Phosphodiesterase 4 in osteoblastic osteosarcoma cells as a potential target for growth inhibition

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Reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed that HOSM-1 cells, an osteosarcoma cell line established from human mandible, expressed mRNA for osteoblastic markers, such as alkaline phosphatase, osteonectin, osteocalcin and parathyroid hormone receptor, thus exhibiting an osteoblastic phenotype. We have investigated a possible role of cyclic nucleotide phosphodiesterases (PDEs) in osteosarcoma cells. RT-PCR analysis revealed that HOSM-1 cells expressed mRNA for PDE4A, 4B and 4C. In addition, rolipram, a specific inhibitor of PDE4, inhibited HOSM-1 cell proliferation. The finding that PDE4 is involved in proliferation of osteosarcoma cells suggests the possibility that PDE4 may be a new target for antitumor therapy. *Anti-Cancer Drugs* **14:377–381 © 2003 Lippincott Williams & Wilkins.**

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Introduction

The incidence of osteosarcoma is quite high among primary bone malignancies [1], but this tumor rarely occurs in the mandible. Furthermore, mandibular osteosarcoma is quite different from those at other sites in terms of prevalent age, frequency of metastasis [2] and prognosis [3]. Since only a few mandible-derived osteosarcoma cell lines have been documented thus far [4,5], it would be useful to establish such cell lines and to characterize their cellular features.

Phosphodiesterases (PDEs) are enzymes that catalyze hydrolysis of intracellular cAMP and/or cGMP [6]. PDE4s are low K_m, cAMP-specific enzymes and sensitive to rolipram, an anti-depressant. PDE4s are expressed relatively abundantly in immune/inflammatory cells and PDE4-specific inhibitors have been proven to exert anti-inflammatory actions [7]. It has been reported that PDE4 is also expressed in several tumor cell lines and that PDE4-specific inhibitors suppress tumor cell proliferation [8,9]. Nevertheless, the roles of PDE4 in human osteosarcoma cells have not been elucidated yet.

We established and maintained the HOSM-1 cell line from osteosarcoma that occurred in human mandible [4]. In the present study, we characterized the osteoblastic phenotype of HOSM-1 cells and demonstrated that PDE4-specific inhibitors suppressed HOSM-1 cell proliferation.

Materials and methods

Cell culture

HOSM-1 [4] and HMG [10,11] cells were maintained in RPMI 1640 (Invitrogen, Grand Island, NY) containing 10% (HOSM-1) and 20% (HMG) fetal bovine serum (Invitrogen) in a humidified atmosphere of 5% CO_2/air at 37°C. Cells were collected using 0.05% trypsin–0.53 mM EDTA solution (Invitrogen) and subcultured for 1 week. HMG cells were used as positive control for reverse transcription-polymerase chain reaction (RT-PCR).

Total RNA isolation

At day 8, cultured cells were harvested with trypsin–EDTA solution (Invitrogen) and stored at –80°C. Total RNA was isolated with the Total RNA extraction kit (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's protocol. First-strand cDNA was synthesized using 1 μg total RNA with the Superscript pre-amplification system (Invitrogen).

PCR for osteoblast-related genes

PCR was performed with specific primer pairs for alkaline phosphatase (ALP), osteonectin (ON), osteopontin (OP), osteocalcin (OC), parathyroid hormone (PTH) receptor and the housekeeping gene GAPDH as shown in Table 1 [12,13]. Two microliters of RT product was added to a PCR reaction which included PCR buffer (pH 8.3, 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% gelatin), 0.2 mM dNTPs, 1 μM primers and 5 U Taq DNA polymerase (Perkin Elmer Applied Biosystems, Foster

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Table 1 Primer sequences for RT-PCR

	Forward primer (5′→3′)	Reverse primer (5′→3′)
ALP	ACG TGG CTA AGA ATG TCA TC	CTG GTA GGC GAT GTC CTT A
ON	GAT GAG GAC AAC AAC CTT CTG AC	TTA GAT CAC AAG ATC CTT GTC GAT
OP	CCA AGT AAG TCC AAC GAA AG	GGT GAT GTC CTC GTC TGT A
OC	AGC GAG GTA GTG AAG AGA CCC	TCA GCC AAC TCG TCA CAG TC
PTH receptor	AGG AAC AGA TCT TCC TGC TGC A	TGC ATG TGG ATG TAG TTG CGC GT
GAPDH	GGT GAA GGT CGG AGT CAA CGG	GGT CAT GAG TCC TTC CAC GAT

Table 2 Primer sequences for PDE4 isoforms

	Forward primer (5′→3′)	Reverse primer (5′→3′)
PDE4A	AAC AGC CTG AAC AAC TCT AAC	CAA TAA AAC CCA CCT GAG ACT
PDE4B	AGC TCA TGA CCC AGA TAA GTG	ATA ACC ATC TTC CTG AGT GTC
PDE4C	TCG ACA ACC AGA GGA CTT AGG	GGA TAG AAG CCC AGG AGA AAG
PDE4D	CGG AGA TGA CTT GAT TGT GAC	CGT TCC TGA AAA ATG GTG TGC

City, CA). Thirty-five PCR cycles were followed by denaturation at 95°C and extension at 72°C with a GeneAmp 2400 thermal cycler (Perkin Elmer Applied Biosystems). Reaction products were analyzed by electrophoresis (5 μl samples in 2.5% agarose gels). The amplified DNA fragments were visualized by ethidium bromide staining.

