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Expression of a $P2X_7$ Receptor by a Subpopulation of Human Osteoblasts

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ABSTRACT

There is now conclusive evidence that extracellular nucleotides acting via cell surface P2 receptors are important local modulators of bone cell function. Multiple subtypes of P2 receptors have been localized to bone, where their activation modulates multiple processes including osteoblast proliferation, osteoblast-mediated bone formation, and osteoclast formation and resorptive capacity. Locally released nucleotides also have been shown to sensitize surrounding cells to the action of systemic factors such as parathyroid hormone (PTH). In nonskeletal tissue recent attention has focused on one particular P2 receptor, the $P2X_7$ receptor (previously termed P2Z), and its ability to form nonselective aqueous pores in the plasma membrane on prolonged stimulation. Expression of this receptor originally was thought to be restricted to cells of hemopoietic origin, in which it has been implicated in cell fusion, apoptosis, and release of proinflammatory cytokines. However, recent reports have indicated expression of this receptor in cells of stromal origin. In this study, we investigated the expression of the $P2X_7$ receptor in two human osteosarcoma cell lines, as well as several populations of primary human bone–derived cells (HBDCs) at the levels of messenger RNA (mRNA) and protein. We found that there is a subpopulation of osteoblasts that expresses the $P2X_7$ receptor and that these receptors are functional as assessed by monitoring ethidium bromide uptake following pore formation. Inhibition of delayed lactate dehydrogenase (LDH) release in response to the specific agonist 2′,3′-(4-benzoyl)-benzoyl-adenosine triphosphate (BzATP) by the nonspecific P2X receptor antagonist PPADS confirmed a receptor-mediated event. After treatment with BzATP SaOS-2 cells exhibited dramatic morphological changes consistent with those observed after $P2X_7$-mediated apoptosis in hemopoietic cells. Dual staining with terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate–biotin nick end labeling (TUNEL) and a $P2X_7$-specific monoclonal antibody confirmed the induction of apoptosis in osteoblasts expressing the $P2X_7$ receptor. These data show for the first time the expression of functional $P2X_7$ receptors in a subpopulation of osteoblasts, activation of which can result in ATP-mediated apoptosis. (J Bone Miner Res 2001;16:846–856)

Key words: **osteoblasts, $P2X_7$, 2′,3′-(4-benzoyl)-benzoyl-adenosine triphosphate, immunocytochemistry, apoptosis**

INTRODUCTION

THE CONCEPT that adenosine triphosphate (ATP) can act as an extracellular signaling molecule via interactions with specific purinergic receptors and mediates a wide variety of processes as diverse as neurotransmission,[1] inflammation,[2] apoptosis,[3] and bone remodeling,[4,5] is now widely accepted. Since the early work of Burnstock et al.,[6] the number of characterized P2 receptors responsive to extracellular nucleotides has increased dramatically. These

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P2 receptors originally were subdivided into two families, P2X and P2Y, based on their pharmacologic profile and mechanism of signal transduction. However, after the recent cloning of genes encoding individual P2 receptors, the basis for subdivision has been superseded by the molecular structure of receptors.[7] The ionotropic P2X receptors are ligand-gated cationic channels, while the metabotropic P2Y receptors form a distinct family of G-protein coupled receptors.[7] The P2 receptor family has widespread tissue distribution and currently is comprised of seven distinct P2X receptors and five distinct P2Y receptors.

The cloning of the $P2X_7$ receptor (previously termed P2Z) has generated considerable interest in this atypical P2X family member.[8] The $P2X_7$ receptor is related structurally to the other members of the P2X family, and as such, when stimulated briefly with low concentrations of agonist, this receptor acts as a nonselective cationic channel. However, when stimulated repeatedly or for prolonged periods with higher agonist concentrations, the $P2X_7$ receptor shows the unique ability among members of the P2X family to form large aqueous pores in the plasma membrane, permeable to hydrophilic molecules as large as 900 Da.[9] Originally, expression of the $P2X_7$ receptor was thought to be restricted to cells of hemopoietic origin, in which receptor activation has been implicated in mediating aspects of the immune response. As such, in either macrophages or microglial cells, $P2X_7$ receptor levels are functionally upregulated by lipopolysaccharide (LPS) or interferon-γ,[10] and stimulation of the receptor leads to the release of mature interleukin-1β (IL-1β).[11] Recent reports have indicated that cells of fibroblastic origin also express the $P2X_7$ receptor,[12] in which its activation may represent a novel pathway for fibroblast activation and their recruitment in the inflammatory response, by triggering release of the proinflammatory cytokine IL-6.[13] However, the best-recognized consequence of prolonged $P2X_7$ receptor activation and pore formation in hemopoietic cells is that of ATP-induced cell death.[14]

Osteoblasts and osteoclasts express multiple P2 receptor subtypes,[15,16] with receptor profile seemingly a function of cellular differentiation.[17] Consistent with this, locally released nucleotides have been shown to have wide ranging and often opposing roles in bone remodeling.[4,5] In osteoblastic cells, extracellular nucleotides induce large transient elevations of intracellular Ca^{2+}, consistent with an increase in inositol 1,4,5-triphosphate.[18] Functional consequences of P2 receptor activation in osteoblasts include induction of the immediate-early gene c-*fos*,[19] an important regulatory gene in skeletal cells,[20,21] stimulation of proliferation,[22] and inhibition of bone formation.[4] One striking consequence of P2 receptor activation in osteoblasts is the potentiation of parathyroid hormone (PTH)–induced responses such as Ca^{2+} signaling[23] and c-*fos* gene expression.[24] These observations and the fact that nucleotides accumulate at sites of inflammation and injury may suggest a role for nucleotides as paracrine regulators of bone cell function in diseases such as rheumatoid arthritis. Similarly, the observations that osteoblasts can constitutively release nucleotides via a nonlytic mechanism and that this release can be modulated positively by shear stress[25] also would imply that an autocrine/paracrine signaling mechanism involving nucleotides may contribute to the physiological response of skeletal tissue to mechanical stimuli. In osteoclasts extracellular nucleotides have been shown to induce elevations of intracellular Ca^{2+}, increase the formation and resorptive activity of osteoclasts,[5] and induce apoptosis.[26] Indeed, we have previously reported that expression of the $P2X_7$ receptor messenger RNA (mRNA) is found in human osteoclastoma tissue and human osteoclastoma stromal cells, and that the specific $P2X_7$ receptor agonist 2′,3′-(4-benzoyl)-benzoyl ATP (BzATP) potently inhibits resorption by a population of human giant cells seeded onto dentine wafers by inducing apoptosis.[27] However, in the absence of specific P2 receptor antagonists it is still unclear which P2 receptors specifically couple to which remodeling processes.

