNOS null mice show imbalances in bone osteogenesis and abnormalities in bone healing[11]. It is interesting to note that iNOS pathway is crucial in bone resorption upon inflammatory stimuli and also mediates the negative effects of estrogen depletion on bones[20]. Indeed, once activated, iNOS is capable of generating high levels of NO locally for many hours. It should be recalled that NO is also an inducer of stress signaling, owing to its ability to damage proteins and DNA. We here show that SaOS2 exposed to prednisolone upregulate iNOS and, because of this, produce higher amounts of NO than untreated cells. Indeed, pharmacological inhibition of iNOS reduced NO release to basal levels and restored the normal proliferation rate. The mechanisms implicated in iNOS induction are still a matter of investigation. It is known that iNOS is regulated through the activation of several signaling pathways among which NF-κB and MAPK. We can rule out a role of NF-κB, since glucocorticoids suppress NF-κB activity. More studies are necessary to reveal the pathways responsible for the increase of iNOS activity.

It is noteworthy that prednisolone does not induce NO in U2OS and this might account for the different behavior of the two cell lines. It is noteworthy that NO impairs also U2OS proliferation as shown in a study that links the increased activity of iNOS and the detrimental effects of benzyl isothiocyanate and phenethyl isothiocyanate on these cells[21]. It is also possible that the different response of SaOS2 and U2OS to prednisolone is due to the many differences of their proteomic profile[9,22]. Alternatively, since the glucocorticoid receptor gene generates several splice and translation protein variants that lead to different genomic and non genomic effects, the different response of U2OS and SaOS2 might result from the expression of various isoforms of glucocorticoid receptors.

We have previously shown that increased iNOS activity mediates SaOS2 growth inhibition by low magnesium[11]. Therefore NO is emerging as a relevant signaling molecule to control SaOS2 cell proliferation.

Our results indicate that prednisolone impairs SaOS2 cell proliferation through the upregulation of iNOS and consequent induction of NO release.

COMMENTS

Background

Glucocorticoids control the growth and differentiation of osteoblasts and bone mesenchymal stem cells. Little is known about the effects of glucocorticoids on osteosarcoma cells. The authors therefore evaluated the response to prednisolone of two human osteosarcoma cell lines, *i.e.*, SaOS2, which show a mature phenotype, and U2OS, which are rather undifferentiated.

Research frontiers

Prednisolone inhibited SaOS2 cell growth through the induction of inducible nitric oxide (NO) synthase with consequent increase of NO production. No effects were observed in U2OS.

Innovations and breakthroughs

NO is emerging as a relevant signaling molecule to control SaOS2 cell proliferation under different experimental conditions. This result also highlights the different sensitivity to prednisolone of osteosarcoma cells with different degree of differentiation.

Applications

More than one cell line should be used when *in vitro* experiments are performed to test the response to various compounds. The possibility of using glucocorticoids in animal models of osteosarcoma should be fostered.

Peer-review

The manuscript by Cazzaniga *et al* analyses the effects of prednisolone on two different osteosarcoma cell lines. The data are novel and the experiments have been competently performed.

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