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Supernatants From Human Osteosarcoma Cultured Cell Lines Induce Modifications in Growth and Differentiation of THP-1 Cells and Phosphoinositide-Specific Phospholipase C Enzymes

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Introduction: Introduction: Molecular components within the microenvironment act upon cell growth, survival/apoptosis, and proliferation. Immune system cells respond to molecules produced by the tumor and released in the surrounding microenvironment, such as cytokines, chemokines, and growth factors. This study aimed to identify the effects of tumor environment on monocyte-macrophage cell lineage.
Methods: We evaluated morphological and functional changes in THP-1 cells cultured in culture medium mixed with the culture supernatant of one of three different osteosarcoma (OS) cell lines, namely 143B, HS888, and MG-63. We analyzed the effect of supernatants from OS cell lines on morphology and growth of THP-1 cells, and mRNA expression of phosphoinositide-specific phospholipase C (PLC) enzymes.
Results: in supernatants from each OS cell line we identified the presence of selected interleukin (IL), TNFα, and GM-CSF. Each OS-derived supernatant differently modified the growth rate of THP-1 cells, depending on the cell line. OS supernatants greatly modified the expression panel of PLC enzymes expressed by THP-1 cells in the in vitro microenvironment. THP-1 cells differently express PLC enzymes, depending on the origin of the supernatant. The differences in PLCs' expression induced by OS supernatants resulted in a statistically significant difference in expression of PLCB1 and PLCG2 genes.
Conclusions: OS supernatants induce the differentiation of THP-1 cells into macrophages. THP-1 cells cultured in OS supernatants expressed different expression panels of PLC enzymes at the mRNA level. The expression panel of PLC enzymes differs during the differentiation of monocyte/macrophage lineage THP-1 cells.

INTRODUCTION

The environment where cells live influences their growth, proliferation, and survival. Knowledge of the molecules contained in the microenvironment, including those belonging to signal transduction systems allowing crosstalk among cells, might help to understand both physiological events and systemic development of diseases [1]. The possibility to modify the microenvironment

might open promising perspectives in terms of targeted molecular therapy approaches. In vitro experiments indicated that modifications of the molecular and biochemical components within the microenvironment can interact and influence the behavior of both cells in the surrounding normal tissue and cells belonging to the immune system [1]. Immune system cells behave differently depending on selected stimuli from signal transduction pathways acting in the microenvironment.

In the present article, we cultured three human osteosarcoma (OS) cell lines and obtained the supernatant culture medium. OS is considered the most common primary bone tumor in children and adolescents [2]. The behavior of OS differs depending on the tumor cell type, probably influenced by different signal transduction pathways [3]. We cultured THP-1 cells in a culture medium; containing supernatant from OS cell lines. We aimed to verify the effect of OS-related microenvironment upon the differentiation of THP-1 cells. THP-1 monocyte-like cell line was derived from the peripheral blood of a one-year-old boy affected with acute monocytic leukemia and was treated with phorbol 12-myristate 13-acetate (PMA) in in vitro condition [4]. THP-1 cells can differentiate into macrophage-like phenotype under appropriate stimuli [5] and behave similarly to the native monocyte-derived macrophages [6]. After incubation with Interferon-γ (IFN-γ) and Lipopolysaccharide (LPS) or with interleukin (IL)-4 and IL-13, THP-1-derived macrophages can be polarized respectively into the M1 or M2 phenotype [7]. After treatment with recombinant human IL-4 (rhIL-4) and recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF), THP-1 cells can differentiate into immature dendritic cells. After treatment with rhIL-4, rhGM-CSF, recombinant human tumor necrosis factor-α (rhTNFα) and Ionomycin, THP-1 cells can also differentiate into mature dendritic cells [8]. Therefore, THP-1 cells are currently used as in vitro model to study the differentiation process of monocytes and macrophages [4].

The role of calcium and triggering elements of many intra- and intercellular events were extensively analyzed in the monocyte-macrophage line [9]. Great interest was reserved for the network of signaling molecules contributing to the complex regulation of calcium metabolism, including the Phosphoinositide (PI) pathway. PIs, minority acidic phospholipids play an instructional role in cell membranes and provide a complex and crucial intracellular signaling system involved in a variety of cell functions [10]. Crucial events are related to the regulation of phosphatidylinositol 4,5-bisphosphate (PIP2), a minor phospholipid located in the inner half-membrane [10] which can be hydrolyzed by enzymes belonging to the PI-specific phospholipase C (PLC) family. Mammalian PLC enzymes are grouped into six subtypes based on the amino acid sequence, domain structure, and mechanism of recruitment: β (1–4), γ (1-2), δ (1, 3, 4), ε, ζ, and η (1-2) [10]. Literature data described the role of the PLC signal transduction in development and differentiation pathway [11-14]. The expression panel of PLCs is tissue-specific [10, 13-22]. Also, the sub-cellular localization of PLC enzymes differs depending on the isoform and varies under different conditions; especially inflammation [10, 19, 22]. The activation of quiescent cells with different stimuli induced variation of the PLCs expression [22-25], and abnormal expression of PLCs was described in nervous anomalies [3, 13, 14, 16, 17, 26-28] and tumors [17, 18, 23, 24, 29]. PLC inhibition [22, 23] or silencing [20, 21, 30] can modify the growth and survival of selected tumors. Recently, PLC-dependent signaling was described to be crucial for macrophage chemotaxis [31]. The expression of PLC enzymes varies in unpolarized (M0), M1, and M2 macrophages in quiescent versus inflammatory-induced stimulation, with a peculiar panel of expression for each cell type [12].