In this study we have investigated $P2X_7$ receptor expression at the mRNA and protein level in two clonal osteosarcoma cell lines, as well as populations of primary human bone–derived cells (HBDCs). In addition, we have assessed receptor function in osteoblasts by monitoring pore formation in response to the potent $P2X_7$ receptor agonist BzATP and linked changes in cellular morphology resulting from receptor activation to the induction of apoptosis.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), RPMI medium, fetal calf serum (FCS), and Superscript II reverse transcriptase (RT) were purchased from Gibco Life Technologies (Paisley, Scotland). *Taq* DNA polymerase was purchased from Advanced Biotechnologies (Epsom, UK). The digoxigenin (DIG) RNA labeling kit, restriction enzymes, deoxynucleoside triphosphates (dNTPs), RNAse inhibitor, and In Situ Cell Death Detection Kit were from Roche Diagnostics (Lewes, UK). NTPs and oligo(dT) were purchased from Pharmacia (St. Albars, UK). Hybond N hybridization membrane was purchased from Amersham (Little Chalfont, UK). Nucleotides, ethidium bromide, Hoechst 33258, and tetramethylrhodamine isothiocyamate (TRITC)-conjugated phalloidin were obtained from Sigma (Poole, UK). Cytotox-96 nonradioactive cytotoxicity assay kit was purchased from Promega (Madison, WI, USA). Vectastain ABC kit was from Vector Laboratories (Burlingame, CA, USA). The monoclonal $P2X_7$ antibody was a kind gift from Dr. Iain Chessell, Glaxo-Wellcome, Cambridge, UK.

Cell culture

HBDCs were isolated and cultured from explants of human bone as previously described.[28] In brief, specimens of human bone were finely minced with a scalpel and then washed free of marrow cells with several volumes of medium. The minced bone was cultured in 9-cm petri dishes containing DMEM supplemented with 10% FCS, 100

μg/ml of streptomycin, 100 U/ml of penicillin, and 2 mM of L-glutamine. The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 until required for assay or preparation of total RNA. SaOS-2 and Te85 cells were maintained in DMEM supplemented as above. THP-1 cells were maintained in suspension culture in RPMI medium supplemented as done previously. As with HBDC, the cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 until required for assay or preparation of total RNA.

RNA isolation and complementary DNA synthesis

Total RNA was isolated using the guanidine isothiocyanate method.[29] Before first-strand complementary DNA (cDNA) synthesis, RNA was DNAse-treated with RNAse-free DNAse 1 (35 U/ml) for 60 minutes at 37°C, and phenol-chloroform was extracted. Five micrograms of DNAse-treated total RNA was used as template for first-strand cDNA synthesis in a 20-μl reaction containing 0.5 mM of dNTPs, 0.5 μg of oligo(dT), 20 U of RNAse inhibitor, 10 mM of dithiothreitol, 6 mM of $MgCl_2$, 40 mM of KCl, 50 mM of Tris-HCl, pH 8.3, and 200 U of Superscript II RT. The reaction mix was incubated at 42°C for 50 minutes, and the reaction was stopped by heating at 70°C for 15 minutes. cDNA was stored at −20°C until required.

Polymerase chain reaction

Fifty-microliter polymerase chain reactions (PCRs) were carried out containing 2 μl of the previously prepared cDNA as template, 1 U of thermostable DNA polymerase, 50 pmol of each sense and antisense primer (see the following list), 0.2 mM each of deoxy-ATP (dATP),deoxycytosine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP), and 1.5 mM of $MgCl_2$ in 1× reaction buffer. Amplification conditions were as follows: denaturation step at 94°C for 2 minutes, followed by 35 cycles at 94°C for 15 s, at 56°C for 30 s, at 72°C for 60 s, and a final 10-minute extension step at 72°C. The following is a list of the primers:

(1) $P2X_7$ sense = TGAAGGGGATAGCAGAGGTGA
(2) $P2X_7$ antisense = TGGGATGGCAGTGATGGA
(3) Nested $P2X_7$ sense = AAGGCCAAGAGCAGCGGTTGT
(4) Nested $P2X_7$ antisense = GCCCTGAATTGCCACATCTGAAA
(5) *gapdh* sense-GGTGAAGGTCGGAGTCAACGG
(6) *gapdh* antisense-GGTCATGAGTCCTTCCACGAT

DIG Riboprobe production and Southern analysis

A 553-base pair (bp) PCR product, amplified from macrophage cDNA using primer pairs 1 and 4 was confirmed by sequencing to be a fragment of the human $P2X_7$ receptor. The 553-bp PCR product was then cloned into pBluescriptII KS using standard procedures.[30] A DIG-labeled riboprobe was then generated by in vitro transcription as previously described.[31] The PCR products were electrophoresed on a 1% agarose gel and transferred to a Hybond N membrane according to the manufacturer's instructions (Amersham). The membrane was hybridized with 100 ng/ml of $P2X_7$ riboprobe in DIG Easy Hyb overnight. After hybridization, the membrane was washed stringently in 0.1% SSC/0.1% sodium dodecyl sulfate (SDS), and detected using disodium 3-(4-methoxyspiro(1,2-dioxetane-3,2′-(5′-chloro)tricyclo-[3.3.1.1^{3,7}decan]-4-yl)phenylphosphate (CSPD), again according to the manufacturer's instructions (Roche Diagnostics).

Immunocytochemistry

SaOS-2, Te85 and HBDC were seeded onto glass coverslips and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 overnight. The media was removed and cells were washed in phosphate-buffered saline (PBS) before fixation by immersion in 3.7% formalin in PBS (PBSF) for 10 minutes. The slides were then washed in PBS and incubated for 20 minutes in blocking serum. Slides were incubated for 1 h in either 10 μg/ml of monoclonal $P2X_7$ antibody[32] or PBS. The slides were washed in PBS, and the immunocomplexes were visualized using the ABC reagent and diaminobenzidine (DAB) substrate according to the manufacturer's protocol (Vector Laboratories and Sigma, respectively). The sections were subsequently counterstained with hematoxylin.

