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Journal of Cellular Biochemistry 115:1138–1146 (2014)

Journal of Cellular Biochemistry

Expression of P2X7 ATP Receptor Mediating the IL8 and CCL20 Release in Human Periodontal Ligament Stem Cells

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ABSTRACT

ATP is released by human periodontal ligament cells (hPDLCs) and has been shown to regulate PDL regeneration and responses to mechanical stress through activation of P2Y receptors. This nucleotide, however, has also been reported to trigger the pro-inflammatory cascade by inducing the maturation and/or release of chemokines/cytokines from various cell types mainly via P2X7 receptors. Much less is known on the possible role of ATP in stem cells deriving from PDL (hPDLSCs) which are considered to be a promising tool for cell-based therapy to restore lesions. Given the role played by P2X7 in pathophysiological conditions, in this study we investigated the expression of P2X7 ATP receptors in hPDLSCs. The results obtained showed that hPDLSCs express P2X7 receptors evaluated by means of cytofluorimetric, immunohistochemistry, reverse transcriptase-PCR, and Western blot analyses. P2X7 ligation by 2′,3′-(benzoyl-4-benzoyl)-ATP (BzATP), a specific receptor agonist, was followed by an increase in intracellular Ca^{2+} and in the uptake of ethidium bromide. These effects were dramatically reduced by oxidized ATP (oATP), the P2X7 irreversible inhibitor, suggesting that the P2X7 is the functional receptor involved. At 24 h treatment of hPDLSCs with BzATP it enhanced the release of the pro-inflammatory agents IL8 and CCL20, without influencing cell viability. These effects were counteracted by pre-treating the cells with oATP or with A-740003, a selective and potent P2X7 competitive antagonist. Collectively, these results indicated that extracellular ATP mediate a pro-inflammatory response via P2X7 receptors in hPDLSCs opening a further approach to control hPDLSCs behavior in their possible application as therapeutic tool. J. Cell. Biochem. 115: 1138–1146, 2014.

KEY WORDS: STEM CELLS; PERIODONTAL LIGAMENT; CYTOKINES; P2X7; INFLAMMATION

Nucleotides, mainly ATP, are known to act as extracellular signaling molecules mediating cell-to-cell interactions by stimulating different specific receptor types.

These include eight P2Y G-protein coupled receptor subtypes (P2Y1, 2, 4, 6, 11, 12, 13, 14) and seven P2X ionotropic receptor subtypes (P2X1–7) [Abbracchio et al., 2006; Jarvis and Khakh, 2009].

In human periodontal ligament (PDL) cells, ATP is released under mechanical stress, and induces the expression of osteopontin via P2Y1/Rho kinase pathway [Wongkhantee and Yongchaitrakul, 2008]. Moreover ATP and ATPγS, a slowly hydrolysable analog, have been reported to modulate cell growth of PDL cells expressing low levels of CD39 [Kawase et al., 2007].

ATP is also released at sites of inflammation, where it can act in a paracrine and autocrine manner mainly by activating P2X receptors, among which the P2X7 subtype a non-desensitizing receptor with the unusual capacity to form membrane pore and to cause membrane permeabilization [Chessell et al., 1997]. P2X7 receptors were firstly recognized in cells of the immune system where it is linked to giant cell formation, degranulation and cytolitic cell death or apoptosis [Di Virgilio et al., 1999; Budagian et al., 2003]. However, in other cell types including glial cells and osteoblasts, these receptors mediate the release of purines and pro-inflammatory factors without causing cell death [Ballerini et al., 1996, 2005; Wei et al., 2008] as well as differentiation and matrix mineralization [Grol et al., 2011; Orriss et al., 2012].

Conflict of interest: none.

Grant sponsor: University of Chieti and Torino PRIN; Grant number: 20102ZLNJ5; Grant sponsor: Ministry of Education University and Research (M.I.U.R.).

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Manuscript Received: 18 December 2013; Manuscript Accepted: 19 December 2013

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 28 December 2013

DOI 10.1002/jcb.24756 • © 2013 Wiley Periodicals, Inc.

The expression of P2X7 receptors has been recently reported in human periodontal ligament cells in which, under mechanical stress, it has been shown to mediate the release of the inflammatory mediator interleukin-1 beta (IL-1β) [Kanjanamekanant et al., 2013].

On the whole, these reports suggest that ATP might influence homeostasis and functions of periodontium, however the expression and activity of ATP receptors in this tissue are still poorly known.

We have recently isolated human mesenchymal stem cells from PDL (hPDLSCs), able to differentiate into osteoblasts and adipocytes and expressing embryonic markers as nanog, OCT-4, SSEA-1, and SSEA-4 [Trubiani et al., 2008, 2010].

The identification of tooth-related stem cells, including those deriving from PDL, has provided the basis for tissue regeneration [Seo et al., 2004] in different pathologies including periodontal disease, which represents a widespread problem of the oral cavity and a potential cause of irreversible destruction of periodontal support tissues.

Although it has been emphasized that paracrine as well as autocrine mechanisms play a crucial role in determining the success of stem cell-based therapies and recent observations indicate that stem cells can spontaneously release ATP, which may be an important factor in determining their functions [Ferrari et al., 2011], few data are still available on the potential role of the purinergic system on stem cell activity.