In the present article, we evaluated the morphological and functional changes occurred in THP-1 cells cultured in a modified culture medium, obtained by replacing part of the Roswell Park Memorial Institute culture medium (RPMI) with supernatant from cultures of one of three different OS cell lines, namely 143B, HS888, and MG-63 cells. Secondarily, we analyzed the effects of supernatants from OS cell lines on enzymes involved in the metabolism of calcium in THP-1 cells. We analyzed the basic expression panel of PLC enzymes at the mRNA level in THP-1 cells by RT-PCR. Then, we analyzed modifications and the possible relationship with the morphological

and functional changes induced in THP-1 cells by partially replacing the culture medium with supernatants derived from OS cell lines.

METHODS

Culture of Osteosarcoma Cell Lines

OS cell lines 143B, HS888, and MG63 were separately cultured as previously described [17]. MG-63, 143B, and Hs888 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). OS cell lines were separately grown under sub-confluent or confluent conditions at 37°C with 5% of CO2 in Dulbecco's minimum essential medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (GIBCO), penicillin (100µg/ml), streptomycin (100U/ml), and sodium pyruvate. Cells grew for 48 hours and reached 40-60% confluence. Then, culture supernatants from each OS cell line were separately collected and stored at -20°C until used for cytokine/chemokine analysis or culture of THP-1 cells.

Biochemical Analysis of Osteosarcoma Supernatants

The cytokine/chemokine profiles of supernatants were analyzed; using the Human Inflammatory Cytokines and Chemokines Multi-Analyte ELISArray Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. The analysis was conducted for the following cytokines and chemokines: IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, IFN, TNFα, and GM-CSF.

THP-1 Cultures

A total of 200,000/well THP-1 cells were seeded into plates contenting 2 ml of RPMI (controls) or 0.5 ml RPMI (25%) plus 1,5 ml of supernatants (75%) obtained from 48 hours cultured 143B or HS888 or MG-63 cells reaching to a final volume of 2 ml/well. The modified culture media would be indicated as RPMI-s143B, RPMI-sHS888, and RPMI-sMG63; respectively. The number of viable cells treated with OS supernatant, pre-trypsin, and attached cells post-trypsin treatment, was evaluated after 24, 48, and 72 hours and normalized for the number of viable THP-1 control cells at corresponding time intervals. After 48 hours, non-attached cells and adherent cells were counted; using a Neubauer hemocytometer. Cell pellets were stored at -20°C until being used for molecular biology. The morphology of THP-1 cells was observed by optic microscopy. All experiments were conducted in triplicate. To evaluate the ability of differently cultured THP-1 cells to react to infections or remove tumor debris in the environment, the phagocytic ability was evaluated; using trypan blue 1:2 in culture medium for 2 hours. Then, cells were fixed in phosphate-buffered formalin and Acid Phosphatase enzymatic assay (Abcam, UK) was conducted following the manufacturer's instructions. The results were observed by optic microscopy. Statistical analysis of the results was conducted; using the ANOVA test. Results were considered statistically significant when $P<0.05$.

PLC Analysis

mRNA from THP-1 cells cultured in RPMI (controls), RPMI-s143B, RPMI-sHS888, and RPMI-sMG63 were separately extracted with the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to manufacturer's instructions. The purity of the RNA was assessed; using a UV/visible spectrophotometer (SmartSpec 3000, Bio-Rad Laboratories, Hercules, California, USA). The procedure, repeated in triplicates, was previously described [3]. mRNA was retro-transcribed into cDNA; using High-Capacity cDNA Reverse Transcription Kit as previously described [3](Life Technologies, Foster City, CA, USA). Two µg of mRNA were incubated with the master mix containing 2µl of 10X Reverse Transcription Buffer, 0.8µl of 25X dNTPs (100mM), 2µl of 10X random primers, 1µl of MultiScribe TM Reverse Transcriptase (50 U/µl), and 3.2µl of DNase-free water to reach a final volume of 20µl. PCR was performed; using the primer pairs (Bio Basic Inc, Amherst, New York, USA) listed in Table 1. PLCZ was not exclusively analyzed in the sperm lineage [10]. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) gene (Bio Basic Inc, Amherst, New York, USA) was used as an internal control; using the following primer pair: forward 5′ -CGAGATCCCTCCAAAATCAA-3′ reverse 5′-GTCTTCTGGGTGGCAGTGAT-3′. The primers were specificity rescued in the NCBI database for homologies to other cDNAs. We mixed 0.2µM of both sense and antisense primers, 1-3µl

