Different Expression and Localization of Phosphoinositide Specific Phospholipases C in Human Osteoblasts, Osteosarcoma Cell Lines, Ewing Sarcoma and Synovial Sarcoma

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Abstract

Background: Bone hardness and strength depends on mineralization, which involves a complex process in which calcium phosphate, produced by bone-forming cells, was shed around the fibrous matrix. This process is strictly regulated. In addition, many signal transduction systems were interested in calcium metabolism, such as the phosphoinositide (PI) pathway and related phospholipase C (PLC) enzymes.

Objectives: Our aim was to search for common patterns of expression in osteoblasts, as well as in ES and SS.

Methods: We analysed the PLC enzymes in human osteoblasts and osteosarcoma cell lines MG-63 and SaOS-2. We compared the obtained results to the expression of PLCs in samples of patients affected with Ewing sarcoma (ES) and synovial sarcoma (SS).

Results: In osteoblasts, MG-63 cells and SaOS-2 significant differences were identified in the expression of PLCδ4 and PLCη subfamily isoforms. Differences were also identified regarding the expression of PLCs in ES and SS. Most ES and SS did not express PLCB1, which was expressed in most osteoblasts, MG-63 and SaOS-2 cells. Conversely, PLCB2, unexpressed in the cell lines, was expressed in some ES and SS. However, PLCH1 was expressed in SaOS-2 and inconstantly expressed in osteoblasts, while it was expressed in ES and unexpressed in SS. The most relevant difference observed in ES compared to SS regarded PLCε and PLCη isoforms.

Conclusion: MG-63 and SaOS-2 osteosarcoma cell lines might represent an inappropriate experimental model for studies about the analysis of signal transduction in osteoblasts.

Keywords: Signal transduction, Phosphoinositide, Phospholipase C, Osteosarcoma, Osteoblast, Ewing sarcoma, Synovial sarcoma, Gene expression, Prognosis.
tumor matrix mineralization of osteosarcoma was not completely highlighted (32).

In the present experiment, we analysed the PLC enzymes in different bone-related cells in order to compare expression panels. We analysed osteoblasts, human cultured osteosarcoma cell lines MG-63 and SaOS-2. We compared the obtained results to the expression of PLCs in few samples of patients affected with Ewing sarcoma (ES) and synovial sarcoma (SS).

Osteoblasts secrete the matrix components in the osseous tissue, playing a crucial role in bone formation and mineralization (33-35).

Both MG-63 (36-38) and SaOS-2 (39-41) human osteosarcoma cell lines used to be considered osteoblast-like cells, as they are currently used as osteoblastic experimental models.

The ES is the second most common primary malignant bone tumor in children and adolescents (42-45). Up to 70% of ESs arises in bone. ES is a heterogeneous family of tumors. Integration of clinical, radiological, immunohistochemical, and molecular data allow the definition of ES, although the diagnosis may result in difficult tumours showing atypical histologic features. ES is characterized by well defined genetic abnormalities, among which the most frequently represented is a translocation involving the chromosome 22 resulting in different fusion genes. The definition of histologic features is important in order to diagnose ES, although the identification of the histologic subtype does not seem to be so crucial, as shared genetic abnormalities characterize the Ewing family of tumors (46-50).

Synovial sarcoma high-grade soft tissue cancer is characterized by local invasiveness and proneness to metastasization. SS affects pediatric, adolescent, and adult population. Despite the name, probably SS arises from primitive mesenchymal cells. The genetic alteration observed up to 95% of cases and considered a specific molecular marker involves chromosome X and chromosome 18 resulting in different fusion genes (SYT-SSX1 or SYT-SSX2) (52-57). The histologic SS subtype does not assume prognostic significance (42,43).

**Objectives**

Our aim was to search for common patterns of expression in osteoblasts, as well as in ES and SS in order to verify differences or similarities that might result in gaining helpful elements for diagnosis and/or possible molecular therapy targets.

**Methods**

RNA extraction from human cell lines experiments: we analyzed cultured human osteoblasts and two osteosarcoma cell lines. MG-63 and SaOS-2 obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The initial seeding number was 250000 cells for each experiment; cells were grown up to 1×10⁶ for molecular biology experiments. Cells were cultured as previously described (26). Briefly, cells were grown under subconfluent or confluent conditions in medium, at 37°C with 5% of CO₂. Cells were cultured in Dulbecco’s minimum essential medium (Sigma) supplemented with 10% fetal bovine serum (GIBCO) with penicillin (100 μg/mL), streptomycin (100 U/mL) and sodium pyruvate. Cells were grown for 24 hours, reaching a confluence of around 40%-60%. After the confluent monolayer was obtained, cells were detached and suspended in TRIzol reagent (Invitrogen Corporation, Carlsbad, CA). Total RNA was isolated following the manufacturer’s instructions. The purity of the RNA was assessed using a UV/visible spectrophotometer (SmartSpec 3000, Bio-Rad Laboratories, Hercules, California, USA).