In this view, the present study reports that hPDLSCs express a functional P2X7 ATP receptor, whose activation is linked to the production of pro-inflammatory molecules IL8 and CCL20.

These findings strengthen the hypothesis that ATP represents an important extracellular signaling molecule also in these cells, where it may become a target for specific therapeutic interventions.

MATERIALS AND METHODS

CELL CULTURES

Human PDL biopsies were carried out after informed consent on five volunteers aged 20–35 years. All subjects were exempt of systemic and oral diseases. Explants were obtained from alveolar crest and horizontal fibers of the PDL by scraping the roots of noncarious third molars with a Gracey's curette as previously reported [Trubiani et al., 2008, 2010]. hPDLSCs were obtained and cultured in MSCM medium (Lonza Verviers Company, Belgium) according to the manufacturer's instructions. Cells migrated from the explants and on Day 7 they were 80–90% confluent as determined by phase contrast microscopy. Adherent cells were isolated using 0.1% trypsin solution and plated in tissue culture polystyrene flasks at 5×10^3 cells/cm^2. hPDLSCs at II passage were used in all experiments.

CYTOFLUORIMETRIC ASSAY

Fluorescein isothiocyanate-conjugated anti-CD13 (CD13 FITC), phycoerythrin-conjugated anti-CD29 (CD29 PE), FITC-conjugated: anti-CD44 (CD44 FITC), anti-CD45 (CD45 FITC), anti-CD105 (CD105 FITC), and anti-CD166 (CD166 FITC) were obtained from Ancell (MN, USA); FITC-conjugated anti-CD14 (CD14 FITC) and PE-conjugated anti-CD133 (CD133 PE) were purchased from Milteny Biotec (Bergisch Gladbach, Germany); PE-conjugated anti-CD73 (CD73 PE), FITC-conjugated anti-CD90 (CD90 FITC), allophycocyanin-conjugated anti-CD117 (CD117-APC), PE-conjugated anti-CD146 (CD146 PE), PE-conjugated anti-CD271 (CD271-PE), Alexa488-conjugated Sox2 (Sox2 Alexa488), FITC-conjugated anti-SSEA4 (SSEA4 FITC) and PE-conjugated anti-OCT3/4 (OCT3/4 PE) obtained from Becton Dickinson (BD, San Jose, CA); FITC-conjugated anti-CD144 (CD144-FITC) was obtained from Acris Antibodies (Herford, Germany); PE-conjugated anti-CD34 (CD34-PE) was purchased from Beckman Coulter (Fullerton, CA, USA); appropriate secondary FITC-conjugated antibody was obtained from Jackson Immunoresearch Laboratories (West Grove, PA, USA).

CELL STAINING FOR FLOW CYTOMETRY

Washing buffer (phosphate buffered saline, PBS; 0.1% sodium azide; and 0.5% bovine serum albumine, BSA) was used for all washing steps (3 ml of washing buffer and centrifugation, 400*g* for 8 min at 4° C). Briefly, 5×10^5 cells/sample were incubated with 100 μl of 20 mM ethylenediaminetetraacetic acid (EDTA) at 37°C for 10 min and washed.

Staining of surface antigens and intracellular antigens was carried out according to Eleuterio et al. [2013].

FLOW CYTOMETRY MEASUREMENT

Quality control included regular check-up with Rainbow Calibration Particles (BD Biosciences). Debris was excluded from the analysis by gating on morphological parameters; 20,000 non-debris events in the morphological gate were recorded for each sample. To assess non-specific fluorescence we used specific irrelevant controls. All antibodies were titrated under assay conditions and optimal photomultiplier (PMT) gains were established for each channel. Data were analyzed using FlowJo™ software (TreeStar, Ashland, OR). Mean Fluorescence Intensity Ratio (MFI Ratio) was calculated dividing the MFI of positive events by the MFI of negative events.

For P2X7 analysis 1×10^6 hPDLSCs were incubated with 16A4, a mouse anti-human P2X7 mAb. Cells were then stained with a PE-conjugated anti-mouse Ig antibody (Becton-Dickinson, San Jose, CA) and analyzed on a FACSCalibur flow cytometer (Becton-Dickinson) using a CellQuest software.

PREPARATION FOR SCANNING ELECTRON MICROSCOPY (SEM)

Cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 30 min at 4°C, post-fixed with 1% osmium tetraoxide, dehydrated in a graded series of ethanol, and immersed in isoamyl acetate, and observed with a Zeiss EVO50 scanning electron microscope.

IMMUNOFLUORESCENCE STAINING AND CONFOCAL LASER SCANNING MICROSCOPE ANALYSIS (CLSM)

hPDLSCs were incubated with the 16A4 anti-human P2X7 and with Alexa Fluor 488 goat anti-mouse Ig(G + M) antibody (Molecular Probes). hPDLSCs were then stained with Alexa Fluor 594 phalloidin probe (Molecular Probes, Invitrogen, Eugene, OR) and the nuclei with TOPRO (Molecular Probes) [Trubiani et al., 2012a]. The samples were observed using a Zeiss LSM510META

confocal (Jena, Germany), connected to an inverted Zeiss Axiovert 200 microscope equipped with a Plan Neofluar oil-immersion objective (40×/1.3 NA). Images were collected using an argon laser beam with excitation lines at 488 nm and a helium–neon source at 543 and 665 nm.