Lactate dehydrogenase measurements

Cells were seeded at equal density into 96-well plates and grown to 75% confluence. The media was replaced with serum-free media overnight so cells would become quiescent. Antagonists were added 2 h before agonists. Treated cells were then incubated for the required time period, after which time the media was removed and assayed for LDH activity using the CytoTox96 nonradioactive cytotoxicity assay kit from Promega. To obtain total LDH values, control cells were lysed in 1% Triton X-100. Values are expressed as percentage of LDH released and represent the fraction of LDH activity found in the supernatants with respect to the total LDH value for control cells.

Ethidium bromide uptake

Subconfluent quiescent cells were trypsinized immediately before assay and resuspended in high potassium salt (HKS) media (125 mM KCl, 1 mM EDTA, 5 mM glucose, and 20 mM HEPES, pH 7.4) containing ethidium bromide to a final concentration of 100 μM and appropriate concentrations of agonist. Cells were then incubated at 37°C with agitation for 60 minutes, after which time the reaction was terminated by the addition of 0.5 ml of ice-cold 5 M NaCl. Ethidium bromide uptake was assessed by monitoring fluorescence intensity using a Flow Cytometer from Coulter Corp (Miami, FL, USA).

Morphological observations

Morphological changes in cells treated for LDH assays were analyzed by phase-contrast microscopy. For immunofluorescence analysis, cells were settled onto coverslips and treated similarly. Cells were subsequently fixed in 3.7% PBSF for 10 minutes, washed in PBS-Triton X-100 (0.1%), and incubated at room temperature for 1 h with the DNA-specific fluorochrome Hoechst 33258 (1 μg/ml) and phalloidin-TRITC (4 ng/ml) to visualize the actin cytoskeleton. The terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate–biotin nick end labeling (TUNEL) staining was performed in accordance with the manufacturer's protocol (Roche Diagnostics). The cells were then analyzed by epifluorescence using a Leitz fluorescence microscope coupled to a Princeton Instruments Micromax CCD camera and captured using Metamorph (Universal Imaging Systems, Inc., West Chester, PA, USA). Images were converted and processed identically using Adobe Photoshop.

Statistical analysis

Statistical analysis was by analysis of variance (ANOVA) or randomized ANOVA; the significance between groups was determined using a Tukey posttest. Logistic curve fitting was performed with standard Excel software and a modified form of the Hill equation.

RESULTS

Expression of P2X$_7$ receptor mRNA by SaOS-2 and primary HBDCs

Osteoblasts have been described to express multiple P2 receptors,[15] and because of the recent report that cells of stromal origin express the P2X$_7$ receptor,[12] we used RT-PCR to investigate whether P2X$_7$ receptor transcripts were expressed by the human osteosarcoma cell lines SaOS-2 and Te85, as well as populations of primary osteoblastic cells derived from explants of human bone. First-round RT-PCR using P2X$_7$-specific primer pair 1 and 2 generated the expected 620-bp amplification product in bone cells, albeit to lower levels than the THP-1–positive control (Fig. 1A). Reamplification of the first-round PCR products with the nested P2X$_7$ primer pair 3 and 4 generated the expected 412-bp amplification product (Fig. 1B). The housekeeping gene *gapdh* was used to confirm the integrity and equal loading of cDNA in each sample (primer pairs 5 and 6; Fig. 1C). Genomic DNA, water, and the *gapdh* PCR product served as negative controls for the P2X$_7$ amplification reactions. The PCR products hybridized strongly with a P2X$_7$ DIG-labeled riboprobe showing that they arose specifically from P2X$_7$ cDNA.

Immunolocalisation of P2X$_7$ receptors

To confirm that the P2X$_7$ receptor mRNA levels described previously reflect receptor protein expression, we performed immunocytochemistry on the same cells using a specific P2X$_7$ monoclonal antibody. In SaOS-2 cells a distinct subpopulation of cells expressed high levels of the P2X$_7$ receptor (Fig. 2A, black arrows), and two populations of HBDC tested showed varying degrees of receptor expression (Figs. 2C and 2D). Interestingly, we were repeatedly unable to detect receptor expression in populations of Te85 cells (Fig. 2B). No staining was observed when the primary antibody was omitted or

