Original Article

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The synergistic effect of SaOS-2 cell extract and other bone-inducing agents on human bone cell cultivation

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ABSTRACT: Human osteosarcoma cell line SaOS-2 is an osteoblastic cell model that contains factors like bone morphogenetic proteins necessary for initiating bone formation. The cell line also expresses high levels of osteoinductive activity. In contrast to highly complicated and expensive ways to identify, purify, and separate specific bone-inducing agents from SaOS-2 cells, lysate can be used as an alternative to isolated bone-stimulating factors. Lysates of SaOS-2 stimulate the activity of the alkaline phosphatase of human osteoblastic cells HOS 58 *in vitro*. In other words, they probably possess osteoinductive activity. Different serial concentrations of substances like dexamethasone and insulin were tested with and without a lysate of SaOS-2 cells to assay their synergistic action. Results showed that a lysate of the SaOS-2 cell line acts as a synergistic agent and increases the osteoinductive activity of known bone-inducing agents. SaOS-2 cell lysate could be used in the future as a clinical agent to promote bone repair and possibly enhance osteointegration. Using SaOS-2 total cellular extract offers the possibility of lowering the effective dose of other bone-inducing agents.

Keywords: Osteoinduction, bone alkaline phosphatase, SaOS-2 cell lysate, osteointegration, human osteosarcoma cells HOS 58, bone morphogenetic proteins, tissue-engineered bone

1. Introduction

The loss of bone tissue can occur through infection, loss of blood supply, diseases such as osteoporosis, or as a complication of a fracture or genetic disorders, *e.g.* osteogenesis imperfecta. Current management of bone defects includes tissue replacement with transplanted autografts or allografts or synthetic devices. However, each of these therapies has its own serious risks and constraints. Harvesting autografts, typically from the iliac crest, is constrained by anatomical limitations and associated with donor-site morbidity (*1*). The problems and risks associated with the use of allografts include not only disease transmission but also the risk of tissue rejection. In addition, the loss of osteoinductive factors during allograft processing may impact tissue quality. A synthetic prosthesis such as bone cement and metal, *e.g.* titanium and its alloys or stainless steel, often results in insufficient osseous integration and stress-shielding of the surrounding bone or fatigue failure of the implant (*1*). These shortcomings highlight the need for greater use and further study of bone-inducing agents to increase the osteogenic character of tissue-engineered bone.

Bone alkaline phosphatase (ALP) is located on the surface of osteoblasts and is thought to play a major role in bone formation and mineralization. Its levels are considered to reflect osteoblastic activity (*2*). Bone ALP levels can therefore be used as a biochemical marker to assess metabolic bone diseases (*e.g.* osteoporosis), bone disorders as a late complication of diabetes, and even bone metastasis. Moreover, bone morphogenetic proteins (BMPs), which are members of the transforming growth factor (TGF)-β superfamily (*3-6*), appear to play an important role in the initiation of osteogenesis during development (*7-10*) and in bone repair (*6,11-14*).

Human osteosarcoma cell line SaOS-2 is an osteoblastic cell model that expresses high levels of tissue ALP activity (*15*). According to reports, its lysate should have osteoinductive activity (*16,17*). SaOS-2 cells may be osteoinductive because they contain several BMPs including BMP-1, 2, 3, 4, and 6, any or all of which may support bone induction (*18,19*). These cells apparently produce factors necessary for initiating bone formation.

The current study sought to investigate the effect of lysate from SaOS-2 cells alone and in combination with other bone-enhancing agents on the ALP activity of the HOS 58 cell line. This investigation should pave the way for more pharmacological, toxicological, and medical studies of this cell lysate to allow its use in medicine as a drug additive.

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2. Materials and Methods

2.1. *Materials*

Cell culture plastics, fetal bovine serum (FBS), phosphate -buffered saline (PBS), L-glutamine, trypsine, and antibiotics were purchased from Biochrom KG (Berlin, Germany). Bovine serum albumin (BSA; fraction V) and Iscove's modified Dulbecco's medium (IMDM) with or without phenol red were purchased from Invitrogen (Karlsruhe, Germany). All other reagents were obtained from Sigma (Deisenhofen, Germany). HOS 58 cells were donated by A. Battmann (University of Giessen, Institute of Pathology, Germany). SaOS-2 cells were purchased from DSZM (Braunschweig, Germany).