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

Total RNA was extracted (RNeasy Plus Mini Kit; Qiagen, Milan, Italy) according to the manufacturer's directions. cDNA was synthesized from 1 μg of total RNA using the SuperScript III reverse transcriptase kit (Invitrogen; Monza, Italy) with random hexamers. The expected size of the amplification products was 430 bp for P2X7 and 203 bp for GAPDH. The primers used for PCR were: P2X7 forward 5′-TGAAGGGAACCAGAAGACC-3′ and P2X7 reverse 5′-AGTACTTGGC GTATCTGAAG-3′;GAPDH forward 5′-GACATCAAGAAGGTGGTG AAGCAG-3′ and GAPDH reverse 5′-CACCCTGTTGCTGTAGCCAAA TTC-3′. PCR was performed with AmpliTaq-Gold DNA polymerase (Applied Biosystems, Carlsbad, CA). Thermocycling conditions were (a) 1 cycle at 95°C for 2 min, 1 cycle at 95°C for 30 s, annealing for 30 s at 54°C, and extension for 1 min at 72°C for 35 cycles, with a final 5 min extension at 72°C for P2X7 and (b) a step of 2 min at 95°C, followed by denaturation for 30 s at 95°C, annealing for 30 s at 68°C, and extension for 1 min at 72°C for 35 cycles, with a final 5 min extension at 72°C for GAPDH.

WESTERN BLOTTING

Confluent hPDLSCs dishes were washed with PBS, lysed in lysis buffer containing 50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycolate, 1 mM EGTA, 2.5 μl/ml leupeptin, 1 μg/ml aprotinin, and 1 mM PMSF, and centrifuged for 10 min (1,100 rpm), and then the pellet was discarded. Supernatant protein concentration was measured by the method of Bradford, and an aliquot of 100 μg of protein was diluted in Laemmli buffer, heated at 100°C for 5 min. Samples and molecular weight markers were electrophoresed on 7.5% Tris–Glycine SDS–PAGE gels and transferred to PVDF membrane (Bio-Rad Laboratories, Milan, Italy) using a mini trans-blot transfer cell (Bio-Rad Laboratories). After a blocking step using Nonfat dry milk 5% in PBS (PBS and Tween-20 0.1%) for 2 h at room temperature, blots were washed three times in PBS, the membranes were incubated overnight at 4°C with anti-P2X7 (Alomone Labs, Jerusalem, Israel) at the appropriate dilution (1:500). The next day blots were washed and reacted with the secondary antibody anti-rabbit diluted 1:2,000. Immunocomplexes were visualized using the enhancing chemiluminescence detection system (ECL) (Amersham Pharmacia Biotech) and analyzed using a Quantity One software (Bio-Rad).

To determine the equal loading of samples per lane, at the end of each experiment the blots were stripped and re-probed with a rabbit anti-β actin polyclonal antibody (Santa Cruz Biotechnology), according to manufacturer's instructions.

MEASUREMENTS OF CYTOPLASMIC FREE CA^{2+}

Intracellular Ca^{2+} concentration was measured in intact cells (0.5×10^6 ml) using the fluorescent probe fura2/AM (Sigma–Aldrich, Milan, Italy) as described in FP-6200 spectrofluorometer, JASCO*Europe* (Milan, Italy) [Orciani et al., 2009]. The fluorescence intensity was monitored at an emission wavelength of 510 nm, while the excitation wavelength between 340 and 380 nm. The increase in $[Ca^{2+}]_i$ was expressed as the ratio of the fluorescence intensity excited at 340 nm to that of 380 nm (F340/380).

MEASUREMENT OF THE PLASMA MEMBRANE PERMEABILITY

The effects exerted by BzATP on pore functions were measured by means of fluorescent dye ethidium bromide (EtBr) (Sigma–Aldrich). hPDLSCs were resuspended in divalent cation-free basal salt solution and incubated at 37°C in a fluorometer cuvette at a final concentration of 0.5×10^6 cells/ml. EtBr was added to a final concentration of 20 μM. For each assay, a maximum ratio of cell permeabilization/EtBr uptake was defined as the fluorescence value achieved by adding digitonin (20 μg/ml) to the cuvette containing the hPDLSCs suspension.

DETERMINATION OF IL8 AND CCL20 LEVELS

The concentrations of IL8 and CCL20 in cell-free media, collected from cultures after exposing hPDLSCs to 150 μM BzATP for 24 h, were measured by an enzyme immunoassay (R&D System, Minneapolis, MN) according to the manufacturer's directions. The results were evaluated by a SpectraMax® 190 (Molecular Devices).

MTT ASSAY

Cell viability was evaluated by MTT assay as previously reported [Eleuterio et al., 2013]. Briefly, the cells were seeded into flat-bottom 96-well plates (3×10^3/well) in 100 μl medium. After 24 h incubation for attachment and starvation in 1% FBS-MSCBM, the medium was replaced with fresh medium for different time intervals (3, 12, 24, and 48 h). At the designated time, 10 μl MTT (Promega, Milan, Italy) were added to each well and incubated for 3 h. Absorbance at 570 nm was measured with a reference wavelength of 630 nm. The cytotoxicity experiments were conducted independently in triplicate for five experiments.