(about 1mg) of template cDNA, 0.2mM of dNTP mix, 2.5U of REDTaq Genomic DNA polymerase (Sigma-Aldrich), and 1X of reaction buffer, MgCl2 at a variable concentration (empirical determination by setting the experiment) to reach a final volume of 50µl. The amplification was started with an initial denaturation step at 94°C for 2' and was followed by 35 cycles consisting of denaturation (30'') at 94°C, annealing (30'') at the appropriate temperature for each primer pair and extension (1') at 72°C. The PCR products were analyzed by 1.5% TAE ethidium bromide-stained agarose gel electrophoresis (Agarose Gel Unit, Bio-Rad Laboratories S.r.l., Segrate, IT). Gel documentation was verified with a PC-mediated CCD camera UVB lamp (VilberLourmat, Marne-la-Vallé France). DNA contamination was excluded; performing PCR cycles without RT (data not shown). The concentration of the amplified cDNA was measured with Agilent 2100 bioanalyzer; using the DNA 1000 LabChip kit (Agilent Technologies, Deutschland GmbH).

Table 1: Primers' Pairs for Polymerase Chain Reaction of PLC Genes [a]

	OMIM	Primers for PLC Genes
PLCB1	*607120	f: 5'-AGCTCTCAGAACAAGCCTCCAACA-3' r: 5'-ATCATCGTCGTCGTCACTTTCCGT-3'
PLCB2	*604114	f: 5'-AAGGTGAAGGCCTATCTGAGCCAA-3' r: 5'-CTTGGCAAACTTCCCAAAGCGAGT-3'
PLCB3	*600230	f: 5'-TATCTTCTTGGACCTGCTGACCGT-3' r: 5'-TGTGCCCTCATCTGTAGTTGGCTT-3'
PLCB4	*600810	f: 5'-GCACAGCACACAAAGGAATGGTCA-3' r: 5'-CGCATTTCCTTGCTTTCCCTGTCA-3'
PLCG1	*172420	f: 5'-TCTACCTGGAGGACCCTGTGAA-3' r: 5'-CCAGAAAGAGAG CGTGTAGTCG-3'
PLCG2	*600220	f: 5'-AGTACATGCAGATGAATCACGC-3' r: 5'-ACCTGAATCCTGATTTGACTGC-3'
PLCD1	*602142	f: 5'-CTGAGCGTGTGGTTCCAGC-3' r: 5'-CAGGCCCTCGGACTGGT-3'
PLCD3	*608795	f: 5'-CCAGAACCACTCTCAGCATCCA-3' r: 5'-GCCA TTGTTGAGCACGTAGTCAG-3'
PLCD4	*605939	f: 5'-AGACACGTCCCAGTCTGGAACC- 3' r: 5'-CTGCTTCCTCTTCCTCATATTC- 3'
PLCE	*608414	f: 5'-GGGGCCACGGTCATCCAC-3' r: 5'-GGGCCTTCATACCGTCCATCCTC-3'
PLCH1	*612835	f: 5'-CTTTGGTTCGGTTCCTTGTGTGG-3' r: 5'-GGATGCTTCTGTCAGTCCTTCC-3'
PLCH2	*612836	f: 5'-AAACTGGCCTCCAAACACTGCCCGCCG-3' r: 5'-GTCTTGTTGGAGATGCACGTGCCCCTTGC-3'

[a] OMIM, online mendelian inheritance in man catalog number of PLC genes.

RESULTS

Analysis of Osteosarcoma Supernatants

IL-2, IL-6, IL8, TNFα, and GM-CSF were identified in the supernatant of all OS cell lines. IL-1α, IL-1β was identified in the supernatant from the 143B cell line. IL-4, IL-10, IL-12, IL-17, and IFNγ were not identified in any supernatant (Table 2).