RNA extraction from ES and SS samples experiments: sample biopsies of 5 patients affected with ES and of 5 patients affected with SS were analyzed. The clinical and radiological diagnosis of ES was confirmed by immunotypization with CD99, FLI1 or ERG, and genetic characterization. About 60% of the patients bore the EWS-FLI1 translocation and 40% the EWS-ERG translocation. The clinical and radiological diagnosis of SS was confirmed by histological features and genetic analyses. The sample biopsies were obtained from tumours bearing the SYT-SSX translocation. 6- to 10-um sections from formalin-fixed, paraffin-embedded specimens were used to extract mRNA using the RecoverAll™ Total Nucleic Acid Isolation kit for FFPE RNA isolation kit (Ambion Inc., Austin, TX) following the manufacturer’s instructions. Briefly, samples were deparaffinized with proteinase K, homogenized and incubated overnight at 55°C. RNA was purified by the addition of RNA extraction buffer. Chloroform was then added, followed by additional incubation and centrifugation. The aqueous phase was removed to fresh tubes and the RNA was precipitated, air-dried and resuspended in 10 μL of RNA storage solution. To remove genomic DNA, all samples underwent DNase treatment. All reagents were purchased from Promega (Promega, Madison, WI, USA). The concentration and quality of the RNA obtained was monitored using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Inc. USA).

One microgram total RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription (RT) kit (Applied Byosystems, Carlsbad, California, USA) according to manufacturer’s instructions. Briefly, RT buffer, dNTP mix, RT random primers, multiscrube reverse transcriptase, RNase inhibitor and DEPC-treated distilled water were added in RNase-
Results

Osteoblasts: PLCB1, PLCB3, PLCB4, PLCG1, PLCG2, PLCD1, PLCD3, PLCD4 and PLCE were expressed. PLCB2 was not expressed. PLCH1 and PLCH2 were inconstantly expressed.

MG-63: PLCB1, PLCB3, PLCB4, PLCG1, PLCG2, PLCD1, PLCD3, PLCE were expressed. PLCB2 was inconstantly expressed. PLCD4, PLCH1 and PLCH2 were not expressed.

SaOS-2: PLCB1, PLCB3, PLCB4, PLCG1, PLCG2, PLCD1, PLCD3, PLCE and PLCH1 were expressed.

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PCR results. (+) absent transcript; (+) detected transcript; (++) inconstantly expressed transcript; (*) not expressed in the samples of patients bearing the EWSR1-FLI1 translocation, expressed in the remaining samples.

PLCB2, PLCD4 and PLCH2 were not expressed.

ES samples: PLCB1, PLCG2, PLCD4 and PLCE were not expressed. PLCB3, PLCB4, PLCD1, PLCD3, PLCH1 and PLCH2 were expressed. PLCB2 was not expressed in the samples of patients bearing the EWSR1-FLI1 translocation, and was expressed in the remaining samples. PLCG1 was not expressed in 20% of the samples (not overlapping).

SS samples: PLCB1, PLCG2, PLCD3 and PLCH1 were not expressed; was very slightly expressed. PLCB and PLCD1 were expressed. PLCB2, PLCG1 or PLCH2 were not expressed in 25% of the samples and expressed in the remaining samples (not overlapping). PLCD4 was expressed in 50% of the samples. PLCD4 was expressed in 25% of the samples.

Discussion

Research efforts were addressed to analyze the metabolism of bones and the signal transduction pathways involved in bone mineralization were actively studied (58-61), with special regard to the systems related to calcium metabolism (62).

Osteoblasts, MG-63 cells and SaOS-2 cells share similar PLC enzymes panel of expression, confirming those observations that have led to use the 2 osteosarcoma cell lines as osteoblasts experimental model. However, slight differences were detected in the expression of selected isoforms.

Confirming our previous reports (26-32), MG-63 expressed a number of PLC enzymes, including PLCB1, PLCB3, PLCB4, PLCG1, PLCG2, PLCD1, PLCD3, PLCE and inconstantly PLCB2. SaOS-2 expressed PLCB1, PLCB3, PLCB4, PLCG1, PLCG2, PLCD1, PLCD3, PLCE and PLCH1. Osteoblasts (26) expressed PLCB1, PLCB3, PLCB4, PLCG1, PLCG2, PLCD1,
PLCD3, PLCδ4 and PLCε, PLCH1 and PLCH2 were inconsistently expressed.

PLCδ4 mRNA was differently detected. The mRNA transcript for PLCδ4 was detected in osteoblasts, while it was absent in both MG-63 and SaOS-2 cells. The PLCδ4 protein was not detected in any of the analyzed cell lines.

