Possible Role of Extracellular Nucleotides in Ectopic Ossification of Human Spinal Ligaments


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Abstract. To reveal the involvement of extracellular nucleotides in the ossification process in ossification of the posterior longitudinal ligament of the spine (OPLL), the mRNA expression profiles of P2 purinoceptors, mechanical stress-induced ATP release, and ATP-stimulated expression of osteogenic genes were analyzed in ligament cells derived from the spinal ligament of OPLL patients (OPLL cells) and non-OPLL cells derived from the spinal ligaments of cervical spondylotic myelopathy patients as a control. The extracellular ATP concentrations of OPLL cells in static culture were significantly higher than those of non-OPLL cells, and this difference was diminished in the presence of ARL67156, an ecto-nuclease inhibitor. Cyclic stretch markedly increased the extracellular ATP concentrations of both cell types to almost the same level. P2Y1 purinoceptor subtypes were intensively expressed in OPLL cells, but only weakly expressed in non-OPLL cells. Not only ATP addition but also cyclic stretch raised the mRNA levels of alkaline phosphatase and osteopontin in OPLL cells, which were blocked by MRS2179, a selective P2Y1 antagonist. These increases in the expression of osteogenic genes were not observed in non-OPLL cells. These results suggest an important role of P2Y1 and extracellular ATP in the progression of OPLL stimulated by mechanical stress.

Keywords: ligament cell, ectopic ossification, P2 purinoceptor, ATP, mechanical stress

Introduction

Ossification of the posterior longitudinal ligament of the spine (OPLL) is a common disease among Japanese and other Asian populations, characterized by ectopic bone formation in the spinal ligament. When ossification enlarges in the spinal canal, the spinal cord becomes compressed, resulting in serious neurological damage, and a high risk of spinal cord injury. Spinal cord decompression surgery has been successfully performed for about 40 years on symptomatic patients. However, there may be many individuals who have ossified lesions without any symptoms and in whom slight trauma can cause damage of the spinal cord by compression of the ossified lesion (1–3). Therefore, not only safe and effective therapy but also diagnosis of the disease at an early stage has been requested. Unfortunately, the natural history of OPLL has not been known and screening methods to detect OPLL have not been established.

The ossification process in OPLL is thought to occur through the endochondral mechanism based on characteristic abnormalities of spinal ligament cells that promote their differentiation into osteogenic cells (4–7). Dynamic factors and active molecular factors acting on human spinal ligaments have been thought to play important roles in this ossification. Multiple etiologic factors, including genetic factors, dietary habits, metabolic abnormalities, and some local factors have been researched. We have reported several factors underlying the ossification process in OPLL such as connective tissue growth factor (CTGF/HCs24) (7), prostaglandin I2 (8), bone morphogenetic protein-2 (BMP-2) (9), runx2/Cbfa1 (10), endothelin-1 (11), and mechanical stress (8–11). Therefore, blocking these
factors may be effective as a therapy for this disease. We are focused on extra cellular substances related to homeostasis of bone tissue and mechanical stress.

Extracellular nucleotides such as ATP and ADP have been reported to play a pivotal role in many human organs and are involved in the bone remodeling via binding to receptors termed P2-purinoceptors (P2) (12–19). These receptors are subdivided into P2X and P2Y subtypes based on the mechanism of signal transduction. Extracellular nucleotides are responsible for homeostasis and differentiation in many human cell lines, including osteoblasts. Furthermore, autonomic release and mechanically-induced release of ATP have been recognized in several cultured cells from human tissue (20–23). If there are significant differences in the expression of these receptors between OPLL and non-OPLL cells, extra cellular ATP may affect the initiation and progression of OPLL.

The purpose of this study was to analyze the mRNA expression profiles of P2 purinoceptors and to explore the roles of extra cellular ATP and P2-purinoceptors in the process of OPLL. We observed extensive expression of P2Y1 in OPLL cells but only slight expression in non-OPLL cells. Addition of ATP induced elevated expression of osteogenic marker genes in OPLL cells, and this elevation was inhibited by a P2Y1-receptor antagonist, suggesting the indispensable role of extra-cellular ATP via P2Y1 receptors in the progression of OPLL.

### Materials and Methods

This study was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine and was conducted according to principles of the Declaration of Helsinki. Informed consent was obtained from each patient.