PDE activity in cell extracts

HOSM-1 cells were seeded at 1×10^6 cell/25 cm^2 flask (Nunc, Roskilde, Denmark). After 3 days, cells were washed twice with phosphate-buffered saline (PBS), harvested with a rubber policeman and homogenized in 2 ml of ice-cold buffer (100 mM TES, pH 7.4, 10 μg/ml each of pepstatin, leupeptin and aprotinin, 1 mM benzamidine, 0.5 mM pefabloc, 1 mM EDTA, 0.1 mM EGTA, 5 mM $MgSO_4$, and 10% glycerol).

cAMP PDE assay

cAMP PDE activity was assayed by a modification of a previously described procedure [14]. Sample were incubated at 30°C for 10 min in a total volume (0.3 ml) containing 50 mM HEPES, pH 7.4, 0.1 mM EGTA, 8.3 mM $MgCl_2$ and 0.1 μM [^{3}H]cAMP (18 000 c.p.m.). PDE4 activity (pmol cAMP hydrolyzed/min) was measured as the cAMP PDE activity inhibited by 10 μM rolipram, a specific inhibitor of PDE4.

RT-PCR for PDE4 isoforms

At day 3, cultured cells seeded at 1×10^6 cells/25 cm^2 flask (Nunc) were harvested with trypsin–EDTA solution (Invitrogen) and stored at −80°C. Total RNA was isolated with the Total RNA extraction kit (Amersham Pharmacia Biotech). First-strand cDNA was synthesized using 1 μg total RNA with Superscript pre-amplification system (Invitrogen). PCR was performed with specific primer pairs for PDE4 isoforms as shown in Table 2 [15]. Two microliters of RT product was added to a PCR reaction which included PCR buffer (pH 8.3, 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% gelatin), 0.2 mM dNTPs, 1 μM primers and 5 U Taq DNA polymerase (Perkin Elmer Applied Biosystems). Thirty PCR cycles were followed by denaturation at 94°C, annealing at 55°C and extension at 72°C with a GeneAmp 2400 thermal cycler (Perkin Elmer Applied Biosystems). Reaction products were analyzed by electrophoresis (5 μl samples in 2.5% agarose gels). The amplified DNA fragments were visualized by ethidium bromide staining.

Growth experiments

HOSM-1 cells were plated at 0. 5×10^4 cells/well in 24-well plate and cultured with different concentrations of rolipram. After 7 days, MTT assays were performed and cell numbers calculated.

Results

RT-PCR for osteoblast-related genes

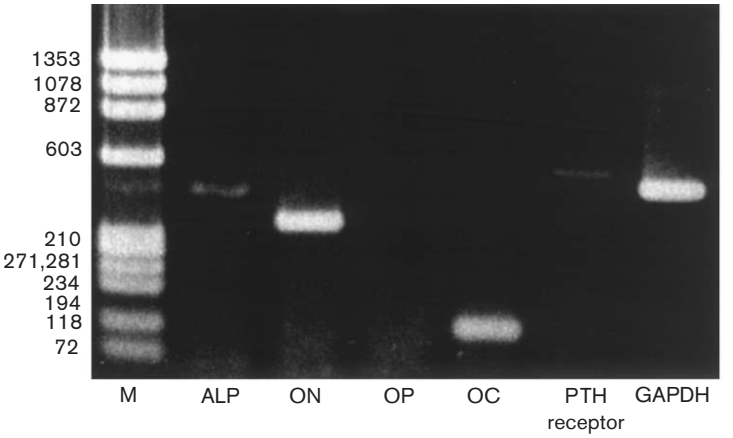
In order to assess the expression of marker genes for the osteoblast phenotype, mRNAs of HOSM-1 at day 8 were measured by RT-PCR on total RNA (1 μg RNA/RT-PCR reaction) from HOSM-1 cells, using primer pairs specific for human osteoblastic markers. With the exception of OP, all osteoblastic genes markers were detected (Fig. 1). No fragments were amplified in the absence of RT or RNA and OP mRNA was detected in total RNA from OP-positive control HMG cells (data not shown).

cAMP PDE activity and expression of PDE4 isoforms

PDE4 activity (assayed with 0.1 μM [^{3}H]cAMP as substrate) was measured in extracts of HOSM-1 cells. PDE activity was inhibited by a PDE4-specific inhibitor, rolipram, indicating the presence of PDE4 isozymes (Fig. 2).