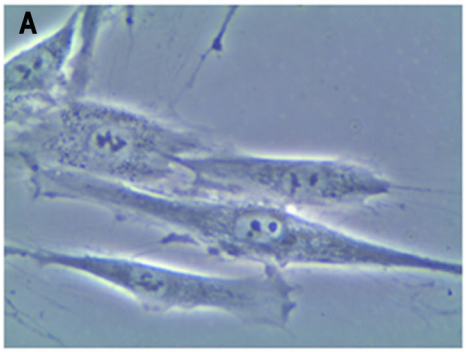
STATISTICAL ANALYSIS

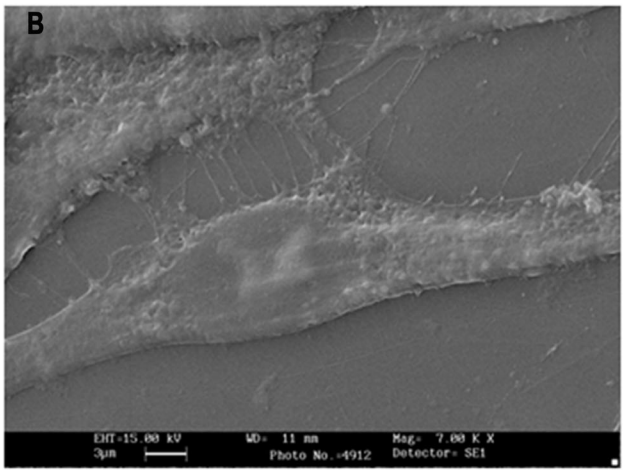
All quantitative results were analyzed by a Student's *t*-test using Graph Pad Prism software. The results were presented as means ± SE. All experiments were performed at least three times. *P* values ≤ 0.05 were considered as statistically significant.

RESULTS

CELL MORPHOLOGY OBSERVATION BY LIGHT AND SCANNING ELECTRON MICROSCOPY

Under light microscopy, cultured hPDLSCs showed a homogeneous fibroblast-like appearance with a stellate shape and long cytoplasmic processes. As usual, the cells adhered to the culture dishes and to each other, forming colonies: the nuclei appeared as round or oval, with one or more nucleoli and a signified secretory apparatus (Fig. 1A). Scanning electron microscopy (SEM) confirmed the fibroblastoid mesenchymal stem cell (MSC) features. At higher magnification, the cytoplasmic processes revealed vesicles of different size and philopodia, overlapping neighboring cells (Fig. 1B).





C

Antigens	Flow cytometry PDLSCs phenotype.																			
	CD13	CD14	CD29	CD34	CD44	CD45	CD73	CD90	CD105	CD117	CD133	CD144	CD146	CD166	CD271	HLA-ABC	HLA-DR	OCT3/4	Sox-2	SSEA-4
	+	-	++	-	++	-	++	+++	+	+/-	-	-	+	+	-	+++	-	+/-	+++	++
MFI Ratio ± SD	26,6±3,3	1,2±0,1	61,6±3,5	1,2±0,1	23,5±1,2	1,2±0,2	41,9±2,6	157,9±9,4	25,7±2,4	4,0±0,6	1,9±0,3	1,3±0,1	11,4±1,1	8,5±0,7	1,4±0,2	121,4±9,8	1,1±0,2	3,7±0,5	104,3±8,1	32,1±2,6

- negative expression; +/- low expression; + moderate expression; ++ positive; +++ high expression; MFI Ratio is the average of five different biological samples ± standard deviation; Cutoff Ratio positivity>2.0.

Fig. 1. Light (A) and Scanning electron microscopy (B) photomicrographs of primary cultures of hPDLSCs, consisting of adherent cells dispersed on the surface of cell culture plates, showing a morphological homogeneous fibroblast-like appearance with a stellate shape, long cytoplasmic processes, and philopodia. The nuclei contain one or more nucleoli. Original magnification (A) 10×, (B) 7,000×. C: Cytofluorimetric analysis of hPDLSCs culture showing the expression of surface molecules (CD13+, CD29+, CD44+, CD105+, CD73+, CD90+, CD146+, CD166+), and of pluripotency associated markers (OCT3/4+, SSEA4+, SOX2+).

FLOW CYTOMETRY ANALYSIS

The homogenous expression of stemness surface molecules (CD13+, CD29+, CD44+, CD105+, CD73+, CD90+, CD146+, CD166+) and of pluripotency associated markers (OCT3/4+, SSEA4+, SOX2+) in hPDLSCs is reported in Figure 1C. As expected the cells were negative for the following markers: CD117, CD133, CD144, CD271, CD14, CD34, CD45 (Fig. 1C). These data confirmed that, as previously reported [Trubiani et al., 2008, 2010; Eleuterio et al., 2013] hPDLSCs used in the present study are of mesenchymal type (MSC). Moreover this pattern of cell surface antigen immunolabeling resulted not to be different among the cells deriving from the tissues obtained by the different donors.

CYTOFLUORIMETRIC AND IMMUNOHISTOCHEMISTRY EVALUATION OF P2X7 RECEPTOR

Indirect immunofluorescence analysis by flow cytometry revealed that P2X7 is localized at the cell surface (Fig. 2A).

Triple-labeled fluorescence preparations of the hPDLSCs analyzed by CLSM confirmed the presence of P2X7 on the cell membrane (Fig. 2B).