Interestingly, in our previous reports PLCδ4 was inconsistently expressed in fibroblasts (13), was expressed in the Hs888 human metastatic cells and was absent in other human osteosarcoma cell lines. In previous reports, the transcription of PLCδ4 was up-regulated after silencing of ezrin, a membrane protein interacting with actin which is involved in the metastatic spread of osteosarcoma (26-31). Moreover, PLCδ4 absence in MG-63 cells did not change after administration of PLC inhibitors, indicating that it was constitutionally untranscribed (31).

PLCδ enzymes, the most primitive and evolutionary conserved, are known to be very sensitive to calcium and were described to play a key role in cell proliferation (9). In yeast and higher plants, PLCδ is involved in nutritional and environmental stresses (63,64). Moreover, recent observations suggested that PLCδ might be involved in the stress-induced response in endothelial cells (12,19-21). In the regenerating liver, PLCδ4 mRNA is expressed at higher levels than in normal resting liver, as well as in hepatoma cells, and in Src-transformed cells (9). PLCδ6 was demonstrated to regulate the liver regeneration in cooperation with nuclear protein kinase C (PKC) alpha and epsilon (65).

Moreover, PLCδ6 expression was abundant in a number of tumours, including astrocytomas (11,15,16). In breast cancer the abnormal expression of PLCδ6 contributes to carcinogenesis by up-regulating ErbB expression and activating the ERK pathway (66). Probably, the expression of PLCδ6 is a response to mitogenic signals or the isoform is expressed more abundantly in high-rate proliferating cells (67). In our present experiments, the transcript of PLCδ4 was detected exclusively in osteoblasts, while it was absent in either the osteosarcoma cell lines. The presence of PLCδ4 transcript in osteoblasts might confirm its role in metabolically active cells. However, the absence of PLCδ4 expression in both MG-63 and SaOS-2 cells indicate that those cell lines cannot be perfectly compared to osteoblasts.

The presence of PLCδ4 transcript in osteoblasts in the absence of the corresponding protein suggests that a complex regulation of PLCδ4 translation occurs in cells. Those aspects and the possible relationship with other PLC enzymes will require further investigations. In fact, up-regulation of PLCδ4 was suggested to be related to the regulation of other PLC enzymes, probably PLCβ1 (16-30). That might be due to the highest sensitivity of PLCδ isoforms to calcium concentration and suggest that an internal regulatory hierarchy might exist among PLC enzymes. That is an old issue, in fact for a long time it was hypothesized that PLCδ4 or some splice variants might act as negative regulators for PLC (68).

That represents an interesting point, in that also for the activity of PLCγ isoforms calcium levels are critical (9,69,70). In the present experiments, osteoblasts differently expressed PLCH1 and PLCH2 compared to MG-63 and SaOS-2 cells.

PLCγ1 acts as a signal amplifier in G protein-coupled receptor (GPCR)-mediated calcium signaling. Knocking down PLCγ1, but not PLCγ2, significantly reduced the PLC pathway. PLCγ1 is efficiently activated by intracellular calcium stores, suggesting that mobilization of calcium from the ER plays a critical role in PLCγ1 activation (71). That might fit with our present observation that PLCδ4 is expressed in osteoblasts. In fact, PLCδ4 is activated by low concentrations of calcium. Once activated, it can increase the intracellular calcium concentration via IP3 production, thus activating PLCγ1 as a positive feed-back. However, despite the presence of PLCδ4 transcript in osteoblasts, PLCδ4 protein was slightly detected within the cell performing immunofluorescence experiments.

Similarly within the cell, PLCγ2 contributes to calcium dynamics (69) by transducing signals from mitochondria calcium. Alterations in the calcium levels modulate the activity of PLCγ2, suggesting that this isoform may contribute to regulate the calcium signaling networking intracellular and extracellular stimuli. The sensitivity of PLCγ2 to calcium might favour the amplification of intracellular calcium transients and/or crosstalk between storing compartments.

The subcellular distribution of the PLC enzymes also showed slight differences in the analysed cell lines. In particular, selected enzymes such as PLCβ1 and either PLCγ1 enzymes were differently stored within the cell. In osteoblasts, PLCβ1 seems to be stored in vesicles partially resembling the endoplasmic reticulum distribution. In SaOS-2 cell, PLCγ subfamily enzymes also seem to be stored in vesicles and the punctate distribution of PLCγ2 results less evident.

The present results confirm that osteoblasts, MG-63 cells and SaOS-2 cells share similar panel of expression of PLC enzymes, with notable exceptions indicating that MG-63 and SaOS-2 osteosarcoma cell lines might not represent an appropriate experimental model to analyse the signal transduction in osteoblasts.