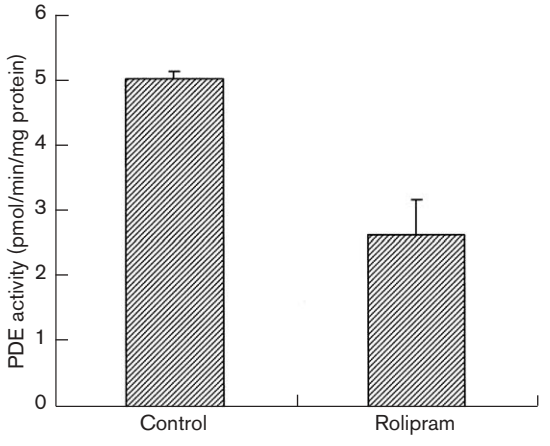
RT-PCR was performed on total RNA (1 μg RNA/RT-PCR reaction) from HOSM-1 cells, using primer pairs specific for human PDE4A, 4B, 4C and 4D. PDE4A, 4B

Fig. 1



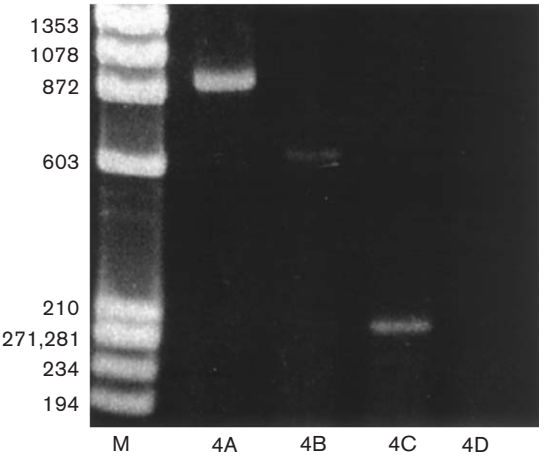
RT-PCR analysis for expression of osteoblast-related genes. Separate PCRs were performed for ALP, OP, OC, PTH receptor and GAPDH as described in Materials and methods. ϕX174 RF DNA/*Hae*III fragments were used as marker (M).

Fig. 2



Effect of rolipram on PDE activity in HOSM-1. Homogenates were assayed with or without 10 μM rolipram as described in Materials and methods. Data are mean ± SD of three experiments.

Fig. 3



RT-PCR analysis for expression of mRNA of PDE4 isoforms. RT-PCRs were performed as described in Materials and methods. PDE4A, PDE4B, PDE4C, PDE4D and marker (M).

and 4C, but not 4D, mRNAs were detected in HOSM-1 cells (Fig. 3). No fragments were amplified in the absence of RT or RNA and PDE4D mRNA was detected in total RNA from PDE4D-positive control HMG cells (data not shown).

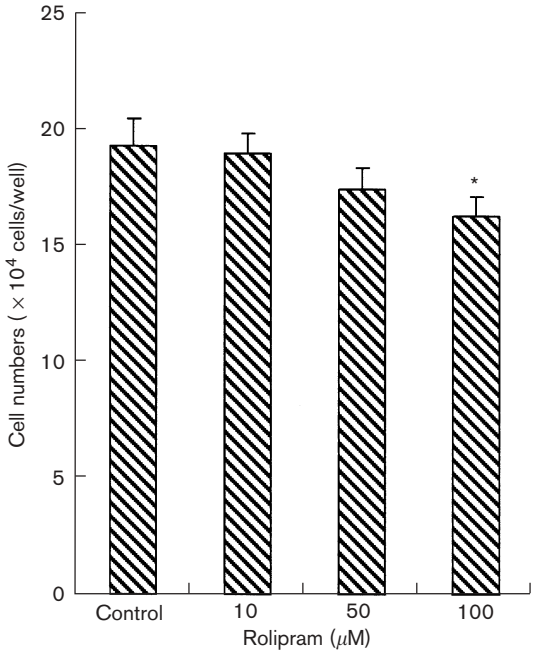
Effect of rolipram on HOSM-1 cells growth

The growth of HOSM-1 cells was inhibited by rolipram, a PDE4-specific inhibitor, at concentration of 100 μM (Fig. 4).