THP-1 Cells' Culture

The growth of THP-1 control cells in RPMI progressively increased after 24 (28%), 48 (130%), and 72 (324%) hours. Percentages are intended to compare the initial number of viable seeded cells (200,000) and cells counted at each time interval (Table 3, Figure 1A). The statistical analysis of the results was performed with the ANOVA test and the $P<0.05$ was considered to be significant. The supernatants differently modified the growth rate of THP-1 cells, depending on the considered OS cell line. In THP-1 cells cultured in RPMI-s143, the growth rate increased similarly to controls (25%) after 24 hours, decreased after 48 hours, and increased after 72 hours (Table 3, Figure 1A). In THP-1 cells cultured in RPMI-sHS888, the growth rate increased less than controls (12.5%)

Table 2: Cytokines in Osteosarcoma Supernatants [a, b]

	IL1A	IL1B	IL2	IL4	IL6	IL8	IL10	IL12	IL17A	INFG	TNFA	GMCSF
C-	0	0	0	0	0	0	0	0	0	0	0	0
143B	0.64	0.01	0.01	0	0.08	0.97	0	0	0	0	0.01	0.78
MG63	0	0	0.014903	0	10.43846	1.223523	0	0	0	0	0.01075	0.516883
HS888	0	0	0.01	0	1.406481	1.151484	0	0	0	0	0.012	0.021
C+	1	1	1	1	1	1	1	1	1	1	1	1

[a] Cytokines/chemokines in supernatants from cultures of 143B cells (143B), MG-63 (MG63), and HS888 (HS888), (C-) negative controls, (C+) positive controls.

[b] Concentrations of cytokines are intended in pg/ml.

after 24 hours, increased after 48 hours, and increased after 72 hours (Table 3, Figure 1A). In THP-1 cells cultured in RPMI-sMG63, the growth rate increased as compared with controls (49%) after 24 and 48 hours and decreased after 72 hours for the next 48 hours. The overall number of live THP-1 cells was greater than time 0 (Table 3, Figure 1A). After trypsin, the survival rates of THP-1 cells greatly differed, both in control cells and in THP-1 cells cultured in the culture medium containing OS supernatants (Table 3, Figure 1B). In control cultures, the survival of THP-1 cells decreased after 24 (-86.75%), and 48 hours, but was almost doubled after 72 hours (Table 3, Figure 1B). In THP-1 cells cultured in RPMI-s143, the survival rate decreased less than controls (-77.5%) after 24 hours, increased after 48 hours, and decreased after 72 hours (Table 3, Figure 1B). In THP-1 cells cultured in RPMI-sHS888, the survival rate decreased less than controls (-54%) after 24 hours, increased after 48 hours, and did not change after 72 hours (Table 3, Figure 1B). In THP-1 cells cultured in RPMI-sMG63, after 24 hours the survival rate decreased less than controls (-77.5%) and similarly to RPMI-s143B cultured cells, increased after 48 and 72 hours (Table 3, Figure 1B). The statistical analysis of the results is available in Table 4, right columns. Although the partial replacement of culture medium with OS supernatant affected the growth and survival rates of THP-1 cells, the statistical analysis of these results was significantly exclusively for the supernatant from the HS888 cell line (Table 3).

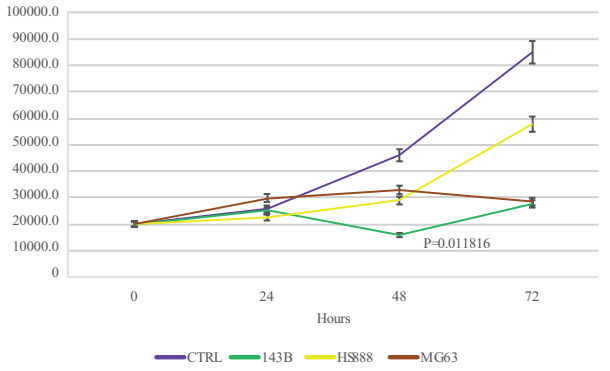


Figure 1: THP-1 Growth Curves
Graphic representations of the growth curve of THP-1 cells (CTRL), of THP-1 cells cultured in RPMI-143B supernatant (S-143), of THP-1 cells cultured in RPMI-HS888 supernatant (S-HS888), and THP-1 cells cultured in RPMI-MG63 supernatant (S-MG63). Standard error bars were indicated for each growth curve.

Table 3: Comparison of THP-1 Viable Cells and of THP-1 Cells Cultured Respectively in RPMI-143B Supernatant, RPMI-HS888 Supernatant, and RPMI-MG63 Supernatant [a, b]

	Seeding Time (T0)	24, hr	48, hr	72, hr	P Value
Pre Trypsin					
CTRL	200,000	257,500	460,000	848,750	0.086
S-143	200,000	250,000	158,750	276,250	0.241
S-HS888	200,000	225,000	288,750	577,500	0.139
S- MG63	200,000	298,750	328,750	285,000	0.370
CTRL/s-143	—	—	—	—	0.086
CTRL/s-HS888	—	—	—	—	0.241
CTRL/s-MG63	—	—	—	—	0.139
CTRL/OOSS	—	—	—	—	0.370
Post Trypsin					
CTRL	—	26,250	23,750	42,500	0.053
S-143	—	45,000	147,500	88,750	0.011
S-HS888	—	91,250	202,500	206,250	0.054
S-MG63	—	45,000	118,750	191,250	0.089
CTRL/s-143	—	—	—	—	0.053
CTRL/s-HS888	—	—	—	—	0.011
CTRL/s-MG63	—	—	—	—	0.054
CTRL/OOSS	—	—	—	—	0.089