Interesting differences were identified relative to the expression of PLCs in ES and SS. In fact, most ES and SS did not express PLCB1, which was expressed in most osteoblasts, MG-63 and SaOS-2 cells. Conversely, PLCB2, unexpressed in the cell lines, was expressed in
some ES and SS. However, PLCβ2 was expressed in SaOS-2 and inconstantly expressed in osteoblasts, while it was expressed in ES and unexpressed in SS.

The main genetic abnormalities described in ES and SS are actually considered as crucial elements for diagnosis (9,72,73). ES is defined by a balanced translocation that involves the EWSR1 gene (OMIM *133450; mapping on 22q12.2) and a member of the E-twenty-six (ETS) family of transcription factors, most frequently FLI1 (OMIM *193067; mapping in 11q24.3) or ERG (OMIM *165080; mapping in 21q22.2) (74,75). Beside the main translocations previously described, either tumour can bear additional secondary chromosomal abnormalities, such as gains of chromosomes and/or loss of chromosome regions (76-78).

Previous studies performed in epigenetically modified stable ES’ cells showed partial loss of endogenous EWSR1-FLI1 due to transfection with antisense EWSR1-FLI1 DNA plasmid (79). Interestingly, the expression of PLCβ2 and PLCβ3 resulted reduced in the transfected cells that showed EWSR1-FLI1 loss. PLCB3 maps on chromosome 11, region 11q13. However, the break point of the EWS-Fli-1 rearrangement usually involves the 11q24 region, probably distal to the PLCB3 locus. Conversely, PLCB2 gene maps in chromosome 15, in a region not commonly involved in the rearrangement. Those findings suggested that the impairment of the G-protein-mediated PI turnover could be responsible for the concomitantly observed suppression of transfected cells growth (79). That supported the hypothesis that signalling through Gq and PLCβ isoforms might represent a crucial pathway in the transformation and growth of tumours (13-20,80).

In the present experiments, PLCB1 gene was not expressed in either ES or SS. Conversely it was not detected in cultured cells. PLCβ1 was suggested to be mainly involved in inflammation (21-25,30), as well as in differentiation (81,82). Moreover, PLCβ1 might be altered in breast cancer (83) and its partial or total lack might influence cancer progression in myeloid tissue (84). Previous studies (24-26) demonstrated that in osteosarcoma, PLCB1 increase is associated with the decrease of ezrin, which is thought to facilitate tumour progression, suggesting that PLCβ1 might play an opposite role.

PLCB2 transcript was detected in 70% of SS samples and in 80% of ES samples. Remarkably, PLCβ2 was not detected in ES samples bearing the EWSR1-ERG translocation. PLCβ2 is mainly expressed in haematopoietic lineage cells (9). PLCβ2 protein was also detected in normal skin fibroblasts (13), as well as in skin fibroblasts from patients presenting with hypertension (85). PLCβ2 is thought to be a part of the IGF2-IGF2R/PLCβ2 axis, which is involved in neovascularization (86). Previous reports also demonstrated a specific role of PLCβ2 in osteosarcoma cells mechano-transduction and attachment, indicating that the presence of PLCβ2 might be correlated with specific stimuli (85). The meaning of PLCβ2 absence in ES carrying the EWSR1-ERG translocation needs further studies.

The most relevant difference observed in ES compared with SS regarded PLCε and PLCγ isoforms. PLCH1 and PLCH2 were expressed in ES samples. PLCH1 was absent and PLCH2 was present in 75% of the SS samples. Conversely, PLCε was not expressed in ES and was weakly expressed in SS. The role of PLCγ subfamily is not fully highlighted. The alternate presence of one/both PLCγ isoforms or PLCε was described in other cell types, suggesting a sort of dualism. The role of PLCγ isoforms needs further studies in order to be elucidated. In fact, different splicing isoforms were described, PLCγ enzymes are considered very highly sensitive to calcium concentrations and probably interact with PLC isoforms belonging to other subfamilies in a complex manner.

Conclusion
Our results indicated that the expression of PLC enzymes differs in osteoblasts, ES and SS. Specific expression panel of PLCs suggests that selected isoforms might play a specific role in calcium metabolism in osteoblasts which differ from ES and SS. Rapid advances in molecular methodologies will allow to discover novel genetic events and help to refine diagnostic criteria, highlighting the biology of a number of tumours. The PI signal transduction pathway might deserve further research effort in order to investigate the role of PLC enzymes in the progression of sarcomas and also paving the way to novel diagnostic elements.

Authors’ Contribution
VRLV: experimental design, results elaboration, discussion, article writing. ML and ASdA: experiments. CDR: critical discussion.

Conflict of Interest Disclosures
All authors declare that there is no conflict of interests.

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