**Clinical diagnosis and spinal ligament samples**

The diagnosis of OPLL or non-OPLL (cervical spondylotic myelopathy) was confirmed by X-ray, computerized tomography, and magnetic resonance imaging of the cervical spine preoperatively. Ligament samples were all from the C3 level. The clinical diagnoses and the spinal ligament tissues used in this study are shown in Table 1.

**Cell culture**

Ligaments harvested aseptically from patients during surgery were rinsed with PBS, after which the surrounding tissue was carefully removed under a dissecting microscope. In all cases, the ligaments were extirpated carefully from a non-ossified site to avoid any possible contamination of osteogenic cells. Collected ligaments were minced into about 0.5 mm³ pieces and washed twice with PBS, then plated in 100-mm culture dishes, and maintained in DMEM (10% FBS, 1% L-glutamine, 100 units/ml of penicillin G sodium, 100 µg of streptomycin sulfate) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Cells derived from explants were harvested with 0.02% EDTA/0.05% trypsin for passage.

SaOS2 cells (human osteogenic sarcoma cell line) were purchased from Dainippon Pharmaceutical Co., Ltd. (Tokyo) and used as a positive control.

**Measurement of extracellular ATP**

Cells from OPLL patients (OPLL cells) and non-OPLL patients (non-OPLL cells) and SaOS2 were grown in 35-mm dishes. When cultures reached confluence, complete DMEM was removed. After

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<th>Code No.</th>
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Table 1. Tissue samples used in this study, including clinical diagnosis, patient gender, and age
washing with warm PBS, cells were incubated with 0.5 ml serum-free and phenol red-free DMEM in the absence or presence of 50 µM ARL67156 (ecto-nuclease inhibitor (24)) at 37°C for 1 h. Culture medium (300 µl) was immediately collected into a 2-ml tube and then 50 µl of ATP detection solution (TOYO B-Net Corp., Tokyo) containing luciferin and luciferase was added. The tube was placed in a luminescence reader BLR-301 (ALOKA, Tokyo) and readings were taken every second for 0.1 s; light emission was detected by a photon counter covering the spectral range of 400–650 nm. Average values were calculated and recorded as relative light units and plotted on a calibration curve. After medium was collected, the cells were harvested from the dish with 0.02% EDTA / 0.05% trypsin and cell numbers were counted using an automatic blood cell counter (Nihon Kohden Corp., Tokyo). Values were divided by cell number and ATP concentrations were expressed as 10⁻⁹ nmol/cell per hour.

**Stretch apparatus and measurement of extracellular ATP**

Cells were placed on a 3.5 × 4.0 cm deformable silicon chamber coated with gelatin (Iwaki Glass, Tokyo) at a density of about 100,000–200,000 cells /cm². After cultures reached confluence, medium was replaced with 0.5 ml of serum-free and phenol red-free DMEM. The silicon chambers were set on a four-point bending apparatus (Scholertec Corp., Osaka) and exposed to motor-driven computer-controlled uniaxial sinusoidal cycle stretch of 120% peak to peak, at 1 Hz in a humidified atmosphere of 95% air and 5% CO₂ at 37°C (9). The time range of stretch was up to 20 min for ATP release experiments and 9 h for mRNA expression. Medium was collected every 10 min and ATP concentrations were calculated as described above.

**Cell viability assay**

A cell viability assay was performed using Cell Counting kit-8 (Dojin, Kumamoto) according to the manufacturer’s protocol.

**RNA preparation and cDNA synthesis**

Cells from OPLL patients (OPLL cells), cells from the spinal ligaments of cervical spondylotic myelopathy patients (non-OPLL cells) and SaOS2 cells were grown in 35-mm dishes. After cultures reached confluence, ATP was added to each dish. Dishes were incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. After different time periods of incubation (0, 1, 4 h), total RNA was extracted simultaneously from cell monolayers using an RNeasy kit (Qiagen, Chatworth, CA, USA) according to the manufacturer’s protocol. Total RNA treated with RNase-free DNase I (Life Technologies, Inc., Rockville, MD, USA) was reverse transcribed into cDNA using random primers (Invitrogen, Co., Carlsbad, CA, USA). One microgram of total RNA was heated at 65°C for 5 min in 10 µl of H₂O supplemented with 100 ng of random primers. The mixture was placed on ice, and cDNA synthesis was performed by reverse transcription (RT) for 1 h at 37°C in a final volume of 20 µl of buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 3 mM MgCl₂) supplemented with 0.5 mM dNTPs (Life Technologies, Inc.), 2 U of RNase inhibitor (Toyobo, Osaka), and 200 U of M-MLV reverse transcriptase (Invitrogen, Inc.). After incubation, the cDNA was heated at 70°C for 15 min and stored at −20°C until used for amplification by the polymerase chain reaction (PCR).