[a] Abbreviations: CTRL, Viable Cells; S-HS888, THP-1 cells cultured in RPMI-HS888 supernatant; S-MG63, THP-1 cells cultured in RPMI-MG63 supernatant; S-143, THP-1 cells cultured in RPMI-143B supernatant

[b] Pre trypsin: P values obtained comparing the number of cells and median of CTRL, S-143, S-HS888, and S-MG63. Post trypsin: P values obtained comparing the number of attached cells counted after trypsin treatment and median of CTRL, S-143, S-HS888, and S-MG63.

Morphology Observations and Functional Assays

The trypan blue phagocytosis assays in THP-1 cells cultured in RPMI and each OS supernatant resulted positive; suggesting that THP-1 cells had acquired phagocytic capabilities (Figure 2). THP-1 cells cultured in RPMI and each OS supernatant showed positive results to the Acid Phosphatase assay (Figure 3); suggesting possible differentiation into macrophages which were confirmed by the positivity to CD68+ immune-characterization (data not shown). THP-1 cells were adhered to culture plates, aggregated, elongated, and formed pseudopodia followed in a time-dependent manner (Figure 2, Figure 3). The ability of THP-1 cells cultured in supernatants from OS cell lines to phagocytize opsonized particles was enhanced compared to THP-1 control cells. The behavior of THP-1 cells cultured in the presence of OS supernatants resembled that of monocyte-derived macrophages for adherence and phagocytosis.

Table 4: Comparison THP-1 Versus Macrophages [a]

	THP	M0	M1	M2
PLCB1	+	+/-	+	+
PLCB2	+	+	+	+
PLCB3	+	+	+	+
PLCB4	+	+/-	-	-
PLCG1	+	+	+	+
PLCG2	+	+	+	+
PLCD1	+	+	+	-
PLCD3	+	-	+	+
PLCD4	+	-	-	-
PLCE	+	-	-	-
PLCH1	+	+	+	+
PLCH2	+	+	+	+

[a] Comparison of the panel of PLCs expression in THP-1 cells (THP), in unpolarized macrophages (M0), M1, and M2 polarized macrophages (data from Di Raimo et al. [12]).

PLC Expression

The mRNA of 12 PLC enzymes was detected in the THP-1 control cells. Cultured in RPMI-s143B, THP-1 cells did not express PLCD4 and PLCE at the mRNA levels (Table 5). Modifications were measured in the expression of the remaining isoenzymes (Table 5). These modifications resulted in a statistically significant difference for PLCB1and PLCG2 (Table 5, Figure 4). Cultured in RPMI-sHS88, THP-1 cells did not express PLCB1, PLCG2, PLCD4, PLCE, PLCH1, and PLCH2 (Table 5, Figure 4). Modifications were measured in the mRNA expression of the remaining isoenzymes (Table 5). The variations of expression of PLCs resulted in a statistically significant difference between PLCB1 and PLCG2 (Table 5, Figure 4). Cultured in RPMI-sMG63, THP-1 cells did not express PLCB1, PLCD4, PLCE, PLCH1, and PLCH2. Modifications were measured in the expression of the remaining isoenzymes (Table 5, Figure 4). The modifications resulted in a statistically significant difference between PLCB1 and PLCG2 (Table 5).