2.2. *Cell lines and culture*

Both the HOS 58 cell line and SaOS-2 cell line were grown as a monolayer in IMDM with 10% FBS, 2 mM L-glutamine, and 1% penicillin-streptomycin solution (penicillin 10,000 IE/mL; streptomycin 10,000 µg/mL). Both cell lines were grown in 95% humidity and 5% CO_2 at 37°C and routinely sub-cultured.

2.3. *Preparation of SaOS-2 lysate*

To prepare a cell lysate, cells were washed with 3 × 10 mL phosphate buffer and scraped from the surface of tissue culture flasks using a cell scraper. Approximately 2.5×10^8 cells were freeze-dried and suspended in 50 mL extraction buffer consisting of 0.1 M Tris-HCl buffer (pH 8.0). Cells were then incubated with gentle stirring for 1 h at room temperature and centrifuged at 4,000 rpm for 15 min to remove cellular debris. The resulting supernatant served as a stock solution (5×10^6 cells/mL).

2.4. *Standardization of the ALP activity of SaOS-2 cell lysate*

The crude lysate was standardized based on its ALP activity. Crude ALP activity was determined by the release of 4-nitrophenol (4-NP) from 4-nitrophenyl phosphate (4-NPP, see Section 2.8).

2.5. *Assay of bone-inducing activity*

For assays, HOS 58 cells were grown to confluence in 96-well plates for 48 h. After they were washed twice with PBS, medium was changed to IMDM without phenol red supplemented with 0.05% BSA, 2 mM L-glutamine, and 1% antibiotics (assay medium). Different concentrations of crude SaOS-2 cell lysate in assay medium were prepared using a stock solution (5×10^6 cells/mL in PBS). The final PBS concentration did not exceed 0.05%. Different serial concentrations of substances like dexamethasone (Dexa) and insulin were used with and without 20 µL of a stock (5×10^6 cells/mL in PBS) crude extract of SaOs-2 cells to assay their synergistic action. Further procedures are indicated below.

2.6. *Cell disruption*

Cultivated HOS 58 cells were washed with PBS and disrupted by adding 100 µL of 0.1% Triton X-100 in 0.1 M Tris-HCl, pH 9.8 (lysis buffer) followed by freeze/thawing and vigorous mixing. The resulting suspension was centrifuged and the supernatant (cell lysate) was assayed for its protein content and ALP activity.

2.7. *Determination of total cellular protein*

Total cellular protein was determined using Roti-Nanoquant reagent (Roth GmbH, Karlsruhe, Germany), a modified Bradford method, according to the manufacturer's instructions (*20*). Briefly, 10 µL cell lysate was diluted with PBS (1:4) in a microtiter plate. Roti-Nanoquant (200 µL) reagent was added and mixed, and the OD was read out at 405 and 620 nm (Anthos Labtec, Salzburg, Austria). The total protein content was calculated from a standard curve using BSA.

2.8. *ALP activity*

Cellular ALP activity was determined by the release of 4-NP from 4-NPP. An aliquot of cell lysate was mixed with 0.2 M amino propanol buffer (pH 9.8, AMP) and 24 mM 4-NPP in AMP. After incubation (37°C), the reaction was stopped with 0.5 M NaOH (50 µL) and the OD was read out at 405 nm. The concentration was calculated from a calibration curve for 4-NP.

2.9. *Cell vitality and cell proliferation assays (MTT assay)*

The MTT assay was used to measure the cell proliferation rate and cell viability. HOS 58 cells (see Section 2.2) were incubated with different concentrations of normalized SaOS-2 cell lysate (0.9-1.1 U/mL – 0.007-0.008 U/mL) for 43 h. After cells were washed, 20 µL MTT in IMDM (5 mg/mL, Sigma, Deisenhofen, Germany) was added to each well. Plates were incubated at 37°C in 95% humidity and 5% CO_2 for another 5 h. Finally, the MTT solution was removed and crystals were dissolved in 200 µL DMSO. After thorough mixing, plates were incubated for 5 min and absorbance was measured at 590 nm. Cell viability was calculated as a percent of vehicle control (*21,22*).

2.10. *Statistical analysis*

For each experiment, three independent experiments were carried out and results were expressed as mean ± SD. Statistical differences were analyzed using single-tailed ANOVA. A p values < 0.05 was considered significant.