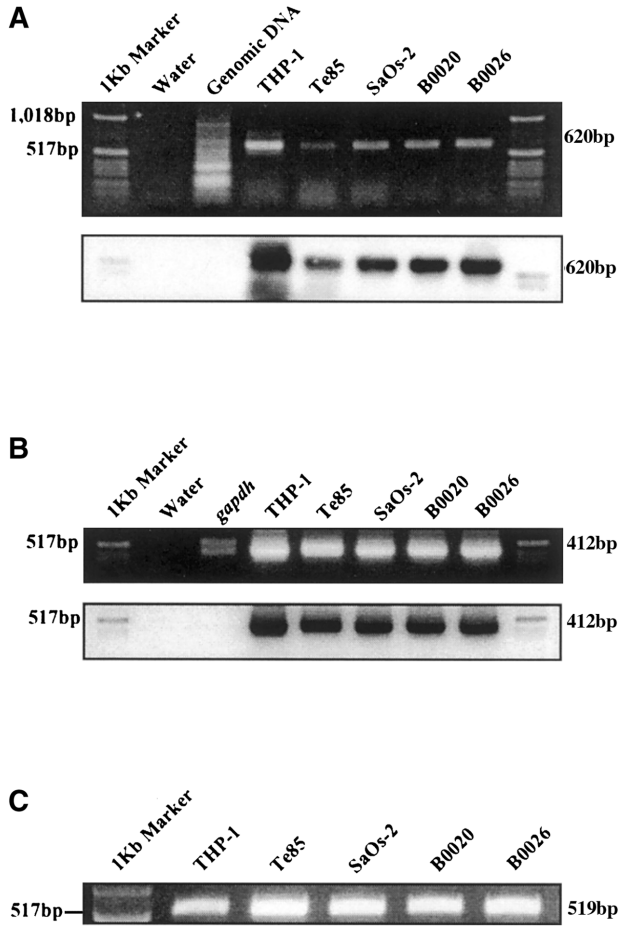

FIG. 1. P2X$_7$ mRNA expression in human bone cells. (A, top panel) RT-PCR analysis of P2X$_7$ mRNA in THP-1 cells, SaOS-2, and Te85 osteosarcoma cell lines and two populations of HBDCs, labeled B0020 and B0026. Thirty-five rounds of amplification with P2X$_7$ primer pair 1 and 2 yielded an amplification product of the predicted size of 620 bp for all cDNAs tested, unlike genomic DNA and water. (bottom panel) Southern analysis of the amplification products shown in the top panel. The membrane was probed with a DIG-labeled riboprobe corresponding to a 553-bp fragment of the human P2X$_7$ receptor to confirm product specificity. (B, top panel). Reamplification of the PCR products with nested P2X$_7$ primer pair 3 and 4 yielded the predicted product of 412 bp, unlike the *gapdh* product amplification reaction. (bottom panel) Corresponding Southern analysis of amplification products as described in panel A. (C) PCR amplification with *gapdh*-specific primer pair 5 and 6 yielded the predicted product of 519 bp for all cDNA tested, confirming integrity and equal loading of cDNA in each sample. Sizes on left refer to marker bands.

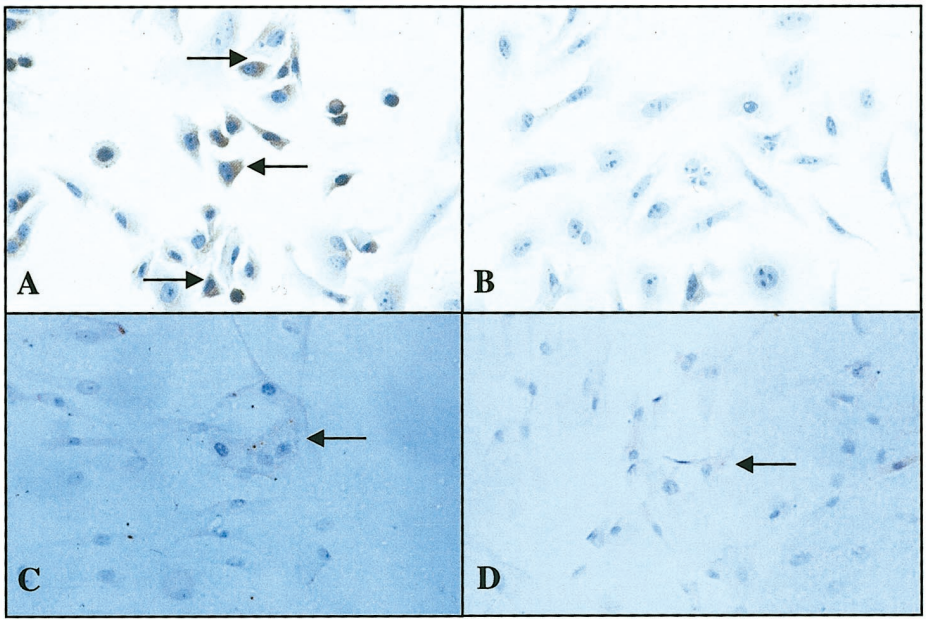

FIG. 2. Immunocytochemical localization of $P2X_7$ receptor in human osteoblasts. SaOS-2 cells, Te85 cells, and HBDCs cultured on glass coverslips were fixed in PBSF and incubated with a monoclonal antibody raised against the $P2X_7$ receptor (10μg/ml). Immunocomplexes were revealed using ABC/DAB staining, and the sections were counterstained with hematoxylin. (A) A distinct subpopulation of SaOS-2 cells, indicated by black arrows, shows $P2X_7$ receptor immunostaining. (B) Te85 cells show no $P2X_7$ receptor immunostaining. (C and D) Two populations of primary HBDCs show varying degrees of $P2X_7$ receptor immunostaining. Original magnification $\times$400 in all panels.

when a control osteoclast–specific antibody was used (data not shown).

$P2X_7$ receptor-induced responses in osteoblasts

A major consequence of $P2X_7$ receptor activation in hemopoietic cells is the formation of large nonselective membrane pores and induction of apoptosis. Therefore, we investigated the responses elicited by osteoblasts after $P2X_7$ receptor agonist treatment. We used several criteria, including the delayed release of the cytoplasmic enzyme LDH, the uptake of the fluorescent dye ethidium bromide, and apoptotic morphological changes.

SaOS-2 and Te85 cells were treated with the potent $P2X_7$ receptor agonist BzATP, the putative partial $P2X_7$ agonist ATP, and the ineffective $P2X_7$ agonist uridine triphosphate (UTP), all at a concentration of 1 mM for 24 h. The $P2X_7$ agonists significantly induced LDH release in SaOS-2 cells, whereas UTP had no effect on LDH release (Fig. 3A). The nonspecific antagonist PPADS,[33] at a concentration of 30 μM, significantly inhibited LDH release from SaOS-2 cells induced by 1 mM of BzATP, 0.5 mM of BzATP, and 1 mM of ATP by 50, 56, and 75%, respectively. In Te85 cells, a miniscule effect on LDH release was observed with BzATP, which was slightly larger with ATP and UTP. Pretreatment with PPADS appeared to augment rather than inhibit this release (Fig. 3B).

LDH release has been used classically as a marker of cytotoxicity, in which the early damage of the plasma membrane would result in the fast release of the cells' cytosolic components into the culture medium. To confirm that the release of the LDH after BzATP treatment occurred because of slow release via the $P2X_7$ receptor pores and/or because of secondary necrosis accompanying $P2X_7$ receptor–induced apoptosis as opposed to general agonist cytotoxicity, a time course for LDH release was performed. Only after 24 h did 100 μM of BzATP induce LDH release (85% relative release at 24 h compared with 2% and 10% at 6 h and 12 h, respectively), while 1 mM of BzATP induced 20% and 37% relative LDH release from SaOS-2 cells at 6 h and 12 h, respectively, with the maximal release of 87% occurring at 24 h (Fig. 3C). Te85 cells showed no increased release of LDH at any of the time points tested (data not shown).