**PCR analysis**

For conventional PCR, specific oligonucleotide primers against human sequences were designed on the basis of sequences in GenBank (9, 25). Reactions were performed using the Taq PCR Master mix kit (Qiagen, Inc.) as follows: 1 µl of cDNA was used as the template in a 20-µl amplification mixture consisting of 1 U of Taq DNA polymerase, 0.5 µM each of the 5’ and 3’ primers, and distilled water. The primer sequences, Tm values, product sizes, and cycle numbers are shown in Table 2. PCR was performed in a Perkin–Elmer 9600 thermal cycler. The standard reaction condition for P2-purinoceptors involved an initial 3-min denaturation step at 94°C, followed by an amplification step (denaturing at 94°C for 10 s, annealing at Tm for 30 s, and extension at 72°C for 30 s) for 40 cycles and an additional extension step at 72°C for 10 min. In the case of G3PDH, an initial 3 min denaturation step at 94°C was followed by the amplification step (denaturing at 94°C for 20 s, annealing at Tm for 30 s, and extension for 90 s at 72°C) for 19 cycles, and an additional extension step at 72°C for 10 min. For analyses of DNA, amplification products were resolved by electrophoresis on a 2.5% w/v agarose gel and were visualized by staining with SYBR Green-I (Molecular Dynamics, Sunnyvale, CA, USA). SYBR Green-I fluorescence was converted to a TIFF image by a CCD camera (C-900ZOOM; Olympus, Tokyo), and the intensity was quantified by QuantiScan software (Biosoft, Ferguson, MO, USA). All products were normalized to glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA levels.

For SYBR-green real-time PCR, PCR primers were designed using Primer Express software Version 1.0 (Applied Biosystems, Foster City, CA, USA). The primer sequences were as follows: G3PDH, sense 5’-
AGATCATCAGCAATGCCTCCCTG-3'; antisense 5'-ATGGCATGGACTGTGGGTGATG-3'; and P2Y1, sense 5'-CGTGCTGGTGTGGCTCATG-3'; antisense 5'-GGA CCCCGGTACCTGAGTGAAGTGA-3'. For Taqman real-time PCR of G3PDH, osteopontin (SPP1) and alkaline phosphatase (ALP), Assays-on-Demands Gene Expression Assay Mix kits (Applied Biosystems) were used. Both types of real-time PCR were conducted using the ABI PRIMER 7000 instrument (Applied Biosystems).

The analysis of PCR results was performed using the ΔCt value (Ct \text{EACH GENE} - Ct \text{G3PDH}). Relative gene expression was obtained by ΔΔCt methods (ΔCt \text{SAMPLE} - ΔCt \text{CONTROL}) for comparison of the gene expression levels of every unknown-sample. The conversion between ΔΔCt and relative gene expression levels is fold induction = \(-2^{-\Delta \Delta Ct}\).

### Western blot analysis

Samples containing $1 \times 10^5$ cells were loaded on 7% sodium dodecyl sulfate (SDS)-polyacrylamide gels, and electroblotted onto Immobilon-FL polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Expression of P2Y1 and actin as an internal standard was detected with specific antibodies for each protein as described previously (26). Anti-P2Y1 antibody was purchased from Alomone Labs, Jerusalem, Israel. Anti-actin C-terminal peptide was purchase from Sigma (Saint Louis, MO, USA).

### Statistical analyses

Ten independent experiments were performed using cell preparations from ten OPLL and ten non-OPLL patients (see Table 1), and reactions were performed in quadruplicate for each cell preparation. The results are expressed as means ± S.E.M. (n = 10), and graphs show
the relative expression levels compared with controls (non-OPLL patients). Data were analyzed by ANOVA and significant interactions were further examined with Dunnett’s post-hoc test for multiple comparisons. *$P<0.05$ was considered to be significant.