EXPRESSION OF P2X7 RECEPTOR IN hPDLSCs

RT-PCR performed with primers specifically designed for P2X7 revealed the presence of a band corresponding to the ionotropic ATP receptor (Fig. 2C). The presence of the P2X7 was also confirmed by Western blotting analysis which showed a major protein band of the expected molecular mass (~70 kDa) (Fig. 2D).

INTRACELLULAR CALCIUM LEVELS ($[Ca^{2+}]_i$) AND ETHIDIUM BROMIDE UPTAKE ARE MODIFIED IN RESPONSE TO P2X7-MEDIATED SIGNALS

BzATP, the most potent and selective P2X7R agonist, was tested for its effect to increase the intracellular calcium levels in hPDLSCs. Figure 3A shows a representative response elicited by BzATP added to the cell suspension at a concentration of 150 µM. This dosage was selected for its potential to increase $[Ca^{2+}]_i$, to elicit the biosynthetic release of cysteinyl leukotrienes and to enhance the expression of P2Y2 mRNA we previously showed in astrocytes cultured from rat brain [Ballerini et al., 1996, 2005; D'Alimonte et al., 2007]. As reported in the figure BzATP induced a rapid rise of $[Ca^{2+}]_i$, followed by a slowly declining value.

$[Ca^{2+}]_i$ increase mediated by the P2X7 agonist was markedly reduced when hPDLSCs were pre-treated (2 h) with oATP (300 µM), a P2X7 antagonist (Fig. 3B). The responses to BzATP treatment in the presence (and in the absence) of oATP are reported in Figure 3C.

The effects exerted by P2X7 on pore formation were measured by means of an EtBr uptake assay (Fig. 3D). The uptake of the fluorescent probe after 5 min exposure to 150 µM BzATP scored approximately 16% of the maximum. The irreversible antagonist oATP (300 µM for 2 h pre-incubation) significantly inhibited the dye uptake as compared to addition of BzATP alone (Fig. 3B). oATP applied alone did not alter the fluorescence levels as compared to the control.

BzATP INCREASES THE IL8 AND CCL20 RELEASE FROM hPDLSCs

LPS-G was recently reported as causing hPDLSCs to increase IL8 and CCL20 release [Trubiani et al., 2012a] (Fig. 4A,B). As expected IL8 and