To further assess pore formation, SaOS-2 and Te85 cells were treated with BzATP, ATP, and UTP in a low divalent ion solution containing ethidium bromide for 60 minutes at 37°C. Flow cytometry revealed increased uptake of ethidium bromide in a subpopulation of agonist-treated SaOS-2 cells (Fig. 4A). In contrast, no significant ethidium bromide uptake was observed in Te85 cells studied (Fig. 4A). Although the response observed for individual HBDC populations was somewhat variable (Fig. 4B), statistical analysis of the mean data (randomized ANOVA) showed that BzATP significantly increased ethidium bromide uptake in HBDC (Fig. 4C). A typical $P2X_7$ receptor agonist potency order curve was obtained for a further HBDC population B0023. As with the cell line THP-1, which is known to homogeneously express high levels of the $P2X_7$ receptor, BzATP was a more potent agonist than ATP (note the right shift of the response curve from BzATP to ATP). The maximal response of B0023 to BzATP was 60%, indicative of a subpopulation expressing the $P2X_7$ receptor. B0023 $P[A]_{50}$ values were BzATP, 5.5, and ATP, 4.13 (Fig. 4D).

BzATP treatment of osteoblasts also induced dramatic changes to several aspects of cell morphology that are consistent with $P2X_7$ receptor–induced apoptosis. In SaOS-2 cells and primary HBDC, but not in Te85 cells,

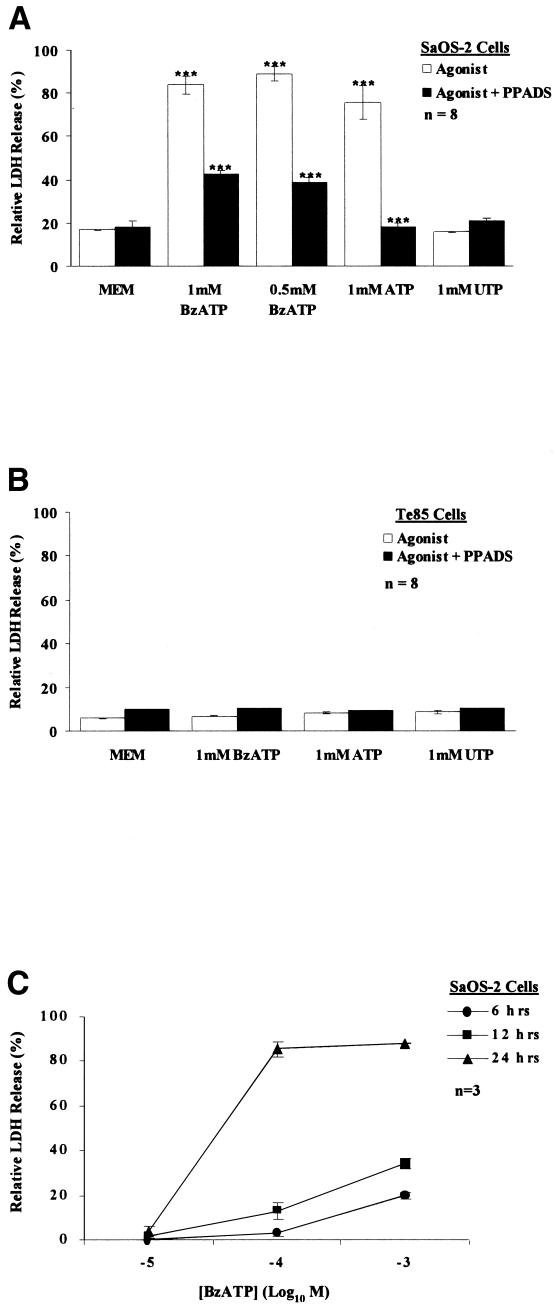

FIG. 3. LDH release in response to P2X$_7$ receptor activation. Cells were seeded equally into 96-well plates and grown to 75% confluence. Quiescent cells were then incubated in serum-free DMEM supplemented with different agonists and the antagonist PPADS as indicated. After the indicated time period, the supernatant was assayed for LDH activity. (A) LDH release from SaOS-2 cells in response to P2X$_7$ receptor agonist stimulation. (B) LDH Release in Te85 cells in response to P2X$_7$ receptor agonist stimulation. Values are expressed as a percentage of the total cellular LDH. Experiments were repeated three times for both cell types. Data represent means ± SEM; $n = 8$; ***$p < 0.001$; significant increase in experimental values compared with MEM only control, or significant inhibition in comparison to the treated sample without PPADS. (C) Time course of LDH release from SaOS-2 cells in response to P2X$_7$ receptor agonist stimulation. Data represent means ± SEM; $n = 3$.

extensive blebbing of the plasma membrane was clearly visible after 40 minutes of treatment with 100 μM of BzATP, which was prevented by pretreatment with the antagonist PPADS (Fig. 5). Three hours post-BzATP treatment, cells were fixed and stained with Hoechst 33258 and phalloidin-TRITC to reveal nuclear and cytoskeleton changes, respectively. Severe disruption of the actin cytoskeleton and nuclear condensation were evident in treated SaOS-2 cells (Fig. 6, compare panel A with panel B and panel C with panel D) but not in Te85 cells (Figs. 6E and 6F). After 12 h in the presence of BzATP, the majority of SaOS-2 cells had rounded up and detached from the substrate, whereas Te85 cells remained unaffected, even when treated with 1 mM of BzATP (data not shown). ATP induced similar changes, whereas UTP had no effect on cell morphology (data not shown).

To further confirm that these observed morphological changes were caused by P2X$_7$ receptor–induced apoptosis, dual staining with the P2X$_7$ receptor antibody and the In Situ Cell Death Detection Kit (TUNEL staining) was performed. In a population of cells highly immunopositive for the P2X$_7$ receptor, positive TUNEL staining was observed in all cells 6 h post-BzATP treatment (Fig. 7 compare panel B with panel A and panel E with panel D). Pretreatment with PPADS before BzATP treatment resulted in levels of TUNEL staining observed with untreated cells (Fig. 7, compare panel C with panel A and panel F with panel D).