**Results**

**Extracellular ATP of three cell types**

The amounts of ATP in the media of OPLL, non-OPLL, and SaOS2 cell cultured 1 h after replacement with fresh DMEM medium (ATP-free and phenol red-free) were measured. OPLL cells released more ATP than non-OPLL cells and SaOS2 cells (Fig. 1).

Ecto-nuclease activity is an important factor to control extracellular ATP concentration and its activity is quite different in different cell types. Treatment with an ecto-nuclease inhibitor (APL67156) increased the ATP level by 2 – 3 times and the difference in ATP amounts observed between OPLL and non-OPLL cells in the absence of the drug disappeared (Fig. 1).

**Extracellular ATP induced by mechanical stress**

Extracellular ATP concentrations in OPLL and non-OPLL cells were increased by uniaxial sinusoidal cyclic stretch and reached almost the same level (Fig. 2). A cell viability assay revealed that the fraction of intact cells before and after cyclic stretch was almost constant (before, $96 \pm 1.5\%$ and after, $97 \pm 1.1\%$ [n = 10]), suggesting that ATP release was not due to stretch damage of the cells.

**Expression profiles of P2 purinoceptor family subtypes in OPLL, non-OPLL, and SaOS2 cells**

Conventional RT-PCR was carried out to evaluate the levels of mRNA expression of P2 receptor subtypes in OPLL, non-OPLL, and SaOS2 cells. Figure 3A is a representative gel image; P2Y1, 2, 4, 6, 12 and P2X4 are expressed in OPLL cells; P2Y4, 6, and P2X4 are expressed in non-OPLL cells; and P2Y1, 2, 4, 12 and P2X1, 4, 6, and 7 are expressed in SaOS2 cells. Figure 3B summarizes the expression levels of these receptors quantified by densitometrical scanning. P2Y1 was intensively expressed in both OPLL and SaOS2 cells, but only weakly in non-OPLL cells. P2X4 receptors were commonly expressed in all cell types.

**P2Y1 expression analysis by Western blotting**

To analyze protein levels, Western blot analysis was carried out. Figure 4 shows the presence of P2Y1 protein in non-OPLL, OPLL and SaOS2 cells, but the levels in non-OPLL seem to be lower than those in the other two cell types. The average of four experiments using 4 independent samples demonstrated that the level of P2Y1 protein is significantly higher in OPLL cells than in non-OPLL cells and similar to that in SaOS2 cells, consistent with the results of PCR.

**Effects of ATP on the expression levels of osteogenic genes in OPLL cells and non-OPLL cells**

ATP at 100 µM is an effective concentration in human cell lines (22 – 25). In the OPLL group, addition of ATP as a P2Y1 agonist increased the ALP mRNA level in a
Time-dependent manner, up to 4 h, and a peak appeared at 1 h. On the other hand, no significant change was found in the non-OPLL group (Fig. 5A). mRNA expression of osteopontin was also stimulated by ATP in the OPLL group, but not in the non-OPLL group (Fig. 5B).

Inhibitory effects of MRS2179 on the expression of ALP and osteopontin induced by ATP addition (A) or cyclic stretch (B). After addition of 100 μM ATP or cyclic stretch for 9 h in the presence or absence of 10 μM MRS2179 as a specific P2Y1 antagonist, the levels of ALP and osteopontin mRNAs in OPLL cells were analyzed by real-time PCR (n = 10). C: control, M: MRS2179, A: ATP, S: cyclic stretch. *P<0.05 versus ATP addition of cyclic stretch in the absence of MRS2179.

Fig. 5. Effects of ATP addition on the level of ALP (A) and osteopontin (B) mRNAs in OPLL and non-OPLL cells. After addition of 100 μM ATP, the levels of ALP and osteopontin mRNAs were analyzed by real-time PCR. Data (means ± S.E.M.) were expressed as the ratio to the level of non-OPLL cells at time 0. *P<0.05 versus time 0 (n = 10).

Fig. 6. Inhibitory effects of MRS2179 on the expression of ALP and osteopontin induced by ATP addition (A) or cyclic stretch (B). After addition of 100 μM ATP (A) or cyclic stretch for 9 h in the presence or absence of 10 μM MRS2179 as a specific P2Y1 antagonist, the levels of ALP and osteopontin mRNAs in OPLL cells were analyzed by real-time PCR (n = 10). C: control, M: MRS2179, A: ATP, S: cyclic stretch. *P<0.05 versus ATP addition of cyclic stretch in the absence of MRS2179.