3. Results and Discussion

3.1. *Extraction of SaOS-2 cells*

The SaOS-2 cell line was established in culture in 1975 (*16*). These cells produce a large amount of ALP but little or no matrix *in vitro* and are unable to grow when transplanted into athymic mice. SaOS-2 cells contain several BMPs including BMP-1, 2, 3, 4, 6, and 7, any or all of which may support bone induction (*18,19*). Moreover, BMPs, which are members of the TGF-β super family (*3-6*), appear to play an important role in the initiation of osteogenesis during development (*7*) and in bone repair (*6,11-14*).

An important question with respect to bone repair or bone tissue engineering is whether a combination of BMPs will be more cost-effective in clinical practice than a single recombinant BMP. Although individual BMPs, and especially recombinant BMP-2 (*23,24*) or recombinant BMP-7 (*25*), have been used to successfully accelerate bone regeneration in large defects, the required concentration of a specific recombinant BMP is up to 1,000-fold higher than that of the native BMP complex (*4*). The combination of several BMPs and other factors might be more cost-effective at enhancing new bone formation than individual recombinant human BMPs. Thus, a total cellular extract of SaOS-2 cells was tested for its bone-inducing ability. The cellular extract was standardized based on its ALP activity.

Cultivation of 10^5 SaOS-2 cells consistently yielded approximately 1 U of bone tissue ALP crude lysate (cALP) (Figure 1 and Table 1), given that one unit (U) of ALP activity is the quantity of enzyme that catalyzes the hydrolysis of 1 μmol substrate in 1 min. Results show that the residual activity of ALP in the crude lysate of SaOS-2 cells (freeze-dried/thawing) was 10.1 U/mL (Figure 1 and Table 1), which was equivalent to the level reported previously (*18*). In comparison to bone ALP made from human bone, the specific activity would be 500 times greater because SaOS-2 cells contain 40-50 times more ALP activity than TE-85 cells (*8,26*).

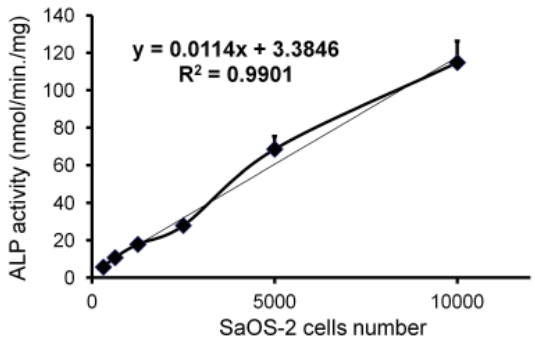

Figure 1. Quantification of the ALP activity in a crude extract of SaOS-2 cells.

3.2. *Effect of SaOS-2 lysate on the ALP activity and total protein content of HOS 58 cells*

ALP activity is commonly used as an indicator of osteoblastic cell maturation. The enzyme is considered to mark the middle stage of bone formation and generally appears during the matrix maturation phase. It plays an unclear but crucial role in matrix mineralization (*2*). Figure 2 clearly shows a significant increase ($p < 0.01$) in the level of ALP activity of HOS 58 cells to almost 200% in the presence of a crude extract of SaOS-2 cells (0.1 U/mL ALP).

Figure 3 shows that the SaOS-2 cell lysate in concentrations up 10 U/mL did not affect protein production by HOS 58 cells. Above that concentration, protein production drops, reaching only 80% of that

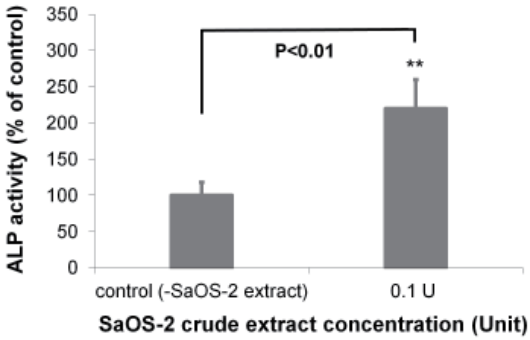

Figure 2. Effect of SaOS-2 cell lysate (normalized to 0.1 U/mL ALP) on the ALP activity of HOS 58 cells.