DISCUSSION

In this study we present for the first time molecular, immunocytochemical, and pharmacologic evidence for the functional expression of the bifunctional P2X$_7$ receptor by human osteoblasts. Using RT-PCR and Southern analysis, we were able to detect P2X$_7$ mRNA transcripts in the human osteosarcoma cell lines SaOS-2 and Te85, as well as several populations of primary HBDCs obtained from patients of varying age, sex, and skeletal pathology. We then used a P2X$_7$-specific monoclonal antibody to confirm that these mRNAs led to the expression of the P2X$_7$ receptor protein. In SaOS-2 cultures, a distinct subpopulation of cells was found to be highly immunoreactive to the P2X$_7$ antibody. This observation is consistent with our previous studies describing the heterogeneous nature of these cells with respect to expression of other P2 receptors.[34] Surprisingly, despite the expression of receptor mRNA, no P2X$_7$ immunoreactivity could be detected in Te85 cultures. Although the exact reason for this is unclear, it may reflect the differentiation state of these osteoblasts before transformation. This lack of P2X$_7$ receptor protein expression by Te85 cells refutes arguments that P2X$_7$ receptor expression in clonal cells is merely a consequence of cellular transformation and a by-product of in vitro culture (Dr. Chessell, personal communication, 1999). The observation that cultured, nontransformed primary human osteoblasts showed varying degrees of P2X$_7$ immunoreactivity provides further support for the notion of differentiation-dependent P2X$_7$ receptor expression in osteoblast populations.

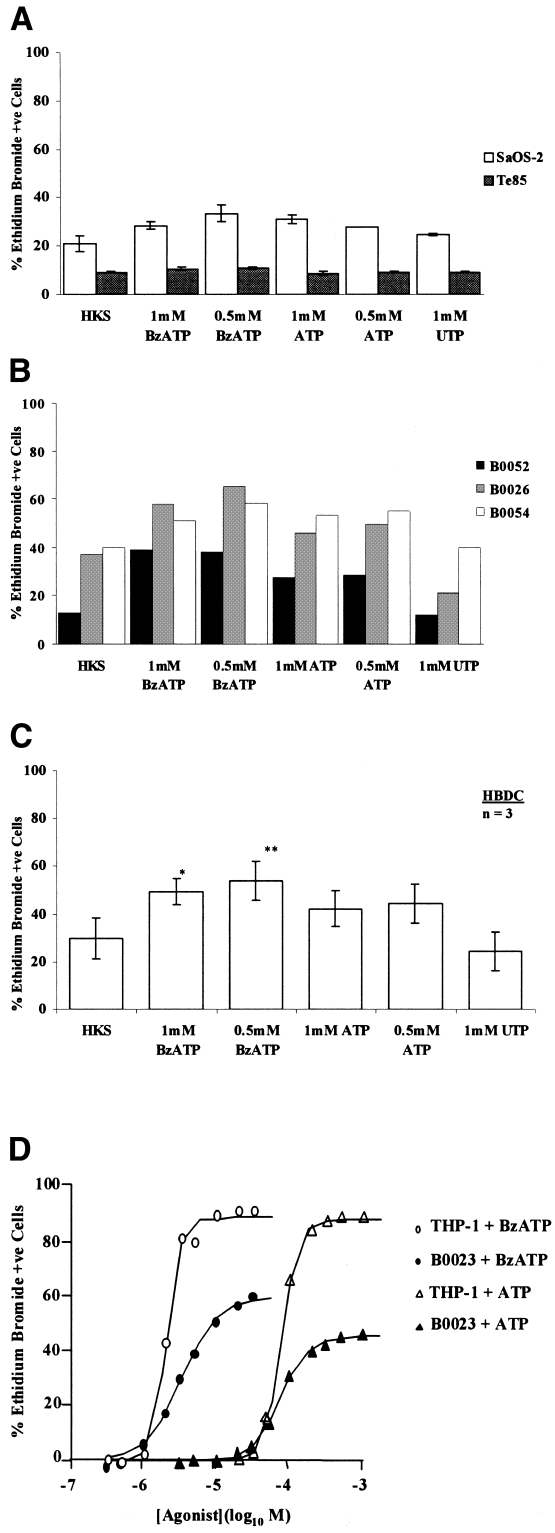

FIG. 4. Ethidium bromide uptake in response to $P2X_7$ receptor activation. Subconfluent, quiescent cells were trypsinized and resuspended in HKS buffer containing 100 μm ethidium bromide and the indicated concentration of nucleotide. After incubation at 37°C for 60 minutes, reactions were terminated by the addition of 0.5 ml ice-cold 5 M NaCl, and the percentage of cells that were ethidium bromide positive was determined by flow cytometry. (A) Percentage of ethidium bromide–positive SaOS-2 and Te85 cells after $P2X_7$ receptor agonist stimulation. (B) Percentage ethidium bromide–positive cells in several populations of HBDC cells in response to $P2X_7$ receptor agonist stimulation. (C) Mean percentage ethidium bromide–positive cells in several populations of HBDC cells in response to $P2X_7$ receptor agonist stimulation. Data represent means ± SEM; $n = 3$; $^{**}p < 0.01$, $^{*}p < 0.5$ significant increase in experimental values compared with HKS only control. (D) Dose-response curve for BzATP or ATP-induced ethidium bromide uptake in THP-1 and B0023 cells. $P[A]_{50}$ values were determined using standard logistic curve fitting and a modified form of the Hill equation.

To assess whether $P2X_7$ receptors expressed by osteoblasts are functional, we next exploited the unique properties of this receptor to form large aqueous pores in the plasma membrane when stimulated with repeated or prolonged applications of higher concentrations of agonist and also to induce apoptosis. Consistent with expression of the $P2X_7$ receptor by SaOS-2 cells, 1 mM of BzATP and 1 mM of ATP induced high levels of LDH release 24 h posttreatment. Conversely, Te85 cells, which do not express the $P2X_7$ receptor protein, showed low levels of LDH release in response to treatment with either agonist. The observed release of LDH probably is caused by the gradual release via $P2X_7$ receptor pores and/or secondary necrosis known to accompany apoptosis and as such is a receptor-mediated event.[35] The fact that the cytosolic enzyme LDH was retained by SaOS-2 cells for up to 6 h after BzATP treatment would suggest an intact plasma membrane and the release was not merely a consequence of general agonist cytotoxicity. In agreement with this, the nonspecific P2X antagonist PPADS reduced nucleotide-induced LDH release from SaOS-2 cells by as much as 75%. Similarly, millimolar concentrations of UTP, which is an ineffective $P2X_7$ receptor agonist, did not induce LDH release from SaOS-2 cells and no significant LDH release could be detected at earlier time points.