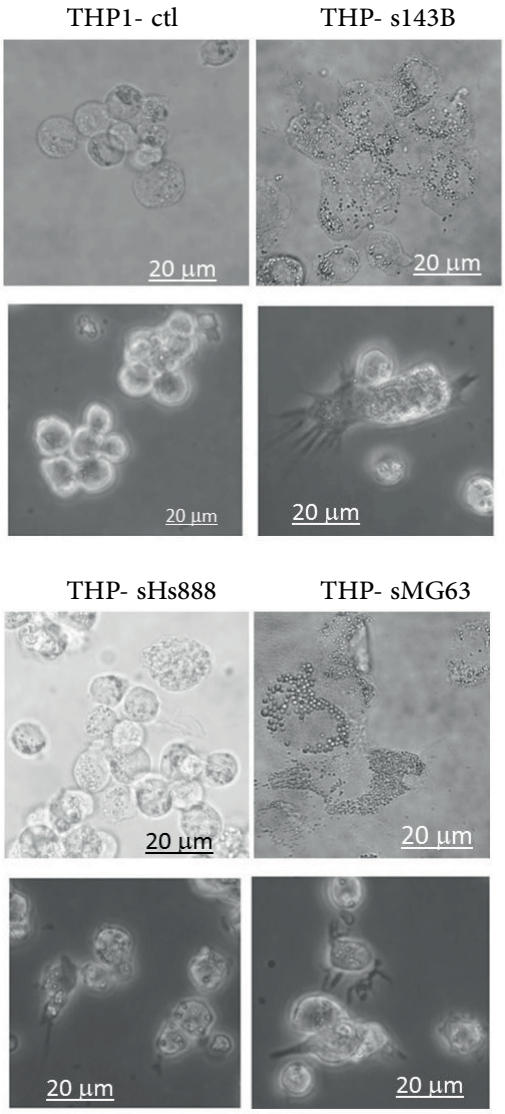
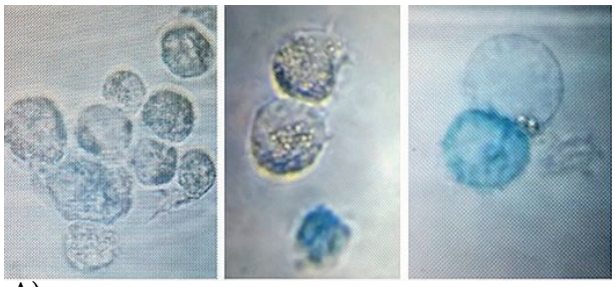
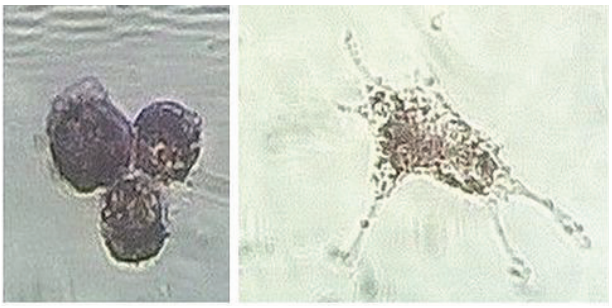


Figure 2: THP-1 Cells Morphology
Optic microscopy. THP-1 cells after 48 hours of culture. THP-1 CTRL: controls grown in RPMI. THP-s143B: THP-1 cells grown in RPMI and supernatant from 143B cells. THP-Shs888: THP-1 cells grown in RPMI and supernatant from hs888 cells. THP-Smg63: THP-1 cells grown in RPMI and supernatant from MG-63 cells (60X).



A)



B)

Figure 3: THP-1 Functional Assays
A) Trypan blue phagocytosis assay after 2 hours. Positive cells (40X); B) Acid Phosphatase enzymatic assay. Positive cells (60X).

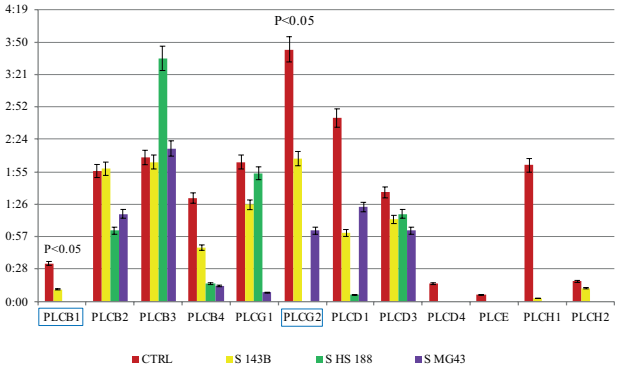


Figure 4: Graphical Representation of the Concentration of PLCs' mRNA in OS Cell Lines
Standard error bars were indicated for each PLC isoform. Isoforms in the box resulted in statistically significant.

DISCUSSIONS

Tissue microenvironment is comprised of factors that directly or indirectly act upon cell behavior via biophysical, biochemical, or other routes [32]. In the present article, we cultured monocyte-macrophage THP-1 cells with culture medium containing supernatant derived from cultures of OS cell lines. We aimed to analyze whether factors released by OS cells within the supernatant could modify the morphology of THP-1 cells and the panel of expression at the mRNA level of PLC enzymes. Many pieces of evidence suggested that the microenvironment can affect the growth, proliferation, and survival of cells in the tissues. Further pieces of evidence suggested that under pathological conditions, additional/abnormal components in the microenvironment, produced as metabolites by pathological cells, might act upon related cells, i.e. inducing modifications in the morphological and functional features of immune system cells [33]. In vitro modifications of the microenvironment by partially replacing the

Table 5: Median Concentration of the Transcripts of PLC Genes (ng/µl) [a, b]