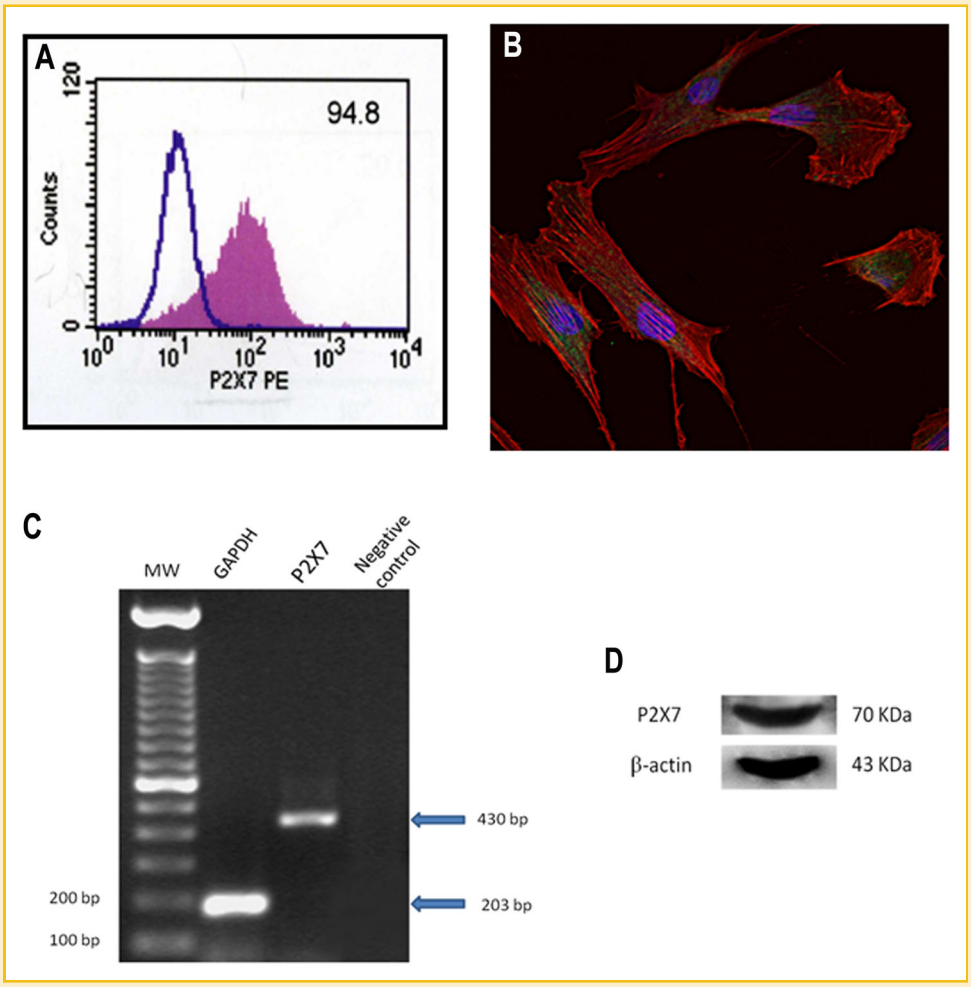


Fig. 2. A: Cytofluorimetric detection of P2X7 in hPDLSCs. Cell surface analysis confirmed that P2X7 is expressed in 94.8% cells. The data are representative of five separate experiments. B: Demonstrative image showing P2X7 immunolocalization in cultured hPDLSCs. The picture shows the cell nuclei in blue (TOPRO), P2X7 protein in green (Alexa 488), and cytoskeleton actin in red (Phalloidin). P2X7 labeling was clearly observed on the cell membrane and diffuse staining was evident in the cytoplasmic structure. Original magnification: 40x. C: Representative RT-PCR for P2X7 in hPDLSCs. The expected size of the amplification products for P2X7 was 430 bp and for GAPDH was 203 bp. No amplification product was found in samples that did not contain template (negative control). Data are representative of the results obtained from RNA of three separate cell preparations. D: P2X7 expression was also evaluated by Western blotting analysis using a specific antibody (anti-P2X7; Alomone Labs, Jerusalem, Israel), which yielded a band of the expected mass (~70 kDa).

CCL20 can be detected in culture supernatants of resting hPDLSCs in the absence of a specific stimulus. This finding indicates that the cells release a small amount of these cytokines in basal conditions. The potential involvement of P2X7 expressed by hPDLSCs in modulating the release of these mediators of the inflammatory response was investigated by means of quantitative immunoenzymatic assay. Treatment with BzATP (150 μM for 24 h) caused increased release of both IL8 and CCL20 (Fig. 4A,B). The release of these cytokines following exposure of these cells to LPS-G (5 μg/ml for 24 h) was considered as a positive control. Pre-treatment (2 h) of hPDLSCs with the P2X7 antagonist oATP (300 μM) significantly reduced the release of IL8 and CCL20 induced by BzATP. Similar results were obtained by using A-740003, a highly selective and potent competitive antagonist for human and rat P2X7 [Donnelly-Roberts et al., 2009].

BzATP MODULATION OF hPDLCs VIABILITY

The results of an MTT assay for estimating cell viability following BzATP exposure are reported in Figure 5. hPDLSCs were stimulated with the P2X7 agonist (30–300 μM) for 3, 12, 24, and 48 h. Control values determined at each time interval were unchanged throughout treatment. Exposure to 150 μM BzATP did not significantly reduce cell viability up to 24 h of treatment. The addition in culture of the agonist BzATP (300 μM) reduced cell viability after only 12 h (Fig. 5).

DISCUSSION

ATP, released after tissue injury and cell death or via activation of regulated release mechanisms, participates in an autocrine as well as paracrine loop with modulatory/regulatory functions. Based on the purinergic receptors involved, strictly related to the activity of a