Another method of determining $P2X_7$ receptor activation and formation of transmembrane pores is to monitor the uptake of the dye ethidium bromide, in which its fluorescent properties change when bound to DNA. When combined with flow cytometry, this method provided data on the proportion of cells within populations that form transmembrane pores. As established by immunocytochemistry, there appeared to be a distinct subpopulation of SaOS-2 cells that formed pores when stimulated with BzATP. Similarly, three populations of HBDC tested showed an ethidium bromide uptake between 20 and 60%. This varied response by different populations of HBDC further supports our observations that these cells are heterogeneous with respect to P2 receptor expression.[34] These varied responses also could reflect the expression of other P2 receptors by these cells, in particular other P2X receptors. As with hemopoietic cells, the ability of P2X receptors to form heterodimers with the $P2X_7$ receptor then would have both positive[36] and negative[37] effects on pore formation.

The $P2X_7$ receptor expressed by osteoblasts has a somewhat atypical pharmacology in that the formation of the

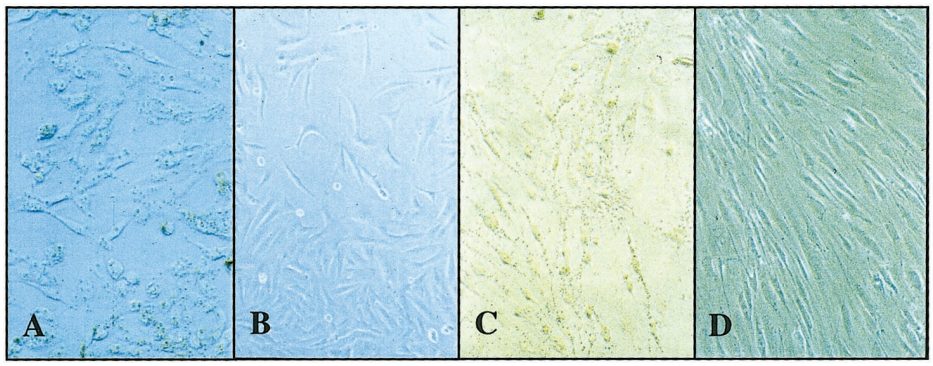

FIG. 5. Morphological changes associated with P2X$_7$ activation in human osteoblasts. SaOS-2 cells and HBDCs were cultured onto glass coverslips and treated with 100 μM of BzATP with and without pretreatment for 1 h with 30 μM of PPADS. Cells were then examined by phase-contrast microscopy 40 minutes posttreatment. (A) BzATP-treated SaOS-2 cells; (B) PPADS-pretreated and BzATP-treated SaOS-2 cells; (C) BzATP-treated HBDCs; (D) PPADS-pretreated and BzATP-treated HBDCs. Original magnification ×200.

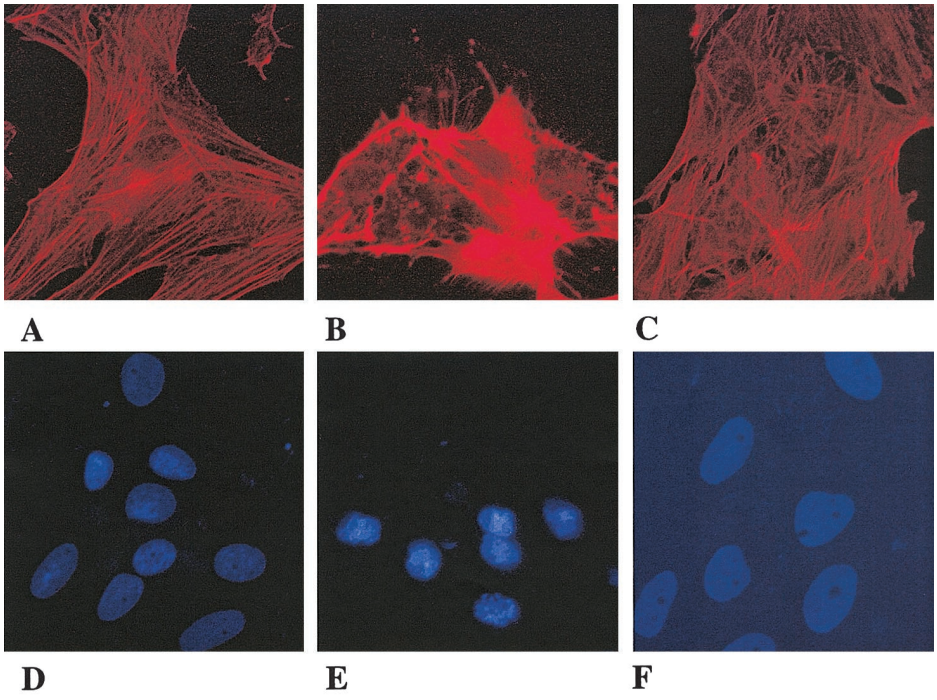

FIG. 6. Morphological changes associated with P2X$_7$ activation in human osteosarcoma cell lines. SaOS-2 and Te85 were seeded onto glass coverslips and treated with 100 μM of BzATP. Three hours posttreatment cells were fixed in 3.7% PBSF, washed in PBS-Triton X-100 (0.1%) and incubated at room temperature for 1 h with (A–C) phalloidin-TRITC and (D–F) the DNA-specific fluorochrome Hoechst 33258. The cells were then analyzed by epifluorescence using a Leitz IF microscope and appropriate filters. (A and D) Untreated SaOS-2 cells; (B and E) BzATP-treated SaOS-2 cells; (C and F) BzATP-treated Te85 cells. Original magnification ×600.

trademark transmembrane pore is delayed. A prolonged incubation of 60 minutes, as compared with 5 minutes with hemopoietic cells, is required before ethidium bromide uptake can be detected in these cells. This delayed onset of pore formation is a phenomenon that also has been reported in human lymphocytes[38] and may account for previous observations that osteoblasts are insensitive to ATP^{4-} in this respect.[26]

Although the expression of this pore-forming receptor on cells of hemopoietic origin is well established and has been implicated in the immune response, its expression by cells of stromal origin, in particular osteoblasts, has not been investigated in detail. One report describes the use of ATP^{4-}, the putative agonist at the P2X$_7$ receptor, as a means of eliminating cells of hemopoietic origin from isolated bone cell populations and fetal bone rudiments to leave an intact stromal cell population.[26] These findings imply that osteoblasts do not express functional P2X$_7$ receptors; however, the data presented in this study clearly show that there is a distinct subpopulation of osteoblasts that do express functional P2X$_7$ receptors.