	CTRL	S-143B	S-HS888	S-MG63	P Value [b]			
					CTRL/S-143B	CTRL/S-HS888	CTRL/S-MG63	CTRL/All Treat
PLCB1	0.34	0.11	0	0	0.020	<0.001	<0.001	0.002
PLCB2	1.56	1.58	1.30	0.78	0.983	0.719	0.298	0.859
PLCB3	2.08	1.64	2.96	2.16	0.503	0.361	0.931	0.466
PLCB4	1.32	0.48	0.16	0.14	0.306	0.16	0.182	0.312
PLCG1	2.04	1.26	1.54	0.80	0.064	0.54	0.051	0.232
PLCG2	3.44	1.67	0	0.63	0.009	0.004	0.014	<0.001
PLCD1	2.43	0.61	0.60	0.84	0.085	0.16	0.223	0.152
PLCD3	1.37	0.73	0.78	0.63	0.381	0.651	0.564	0.765
PLCD4	0.16	0	0	0	0.117	0.221	0.221	0.133
PLCE	0.60	0	0	0	0.189	0.308	0.308	0.256
PLCH1	1.61	0.03	0	0	0.392	0.494	0.494	0.624
PLCH2	0.18	0.12	0	0	0.787	0.495	0.495	0.685

[a] Abbreviations: CTRL, untreated THP cells; CTRL/All Treat, comparison of untreated THP and THP treated with overall treatments; S, supernatant from the OS cell line
[b] Statistical analysis of PLCs' in controls compared to THP-1 cultured in OS supernatants. ANOVA test was used to compare concentrations of PLC genes' transcripts.

culture medium with supernatants from different OS cell lines acted upon THP-1 cells. The results of our experiments slightly differed depending on the OS cell line. Partial replacement of culture medium with OS supernatants acted upon the survival and proliferation rate of THP-1 cells. The growth curves indicate the most relevant differences after 48 hours from seeding; suggesting that the effects of OS supernatants last more than one day. Although statistically not significant, modifications were also induced by RPMI-sHS888, which slowly reduced the growth of THP-1 cells for controls after 24 hours (Table 3, Figure 1A). Statistically not significant, modifications were also induced in THP-1 cells by RPMI-sMG63. The number of live and attached THP-1 cells cultured in RPMI and OS supernatant was greater than THP-1 control cells (Table 3, Figure 1); suggesting that THP-1 cells grown in RPMI-OS supernatant become more resistant to trypsin digestion and detachment. Our results suggest that OS supernatants can modify the growth rate of THP-1 cells with the most significant effect played by the supernatant from the 143B cell line. That is not surprising, as 143B cells are known to be highly aggressive and migrating [34].

Microenvironment changes due to OS supernatants modified the morphological and functional features of THP-1 cells, inducing differentiation. The comparison of trypan blue phagocytosis assay in THP-1 cells cultured in RPMI and OS supernatant from each OS cell line demonstrated that THP-1 cells had acquired phagocytic capabilities (Figure 2). Similarly, cultured THP-1 cells also showed positive results to the Acid Phosphatase assay (Figure 3); suggesting their differentiation into macrophages, then confirmed by the positivity to CD68+ immune-characterization (data not shown). Morphology observations and functional tests indicated that THP-1 cultured with RPMI-OS supernatant differentiated into cells behaving similarly to THP-1-derived macrophages. In this perspective, the cytokines and chemokines identified in the supernatants deserve consideration. The content of cytokine/chemokine in each OS supernatant differed; depending on the OS cell line (Table 2). In each OS cell line, we identified IL-2, IL-6, IL-8, TNFα, and GM-CSF. IL-1α and IL-1β were exclusively detected in the supernatant derived from cultures of 143B cells (Table 2). The differences among the cytokines contained in the three supernatants might be related to the different features of OS cells they were derived from. The MG-63 cell line is currently used as an experimental model for human osteoblasts [35]. The 143B cell line and HS888 cell line derives from lung metastases of OS develop osteolytic tumors, sharing migration abilities and aggressiveness [36](Atcc.org). Notably, IL-2 is a central factor in controlling the immune response [37]. The remaining cytokines we identified in the supernatant of OS cell lines bear pro-inflammatory features [37]. GM-CSF and TNFα are known to induce the differentiation of THP-1 into dendritic cells [4, 8]. By contrast, IL-4, which can induce dendritic differentiation of macrophage polarisation, was not detected. Also, IL-17, known to bear regulatory role upon the activity of the immune system [38], was not detected in the supernatant from OS cell lines. The trypan blue phagocytosis assay suggested that THP-1 cells cultured in RPMI-OS supernatants had acquired phagocytic capabilities (Figure 2). Such THP-1 cells also showed positive results to the Acid Phosphatase assay (Figure 3); suggesting their differentiation into macrophages. This hypothesis was confirmed by the positivity to CD68+ immune-characterization (data not shown). THP-1 cells adhered to culture plates and aggregated. Elongation and formation of pseudopodia were detected in a time-dependent manner (Figure. 2, Figure 3) and the ability to phagocytize was enhanced compared to THP-1 control cells. Therefore, the behavior of THP-1 cells cultured in the presence of OS supernatants resembles that of monocyte-derived macrophages for adherence and phagocytosis. That might partially fit with the presence of cytokines such as GM-CSF, TNFα, and IL-1 in the OS supernatants that are known to act upon monocyte-macrophage lineage differentiation [37, 39]. The role of the cytokines in the OS microenvironment and their effect upon tumor growth and spreading will require further studies.