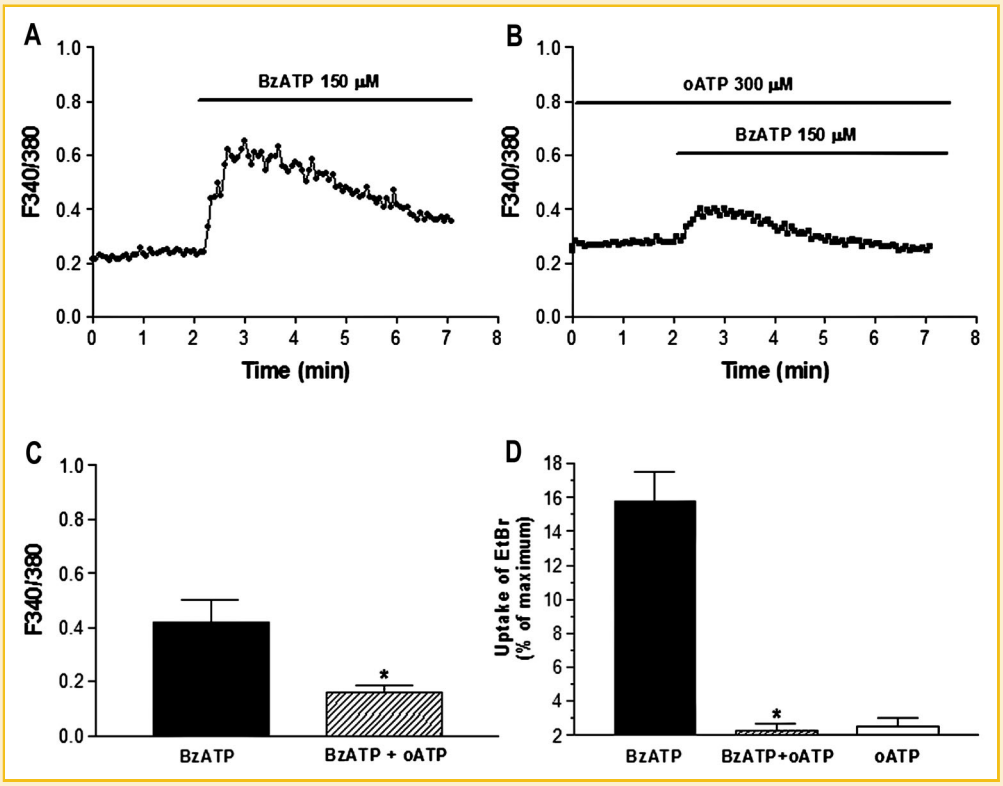


Fig. 3. Increase of intracellular $[Ca^{2+}]_i$ (A–C) and of ethidium bromide (EtBr) uptake (D) upon P2X7 stimulation in hPDLSCs. The upper traces show representative Ca^{2+} responses caused by BzATP (150 μM) alone (A) or (B) in the presence of oATP, a P2X7 antagonist, added at 300 μM for 2 h before BzATP application. C: Ranges in $[Ca^{2+}]_i$ responses for different treatments are shown as the mean ± SE (n = 5 independent experiments for each group; $^*P < 0.05$ vs. BzATP treated hPDLSCs). D: EtBr uptake by hPDLSCs cultures induced by BzATP(150 μM) alone or in the presence of oATP (300 μM) added 2 h before BzATP application. The lack of detectable effect of oATP on EtBr uptake is also shown. Data are reported as the mean ± SE (n = 5 independent experiments for each group; $^*P < 0.05$ vs. BzATP treated hPDLSCs).

complex pool of ecto-enzymes which control the extracellular formation of adenosine, ATP, via P2 receptors mainly P2X7 subtype, has been reported to mediate pro-inflammatory effects while the nucleoside, via P1 receptors, to induce anti-inflammatory responses.

Although there is a growing body of evidence indicating the importance of stem cell-mediated paracrine effects in the mechanisms underlying tissue repair and regeneration, the contribution given by the purinergic system in this process is still poorly known.

The attention on P2X7 was suggested by its multiple functional roles. It was, initially, thought that only high levels of ATP outside the cells could activate the receptor, making it controversial as to whether it could play a pathophysiological role in vivo. However, more recent data indicate that ATP is released from cells at concentrations sufficient to activate P2X7 [Pellegatti et al., 2005]. Another report supporting this view shows that signals mediated by pattern recognition receptors (including TLR4) induce human monocytes to release ATP which mediates an autocrine stimulation of P2X7, and causes the maturation and the secretion of pro-inflammatory cytokines (e.g., IL1 and IL18) [Piccini et al., 2008].

To our best knowledge the expression of P2X7R has not yet been reported in hPDLSCs. Cellular characterization showed that the primary cultures from PDL used in the present study exhibited the expression of embryonic and mesenchymal markers and a fibroblast-like morphology after in vitro expansion as previously described [Eleuterio et al., 2013].

The first step in the analysis of P2X7 in these cells was the evaluation of the presence of the specific mRNA and the expression of the receptor on the surface of hPDLSCs, performed following multiple approaches.

Our results provide evidence for the expression, at both mRNA and protein level, of P2X7 receptor in this kind of stem cells.

P2X, including P2X7, are membrane cation channels which, once gated by ATP, causes Ca^{2+} and Na^{+} influx as well as K^{+} efflux. As expected the stimulation of PX7 receptor in cultured hPDLSCs with BzATP, the most potent P2X7 agonist, induced a rapid increase of $[Ca^{2+}]_i$.

Activation of P2X7 receptor has also been linked to other functional responses including pore formation in dye loading protocols. We have shown that the stimulation of hPDLSCs with the P2X7 agonist is linked to a membrane permeabilization leading to the influx of EtBr.

Both the effects reported above were confirmed by the use of P2X7 receptor antagonist oATP.

Even though rapid changes in ionic currents and membrane permeabilization are the best characterized cellular responses following the activation of P2X7, this receptor is known to be

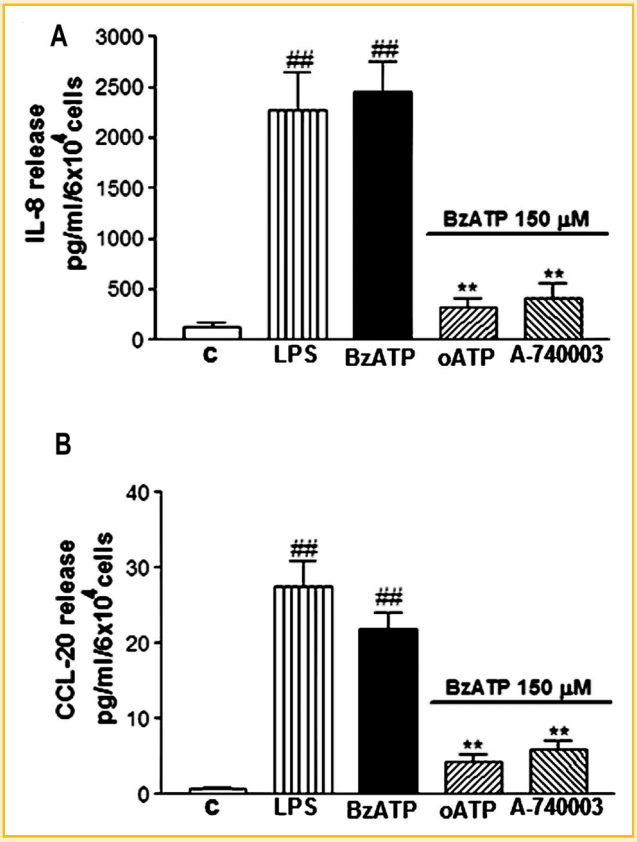


Fig. 4. Involvement of P2X7 in the release of (A) inteleukin8 (IL8) and (B) CC chemokine ligand 20 (CCL20) from hPDLSCs. hPDLSCs cultures were treated with BzATP (150 μM) for 24 h alone or in the presence of the P2X7 receptor antagonists oATP (300 μM) or A-740003 (0.1 μM) added 2 h or 30 min before BzATP application, respectively. LPS-P.Gengivalis (LPS-G) added at 5 μg/ml for 24 h was used as positive control. The IL8 and CCL20 levels in cell-free culture supernatants were measured using an ELISA, as described in Materials and Methods section. Each value represents the mean ± SE of three independent experiments performed in triplicate. $^{\#\#}P < 0.005$ significantly different from the untreated hPDLSCs (control), $^{**}P < 0.005$ significantly different from the BzATP treated hPDLSCs.