The exact role of this receptor in modulating osteoblast cell function is unclear. What is clear is that repeated or prolonged exposure to higher concentrations of agonist leads to the formation of transmembrane pores and results in eventual cell death. The process of bone remodeling is essential for the anatomical integrity and homeostasis of the skeleton, and critical to this is the

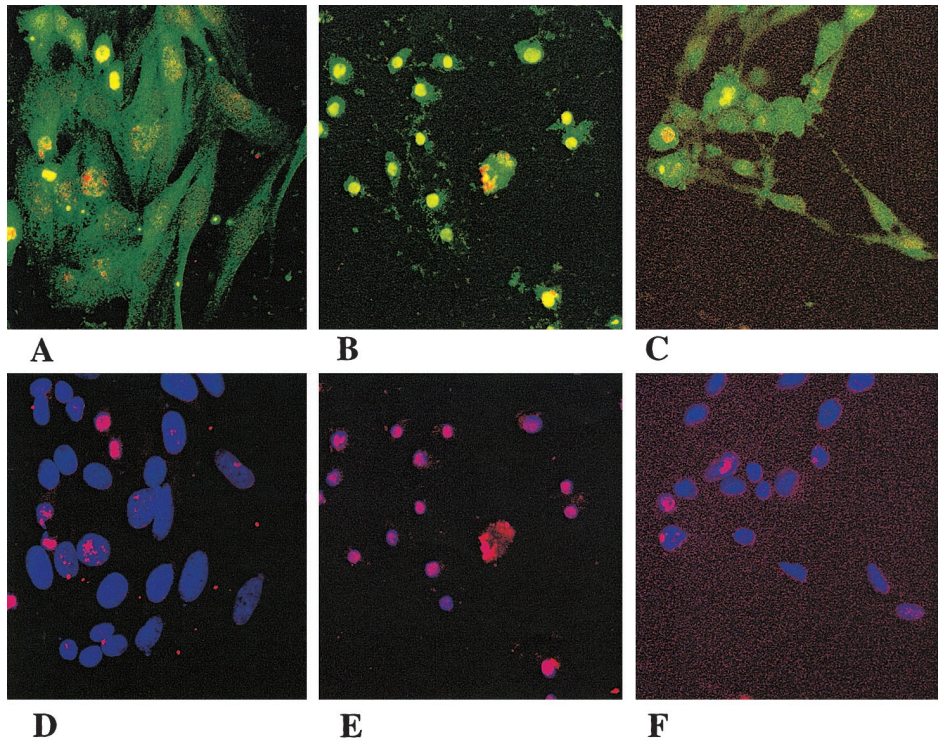

FIG. 7. $P2X_7$ receptor –induced TUNEL staining in SaOS-2 cells. SaOS-2 were seeded onto glass coverslips and treated with BzATP, with and without pretreatment for 1 h with 30 μM of PPADS. Six hours posttreatment cells were fixed in 3.7% PBSF, washed in PBS-Triton X-100 (0.1%), and incubated at 37°C for 1 h with the TUNEL mix. The cells were subsequently washed in PBS-Triton X-100 (0.1%), incubated in blocking serum, and then incubated with the $P2X_7$ receptor antibody (10 μg/ml). After incubation with fluorescein isothiocyanate (FITC)–labeled secondary antibody and the DNA-specific fluorochrome Hoechst 33258, the coverslips were rinsed in water, mounted in Mowiol, and then analyzed by epifluorescence using a Leitz IF microscope and appropriate filters. (A and D) Untreated SaOS-2 cells; (B and E) BzATP-treated SaOS-2 cells; (C and F) PPADS-pretreated and BzATP-treated SaOS-2 cells. Green = FITC labeled $P2X_7$ monoclonal antibody (mAb); blue = Hoechst nuclear stain; purple = TUNEL-positive nuclei; yellow = TUNEL-positive $P2X_7$-positive cells. Original magnification ×400.

orderly genesis of osteoblasts and osteoclasts from their precursors, as well as the removal of existing osteoblasts and osteoclasts from the bone surface.[39] Apoptosis, or programmed cell death, is a widely recognized method of limiting the life span of cells in regenerative tissues,[40] a process that recently has been shown in osteoblasts both in vitro and in vivo.[41] Prolonged exposure of SaOS-2 cells to BzATP results in several morphological changes typical of apoptosis, such as membrane blebbing, nuclear condensation, and cytoskeletal alterations. Apoptosis in $P2X_7$ receptor–positive osteoblast cells was further shown by dual labeling with the $P2X_7$ monoclonal antibody and the apoptotic marker TUNEL. Therefore, as observed with cells of hemopoietic origin, prolonged $P2X_7$ activation in osteoblasts would appear to be a suicide response. The sustained high in vivo concentrations of ATP, in particular the tetrabasic ATP^{4-} species, required for receptor activation and induction of apoptosis in osteoblasts would appear unlikely in the bone microenvironment. However, release of ATP from intracellular stores in response to trauma or stress or by neighboring osteoblasts via a constitutive nonlytic mechanism,[25] could result in the accumulation of high concentrations of ATP in very localized pockets of the bone microenvironment, and as such could facilitate targeted osteoblast apoptosis.

With a low prevalence of apoptosis in osteoblasts,[41] conservation of a receptor in which its only function was to induce cell death would appear extravagant and as such would evolve to redundancy. A much more pertinent function of this receptor therefore would seem to be the controlled release of cytoplasmic components via rapid reversible pore formation in response to transient high concentrations of ATP. Such transient high ATP concentrations would exist more readily throughout the bone microenvironment, and the release of proresorption cytokines such as IL-1β and IL-6, which has been reported to occur as a consequence of $P2X_7$ receptor activation in other cell types abundantly expressing this receptor,[11,13] would prove to be a more physiological relevant consequence of receptor activation in skeletal tissues.

These data provide compelling evidence for the expression of the $P2X_7$ receptor in a subpopulation of human osteoblasts and suggests a possible role for this receptor in osteoblast apoptosis. However, $P2X_7$ receptor activation

and pore formation is almost certainly much more complex and subtle in nature and as such requires further investigation.

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