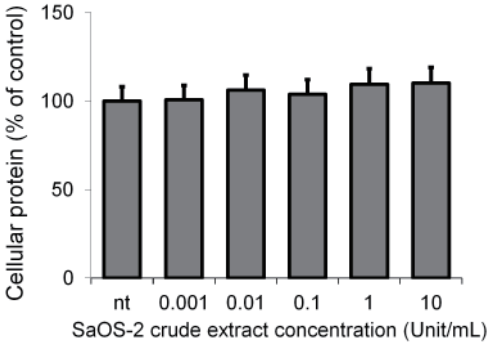

Figure 3. Effect of different concentrations of an extract of SaOS-2 cells on the total protein activity of HOS 58 cells. No significant toxicity was observed up to 10 unit/mL, suggesting that the cells continued to be viable in the experimental setup. Three independent experiments were performed.

Table 1. ALP activity from a crude extract of SaOS-2 cells

	Working solution volume (mL)	Cell number/mL of working solution	ALP (U/mL)	Average protein (mg/mL)
Crude extract	49	0.9-1.1 × 10^6	10.1	1.86

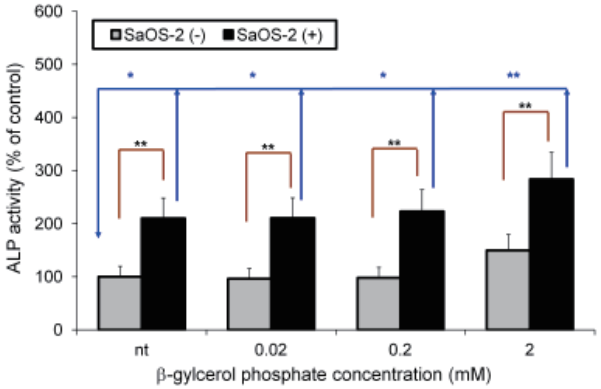

Figure 4. Synergistic effect of SaOS-2 cell lysate (normalized to 0.1 U/mL ALP) with β-glycerol phosphate on the ALP activity of HOS 58 cells. * $p < 0.05$, ** $p < 0.01$.

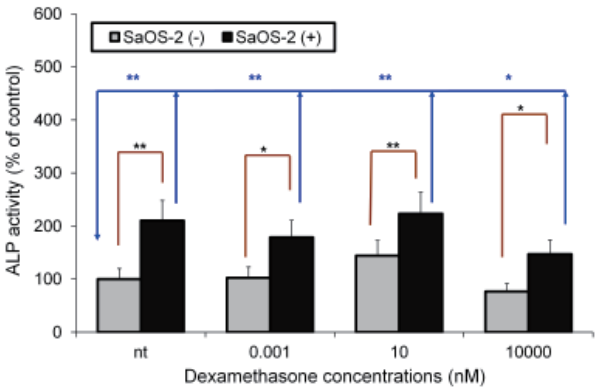

Figure 5. Synergistic effect of SaOS-2 cell lysate (normalized to 0.1 U/mL ALP) with Dexa on ALP activity of HOS 58 cells. * $p < 0.05$, ** $p < 0.01$.

of the vehicle control (data not shown). Because cytotoxicity is lacking, cell maturation must have been stimulated. Had it not, extracellular matrix (ECM) production would have decreased and thus the protein content of the cell (and matrix) lysate would have decreased. No increase in cellular protein was noted at any of the concentrations tested. This corroborates the contention that SaOS-2 cell lysate promotes cell maturation and reduces cell proliferation and ECM production.

3.3. *Synergistic effect with β-glycerol phosphate*

Figure 4 clearly shows a significant increase ($p < 0.01$, *vs.* vehicle control) in the level of cellular ALP activity of HOS 58 cells to almost 130% at a 2 mM β-glycerol phosphate (bGP) concentration alone. Synergistic use of SaOS-2 cell lysate (normalized to 0.1 U/mL ALP) respectively increased ALP activity to 210%, 225%, and 280% with 0.02, 0.2, and 2 mM bGP (+ 0.1 U cALP).

The most rational explanation for the observed synergistic action of bGP with SaOS-2 extract is that bGP hydrolyzed to glycerol and inorganic phosphate ions (Pi). Glycerol inhibits cell proliferation (*27*), so bone-inducing agents from SaOS-2 extracts accelerate cell maturation and lead to increased ALP activity. The hydrolysis of bGP also leads to a greater concentration of inorganic Pi in the culture medium, which may react with more BMPs from the SaOS-2 extract (phosphorylation) and result in increased formation of phosphoproteins (*28,29*). These phosphoproteins increase bone-inducing activity.

