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Research Article

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, and a number of 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 PLC δ4 and PLC η 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 PLC ε and PLC η 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.

Background

Calcium plays a key role within cells, and it is involved in many signal transduction pathways, acting as a second messenger or as an enzyme cofactor. Extracellular calcium is also crucial for excitable cell membranes activity, as well as proper bone formation. Bone hardness and strength depend on mineralization, which involves a complex process in which bone-forming cells produce crystals of calcium phosphate, which were shed around the fibrous matrix. This process is strictly regulated. In addition, many signal transduction systems were interested in calcium metabolism, such as phosphoinositide (PI) pathway and related phospholipase C (PLC) enzymes. In mammals, bones represent the major calcium storage, and its capture from or release in the blood is regulated by a number of molecules, including hormones and vitamins.

Functional studies demonstrated that increase of calcium levels activates PLC, resulting in calcium increase (1). PLC cleaves phosphatydil inositol (4,5) bisphosphate (PIP2), a phosphorylated derivative of phosphatydil inositol standing in the inner half of the plasma membrane (2-7), into inositol trisphosphate (IP3) and diacylglycerol (DAG), 2 further signaling molecules. In mammalian species, the PLC family comprises modular, multi-domain enzymes covering a wide network, including direct binding to G protein subunits, small GTPases, tyrosine kinases and membrane lipids (8-10). PLC enzymes are thirteen isoenzymes subdivided into 6 subfamilies on the basis of amino acid sequence, domain structure and recruitment: β(1–4), γ(1-2), δ(1, 3, 4), ε(1), ζ(1), and η(1-2) (9,10). Our previous studies suggested that selected PLC enzymes are involved in osteosarcoma progression (11-25), and probably networking ezrin, a molecule acting during the metastatic spread (26-31). However, the

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 tumour in children and adolescents (42-45). Up to 70% of ESs arises in bone. ES is a heterogeneous family of tumours. 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 250 000 cells for each experiment; cells were grown up to 1×10^6 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 CO_2. 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 deparaffined 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, multiscribe reverse transcriptase, RNase inhibitor and DEPC-treated distilled water were added in RNase-

free tubes on ice. The RNA sample was added. The thermal cycler was programmed as follows: 25°C for 10 minutes; 37°C for 120 minutes; the reaction was stopped at 85°C for 5 minutes. The final volume was 20 µL. For polymerase chain reaction (PCR) reactions, the primer pairs (Bio Basic Inc, Amherst, New York, USA) were listed in Table 1. To amplify glyceraldehyde 3 phosphate dehydrogenase (GAPDH) (Bio Basic Inc, Amherst, New York, USA), used a constitutive positive control, the following primer pair was used: forward 5' -CGAGATCCCTCCAAAATCAA-3' reverse 5'-GTCTTCTGGGTGGCAGTGAT-3'. The specificity of the primers was verified by searching in the NCBI database for possible homology to cDNAs of unrelated proteins. The specificity of the primers was verified by searching in the NCBI database for possible homology to cDNAs of unrelated proteins. Each PCR tube contained the following reagents: 0.2 µM of both sense and antisense primers, 1-3 µL (about 1 µg) template cDNA, 0.2 mM dNTP mix, 2.5 U REDTaq genomic DNA polymerase (Sigma-Aldrich) and 1X reaction buffer. MgCl2 was added at a variable final concentration (empirical determination by setting the experiment). The final volume was 50 µL. The amplification was started with an initial denaturation step at 94°C for 2 minutes and was followed by 35 cycles consisting of denaturation (30 seconds) at 94°C, annealing (30 seconds) at the appropriate temperature for each primer pair and extension (1 minutes) at 72°C. The PCR products were analysed by 1.5% TAE ethidium bromide-stained agarose gel electrophoresis (Agarose Gel Unit, Bio-Rad Laboratories S.r.l., Segrate, IT). A PC-assisted CCD camera UVB lamp (Vilber Lourmat, Marne-la-Vallé France) was used for gel documentation. Gel electrophoresis of the amplification products revealed single DNA bands with nucleotide lengths as expected for each primer pair. RNA samples were also amplified by PCR without RT. No band was observed, excluding DNA contamination during the procedure (data not shown). The reaction products were further quantified with the Agilent 2100 bioanalyzer using the DNA 1000 LabChip kit (Agilent Technologies, Deutschland GmbH).

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. *PLCB2, PLCD4* and *PLCH2* were not expressed.

Table 1. PCR Results

	Osteoblasts	MG-63	Sa-OS2	ES	SS
PLCB1	+	+	+	-	-
PLCB2	-	-+	-	+- *	-+
PLCB3	+	+	+	+	-+
PLCB4	+	+	+	+	-+
PLCG1	+	+	+	-+	-+
PLCG2	+	+	+	-	-
PLCD1	+	+	+	+	+
PLCD3	+	+	+	+	-
PLCD4	+	-	-	-	+
PLCE	+	+	+	-	+
PLCH1	-+	-	+	+	-
PLCH2	-+	-	-	+	-+

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.

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). *PLCB4* 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, PLCD4 and *PLCE*, *PLCH1* and *PLCH2* were inconstantly expressed.