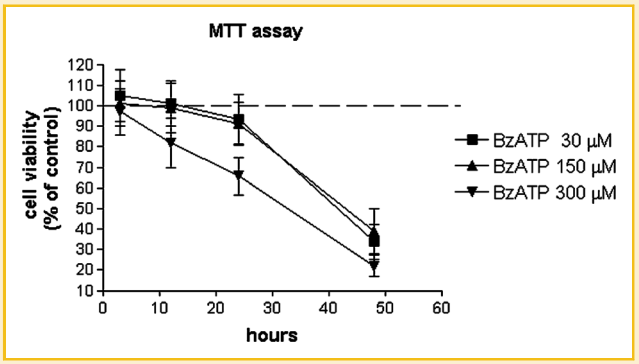


Fig. 5. hPDLSCs were treated with BzATP at various concentrations (30–150–300 μM) for 3, 12, 24, and 48 h. Cell viability was measured by MTT assay and expressed as percentage of untreated control. Each value represents the mean ± SE of five independent experiments performed in triplicate $^{*}P < 0.05$ versus untreated cells. Absorbance at 570 nm was measured with a reference wavelength of 630 nm.

involved in several immune functions including the killing of intracellular bacteria and the release of pro-inflammatory cytokines.

The addition of BzATP to our hPDLSCs cultures (in concentrations not modifying cell viability) was followed by a marked increase in the secretion of IL8 and CCL20. These effects were dramatically reduced when cells were pre-treated with the P2X7 antagonist oATP. However, it has been reported that oATP may exert anti-inflammatory effects independently from P2 receptor-mediated mechanisms [Beigi et al., 2003]. A-740003, a more selective and potent competitive antagonist of these ATP receptor sites [Donnelly-Roberts et al., 2009], has been also used. A-740003 markedly reduced the BzATP-mediated overproduction of the above-mentioned cytokines, thus confirming a role of P2X7 in this mechanism.

The production of IL8 is increased in oral stem cells in response to different stimuli [Trubiani et al., 2012a,b]. Roles of IL8 in enhancing bone resorption and stimulating angiogenesis [Tartour et al., 2011] are known. In periodontitits, IL8 levels are higher than those observed in healthy individuals when measured in the crevicular fluid and periodontal tissues and they seem to parallel disease progression [Gramonal et al., 2000].

CCL20 is a chemokine that exerts its activity through the interaction with CCR6, a specific chemokine receptor. The CCL20/CCR6 complex plays a crucial role in trafficking and homing of T lymphocytes (including T helper-17), a primary source of RANK ligand in periodontal disease [Cardoso et al., 2009] and of leucocytes into inflammatory sites in different pathological conditions, including periodontitis [Hosokawa et al., 2002].

The conclusion of this study is that hPDLSCs express a functional P2X7, whose activation leads to the production of pro-inflammatory and angiogenic factors. The intracellular mechanisms underlying the effects observed in hPDLSCs after P2X7 activation are not known at present and are under investigation.

Pre-treating cells with oATP or A-740003 reduced, but not abolished the observed effects on $[Ca^{2+}]_i$ or the cytokine release mediated by P2X7 agonists. These findings suggest that P2X7 may be one of the players in the purinergic system controlling these biological events and it is reasonable to assume that other P2 receptors are involved. Another possibility to be considered deals with the roles exerted by the ecto-enzymes involved in the metabolism of the extracellular nucleotides which influence the interplay among released ATP and purinergic receptors [Malavasi et al., 2010; Serra et al., 2011].

The absence or the inhibition of P2X7 is followed by a less severe outcome in chronic inflammatory diseases and at the same time it enhances a functional recovery [Carroll et al., 2009]. This makes P2X7 an emerging pharmacological target as shown by the growing interest in new molecules able to selectively antagonize its mediated effects such as CE-224535 and EVT (currently undergoing phase I or II clinical trials).

Thus, given the recognized importance of the contribution of stem cells in the control of microenvironment where they engraft when used for tissue repair therapies, a study of the effects mediated by

P2X7 in hPDLCs cells, flanked by further analysis of the roles played by the purinergic system and its related ecto-enzymes in this tissue might open new pharmacological approaches in the treatment of periodontal disease [Stock et al., 2012].

ACKNOWLEDGMENTS

This work has been supported from ex 60% University of Chieti and Torino, and partly by PRIN 20102ZLNJ5 "Stem cells and 3D scaffolds: a novel construct in bone regeneration" financed by the Ministry of Education, University and Research (M.I.U.R.), Rome, Italy.

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