We analyzed the expression of PLC enzymes at the mRNA level which is known to play a role in macrophage differentiation [12]. All PLC isoforms were expressed in THP-1 cells that were grown in RPMI, except for PLCζ which is

exclusively present in the sperm lineage [10]. The presence of OS supernatants in the in vitro microenvironment greatly modified the mRNAs of PLC enzymes expressed by THP-1 cells (Table 1). Partial replacement of RPMI with supernatants from cultured OS cell lines, such as 143B, HS888, or MG-63, reduced the overall mRNAs level of PLCs, with special regard to some isoforms, with the notable exception of PLCB3. The partial replacement of the RPMI culture medium with supernatant obtained from cultures of 143B or MG-63 cells did not modify the expression of PLCB3. Furthermore, PLCB3 expression at the mRNA level increased partially and substituted RPMI with supernatant from HS888 cultures. This observation might accord to previous literature data indicating that the activity of PLCβ3 enzyme is essential to promote macrophage survival [40]. PLCβ3 is expressed in several tissues [10] and constitutes the upstream target of protein kinase Cε, involved in the inflammatory response [41]. Recently, PLCB3 silencing was demonstrated to potentiate the Toll-like receptor inflammatory signaling cascade in cystic fibrosis bronchial epithelial cells that induce IL-8 release [42]. Along with morphological changes, indicating macrophage transition, THP-1 cells cultured in the presence of OS supernatants did not transcribe the mRNAs of PLC genes, depending on the OS cell line. In THP-1 cultures, the supernatant from 143B cell lines reduced the expression at the mRNA level of all PLC isoforms; while PLCD4 and PLCE were not expressed (Tables 1, 4, and 5).

Notably, THP-1 cells, which constitutively express PLCδ4 and PLCε, did not transcribe the corresponding genes when cultured with OS supernatant (Table 1). In our previous work, we described that macrophages, including M0, M1, and M2, did not express PLCD4 and PLCE, both in a physiological state and after inflammatory stimuli [12](Table 4). PLCδ4 is expressed in response to mitogenic stimulation and plays an important role in cell growth and tumorigenesis. PLCε, a peculiar PLC isoform is involved in several signaling pathways, such as Ras and Rho signaling [43]. The administration of OS supernatants to THP-1 cultures seemed to block the expression at the mRNA level of both PLCD4 and PLCE. That might suggest that OS supernatants, as inducing THP-1 to differentiate into macrophages, modify the expression panel of PLCs at the mRNA level, which mutually changes. The different expressions of PLCD4 and PLCE might represent a further marker to discriminate the differentiation into macrophages.

The differences of PLCs expression at the mRNA level induced by adding OS supernatants to the cultures of THP-1 cells resulted in a statistically significant difference for PLCB1 and PLCG2 genes, which codify isoforms belonging to the so-called primary PLCs. PLCβ1 enzyme was demonstrated to be involved in myocyte [44] and adipocyte differentiation [11]. Moreover, PLCβ1 is related to Cyclin D3, which was described to control important signaling pathways in macrophages [45]. PLCγ2 is highly expressed in hematopoietic lineage cells and plays a crucial role in immune responses [46, 47]. Mutations in the PLCG2 gene lead to dominantly inherited auto-inflammatory disease with immunodeficiency [48, 49]. In conclusion our results indicated that supernatants from cultures of OS cell lines contain cytokines/chemokines, which differ depending on the OS cell type. OS supernatants can induce the differentiation of THP-1 cells into macrophages. THP-1 cells cultured in OS supernatants express different panels of PLC enzymes. The expression panel of PLC enzymes differs during the differentiation of monocyte/macrophage lineage THP-1 cells. Further studies are required to elucidate the role of PLCδ4 and PLCε both in the differentiation and in the immune system cells. More pieces of evidence might help to confirm whether the absence of these two PLC enzymes might be used as markers of macrophage differentiation. Selected PLC enzymes are involved in the signal transduction pathways; inducing the production of specific ILs. Inconsistent data are available concerning the effect of ILs on the expression of PLC enzymes. The role of the cytokines in the OS microenvironment, their effect upon tumor growth and spreading, as well as the possible relationships among selected cytokines and PI signal transduction pathway, deserve further observations.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

ETHICS APPROVAL

Not applicable.

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