PLCD4 mRNA was differently detected. The mRNA transcript for *PLCD4* was detected in osteoblasts, while it was absent in both MG-63 and SaOS-2 cells. The PLC δ4 protein was not detected in any of the analyzed cell lines.

Interestingly, in our previous reports PLC δ4 was inconstantly 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 *PLCD4* 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, *PLCD4* absence in MG-63 cells did not change after administration of PLC inhibitors, indicating that it was constitutionally untranscribed (31).

PLC δ 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, PLC δ4 is involved in nutritional and environmental stresses (63,64). Moreover, recent observations suggested that PLC δ4 might be involved in the stress-induced response in endothelial cells (12,19-21). In the regenerating liver, PLC δ4 mRNA is expressed at higher levels than in normal resting liver, as well as in hepatoma cells, and in Src-transformed cells (9). PLC δ4 was demonstrated to regulate the liver regeneration in cooperation with nuclear protein kinase C (PKC) alpha and epsilon (65).

Moreover, PLC δ4 expression was abundant in a number of tumours, including astrocytomas (11,15,16). In breast cancer the abnormal expression of PLC δ4 contributes to carcinogenesis by up-regulating ErbB expression and activating the ERK pathway (66). Probably, the expression of PLC δ4 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 *PLCD4* was detected exclusively in osteoblasts, while it was absent in either the osteosarcoma cell lines. The presence of *PLCD4* transcript in osteoblasts might confirm its role in metabolically active cells. However, the absence of *PLCD4* expression in both MG-63 and SaOS-2 cells indicate that those cell lines cannot be perfectly compared to osteoblasts.

The presence of *PLCD4* transcript in osteoblasts in the absence of the corresponding protein suggests that a complex regulation of PLC δ4 translation occurs in cells. Those aspects and the possible relationship with other PLC enzymes will require further investigations. In fact, up-regulation of *PLCD4* was suggested to be related to the regulation of other PLC enzymes, probably PLC β1 (16-30). That might be due to the highest sensitivity of PLC δ 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 PLC δ4 or some splice variants might act as negative regulators for PLC (68).

That represents an interesting point, in that also for the activity of PLC η 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.

PLC η1 acts as a signal amplifier in G protein-coupled receptor (GPCR)-mediated calcium signaling. Knocking down PLC η1, but not PLC-η2, significantly reduced the PLC pathway. PLC η1 is efficiently activated by intracellular calcium stores, suggesting that mobilization of calcium from the ER plays a critical role in PLC η1 activation (71). That might fit with our present observation that *PLCD4* is expressed in osteoblasts. In fact, PLC δ4 is activated by low concentrations of calcium. Once activated, it can increase the intracellular calcium concentration via IP3 production, thus activating PLC η1 as a positive feed-back. However, despite the presence of *PLCD4* transcript in osteoblasts, PLC δ4 protein was slightly detected within the cell performing immunofluorescence experiments.

Similarly within the cell, PLC η2 contributes to calcium dynamics (69) by transducing signals from mitochondria calcium. Alterations in the calcium levels modulate the activity of PLC η2, suggesting that this isoform may contributes to regulate the calcium signaling networking intracellular and extracellular stimuli. The sensitivity of PLC η2 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 PLC β1 and either PLC η enzymes were differently stored within the cell. In osteoblasts, PLC β1 seems to be stored in vesicles partially resembling the endoplasmic reticulum distribution. In SaOS-2 cells, PLC η subfamily enzymes also seem to be stored in vesicles and the punctuate distribution of PLC η2 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, *PLCH1* 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 PLC β2 and PLC β3 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 PLC β 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. PLC β1 was suggested to be mainly involved in inflammation (21-25,30), as well as in differentiation (81,82). Moreover, PLC β1 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 PLC β1 might play an opposite role.

PLCB2 transcript was detected in 70% of SS samples and in 80% of ES samples. Remarkably, PLC β2 was not detected in ES samples bearing the *EWSR1-ERG* translocation. PLC β2 is mainly expressed in haematopoietic lineage cells (9). PLC β2 protein was also detected in normal skin fibroblasts (13), as well as in skin fibroblasts from patients presenting with hypertension (85). PLC β2 is thought to be a part of the IGF2-IGF2R/PLC β2 axis, which is involved in neovascularization (86). Previous reports also demonstrated a specific role of PLC β2 in osteosarcoma cells mechano-transduction and attachment, indicating that the presence of PLC β2 might be correlated with specific stimuli (85). The meaning of PLC β2 absence in ES carrying the *EWSR1-ERG* translocation needs further studies.

The most relevant difference observed in ES compared with SS regarded PLC ε and PLC η isoforms. *PLCH1* and *PLCH2* were expressed in ES samples. *PLCH1* was absent and *PLCH2* was present in 75% of the SS samples. Conversely, *PLCE* was not expressed in ES and was weakly expressed in SS. The role of PLC η subfamily is not fully highlighted. The alternate presence of one/both PLC η isoforms or PLC ε was described in other cell types, suggesting a sort of dualism. The role of PLC η isoforms needs further studies in order to be elucidated. In fact, different splicing isoforms were described, PLC η 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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