Discussion

Osteoblasts are characterized by high ALP activities and enhanced synthesis of matrix proteins, such as type I collagen, ON, bone sialo-protein, OP, OC and other adhesion molecules [16]. In addition, osteoblasts respond to a number of hormones, PTH, vitamin D and estrogen via receptor pathways [17]. RT-PCR analysis has revealed that HOSM-1 cells express marker genes for the osteoblast phenotype, including ALP, ON, OC and PTH receptor, thus demonstrating that HOSM-1 is an osteoblastic osteosarcoma cell line. Since OC is expressed at later stages of osteoblast differentiation, HOSM-1 represents a more differentiated phenotype. However, HOSM-1 cells did not express OP. RT-PCR and Western blot analysis have revealed that OP is detectable in other osteoblastic cell lines. Nevertheless, osteogenesis is

Fig. 4



Effect of rolipram on growth of HOSM-1. Cells were plated in 24-well plates and cultured with or without the indicated concentrations of rolipram for 7 days. The final concentration of vehicle (DMSO) was 0.05%, which did not affect growth of HOSM-1. Cell numbers were calculated with MTT assays. Data are mean ± SD of three experiments. Significantly different from the control without rolipram (*$p < 0.05$).

normal in OP knockout mice [18]. Taken together, these findings indicate that OP may not be essential for osteogenesis. Since the roles of OP in osteoblasts still remain unknown, further studies will be needed to address this issue.

Cyclic nucleotide PDEs belong to a large superfamily of enzymes that are expressed in a variety of tissues and cells. In addition, it has been proven that several types of tumor cells express different PDEs and PDE inhibitors have been reported to suppress proliferation of normal cells as well as tumor cells [15,19]. However, no investigators have reported that PDE inhibitors suppress osteosarcoma cell proliferation. Here, we report, for the first time, that a cultured human osteosarcoma cell line (HOSM-1) expresses PDE4 and that HOSM-1 cell proliferation is inhibited by rolipram, a specific inhibitor of PDE4.

Similarly, rolipram has been reported to inhibit proliferation of other malignant tumor cells, including human megakaryoblastic leukemic Dami cells [20] and human neuroepithelioma SK-N-MC cells [9]. Taken together, these findings suggest that PDE4 may be involved in tumor cell proliferation and can be a target for antitumor therapy for human osteosarcoma. Another PDE4 inhibitor, DC-TA-46 (7-benzylamino-6-chloro-2-piperazino-4-pyrrolidinopteridine), inhibits proliferation of human mammary carcinoma cell lines, MCF-7 [8] and SK-N-MC cells [9], at low concentrations. In addition, the concomitant use of PDE3 and PDE4 inhibitors suppressed proliferation of rat vascular smooth muscle cells at a lower concentration than that required for PDE3 or PDE4 inhibitors alone [21]. Taken together, these findings indicate that the concomitant use of rolipram and other PDE inhibitor(s) may suppress proliferation of HOSM-1 cells at lower concentrations.

Binding of PTH to its receptor causes activation of adenylate cyclase, the enzyme responsible for the formation of cAMP. cAMP plays an essential role in osteoblast proliferation and differentiation. Intracellular cAMP content is regulated by cAMP production via adenylyl cyclases and degradation by PDEs [22,23]. Ahlstorm *et al.* [22] reported that in the rat osteoblastic osteosarcoma cell line UMR-106, PTH-stimulated the activity of PDE4, the predominant cAMP-hydrolyzing PDE isozyme in these cells [23]. In osteoblast differentiation of the mouse bone marrow stromal cell line ST2, use of PDE4-specific inhibitors indicated that PDE4 was involved in regulation of biological action of BMP-4 [24]. The PDE4-specific inhibitors denbufylline and Ro-20-1724 increased the number of mineralized nodules and decreased the number of osteoblastic cells in bone marrow cultures, and in Walker 256/S carcinosarcoma-bearing rats denbufylline inhibited the decrease in the bone density of femurs [25]. Furthermore, the non-specific PDE inhibitor pentoxifylline and the PDE4-specific inhibitor rolipram increased bone mass by enhancing physiological ossification in normal mice [26]. Since PDE4s were expressed in the mandibular osteoblastic cell line HOSM-1, this cell line may be a useful model to study the role of PDE4 in osteoblast differentiation in the mandible. Whether PDE4s are involved in osteoblastic differentiation of HOSM-1 cells is unclear and changes in osteoblast markers in the presence PDE4 inhibitors will be examined in future studies.

Conclusion

We have characterized the osteoblastic phenotype of human mandibular osteosarcoma HOSM-1 cells. HOSM-1 cells exhibited PDE4 activity, PDE4s mRNAs were detected by RT-PCR and the PDE4-specific inhibitor, rolipram, inhibited proliferation. Our results indicate that PDE4 is involved in the proliferation of HOSM-1 cells and therefore that PDE4-specific inhibitors may be useful as antitumor agents against osteosarcoma in the mandible.

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