3.4. *Synergistic effect with Dexa*

The ALP activity of HOS 58 cells increased with greater Dexa concentrations from 10^{-12} M to 10^{-8} M. Higher concentrations (from 10^{-7} M to 10^{-5} M) led to decrease in ALP activity. Figure 5 shows that synergistic use of SaOS-2 cell lysate (normalized to 0.1 U/mL ALP) and Dexa respectively increased ALP activity from 102%, 144%, and 77% with 10^{-12} M, 10^{-8} M, and 10^{-5} M Dexa to 178%, 223%, and 147% with 10^{-12} M, 10^{-8} M, and 10^{-5} M of Dexa plus SaOS-2 cell lysate.

Results show that Dexa and the SaOS-2 cell extract act synergistically on ALP activity. Dexa concentrations that decrease ALP activity have less impact when combined with the SaOS-2 cell extract. Concentrations of Dexa that increase ALP activity further increase ALP activity when combined with the SaOS-2 cell extract.

Given these findings, lower concentrations of Dexa appear to stimulate the proliferative activity of bone-like cell cultures and increase the number of osteoblastic cells in culture. Osteoblastic cells in the presence of excessive amounts of BMPs from an SaOS-2 extract promote early maturation of cells and therefore cause an increase in ALP activity. In addition, higher concentrations of Dexa appear to inhibit cell proliferation and reduce the number of osteoblastic cells, thus decreasing ALP activity.

Prolonged physiological levels of Dexa are clearly associated with deleterious effects on the skeleton and are a major cause of osteoporosis (*30,31*). One advantage of the synergistic effect of Dexa and an SaOS-2 cell extract would be that lower concentrations of Dexa could be used to avoid prolonged exposure and avoid its negative impact on bones.

3.5. *Synergistic effect with insulin*

Figure 6 clearly shows a significant increase ($p < 0.01$, *vs.* vehicle control) in the level of ALP activity of HOS 58 human cells to almost 190% at an insulin concentration of 20 µg/mL. ALP activity is affected by the insulin concentration and respectively increased to 120%, 155%, and 190% with 0.2, 2, and 20 µg/mL insulin. Synergistic use of SaOS-2 cell lysate (normalized to 0.1 U/mL ALP) respectively increased ALP activity to 190%, 205%, and 260% with 0.02, 0.2, and 2 mM insulin (+ 0.1 U cALP).

Synergistic action with insulin may be because insulin, like insulin-like growth factor (IGF)-I, increases

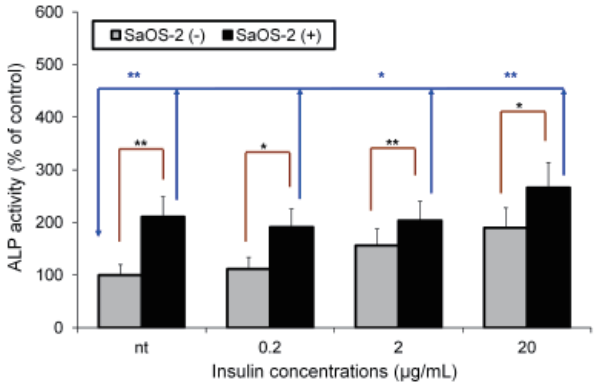

Figure 6. Synergistic effect of SaOS-2 cell lysate (normalized to 0.1 U/mL ALP) with insulin on ALP activity of HOS 58 cells. * $p < 0.05$, ** $p < 0.01$.

cell proliferation. The combinations of IGF and TGF-β, IGF and platelet-derived growth factor (PDGF), and PDGF and TGF-β enhance murine osteoblast activity and proliferation (*32*). As mentioned earlier, BMPs in SaOS-2 extract caused early maturation of an increased number of osteoblastic cells, changing their phase of differentiation phase and causing ALP to accumulate in cell culture.

4. Conclusion

In conclusion, SaOS-2 lysate increases the ALP activity of human osteoblastic cells *in vitro*, presumably indicating that it has bone-inducing activity. SaOS-2 lysate may have the potential to serve as a clinical agent to promote bone repair and possibly enhance osteointegration. Evidence is not clear as to whether a single factor is responsible for SaOS-2 osteoinductivity. The mechanism of osteoinductivity seems to be multifactorial, so a total lysate of SaOS-2 cells may be better than isolated compounds. The mechanism of its activity (both *in vitro* and *in vivo*), its possible toxicity, its chemical composition, and its standardized quality must be studied further.

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