Time-dependent manner, up to 4 h, and a peak appeared at 1 h. On the other hand, no significant change was found in the non-OPLL group (Fig. 5A). mRNA expression of osteopontin was also stimulated by ATP in the OPLL group, but not in the non-OPLL group (Fig. 5B).

Inhibitory effects of a P2Y1 antagonist on ATP-induced and cyclic stretch–induced expression of osteogenic genes in OPLL cells

MRS2179, a representative P2Y1 antagonist (27), restored ATP-induced increases in the expression of ALP and osteopontin mRNAs in OPLL cells, to the
levels seen before the addition of ATP (Fig. 6A). As shown in Fig. 6B, cyclic stretching for 9 h significantly induced increases in ALP and osteopontin mRNA expressions, confirming the previous report (9). MRS2179 also inhibited the increases in expression of these genes induced by mechanical stress.

Discussion

**Basal and mechanical stress–induced extracellular ATP levels**

Pathophysiological stages of cells are thought to be controlled by the environment and various local factors. In the case of OPLL, mechanical stress has been thought to be an important environmental factor (25, 28 – 30). Mechanical stress induces the releases of various local factors that have been reported to play a pivotal role in bone metabolism (7 – 11, 28).

Several lines of evidence have demonstrated that extracellular nucleotides affect many of the physiological and pathophysiological activities of human cell lines via P2 purinoceptors (12, 13). Extracellular nucleotides have been reported to affect differentiation, proliferation, and homeostasis of osteoblasts, osteoclasts, and osteosarcoma in vitro (15 – 18). Extracellular ATP advances the mitogenic action of several growth factors in a variety of cell types including osteoblasts through the control of transcriptional activators such as c-fos, egr-1, and runx2 (24, 31 – 33). According to the best of our knowledge, however, studies on the physiological and pathophysiological roles of extracellular ATP in human spinal ligament tissue and cells have not been conducted.

The levels of basal extracellular ATP levels of cultured ligament cells and SaOS2 cells did not contradict those reported previously (22, 32 – 36). The basal extracellular ATP level of OPLL cell cultures was clearly high compared with those of non-OPLL cells and SaOS2 cells. ARL67156, an ecto-nuclease inhibitor, increased the basal ATP level of non-OPLL and SaOS2 cells by about 2 – 5 times, up to almost the same level as OPLL cells, and diminished the difference among these cell types. These results suggest that the high basal ATP level in OPLL cells compared with other cell lines may be due to a low ecto-nuclease activity. Ecto-nuclease hydrolyzes ATP to yield pyrophosphate, a physiological inhibitor of mineralization. The concentration of pyrophosphate may be lower in OPLL cells than in non-OPLL cells. This may explain the mineralization of OPLL cells observed during a long term static culture (K.I. Furukawa et al., unpublished observation). It is necessary to investigate the role of pyrophosphate. On the other hand, cyclic stretch as a mechanical stress dramatically increased ATP level by about 10 times during a 10-min stretch, and the ATP level reached almost the same level in both OPLL and non-OPLL cells. The dynamic level of extracellular ATP during exposure to mechanical stress may be dependent on the ATP-releasing activity that exceeds the ecto-nuclease activity, suggesting that the ATP-releasing activities of ligament cells are not dependent on the pathophysiological stage. The possibility that ATP release is due to a leak from cells damaged by cyclic stretch was excluded because the cell viability assay indicated that the number of intact cells did not decrease after exposure to cyclic stretch.

The concentration of ATP released by cyclic stretching seems to be lower than that of ATP added exogenously. However, it is possible that the local concentration of ATP around its receptor reaches up to 100 µM, either due to the presence of ATP at the cell surface, membrane trapping, and/or unstirred layer effects due to the autocrine and/or paracrine release of ATP. This concept was confirmed previously (37). As an alternative explanation, there may be a supply of ATP from another source. Clinical reports indicate that patients with OPLL have a tendency to bleed in the perioperative period and show significantly greater blood loss after surgery (38, 39), suggesting invasion of blood vessels into spinal ligament tissue. Furthermore, repeated episodes of inflammation underlie ectopic bone formation by spinal ligaments (40). Cyclic stretching of OPLL tissues resulted in increases in the levels of many cytokines related to inflammation (K.I. Furukawa et al., unpublished observation). Therefore, ATP may be delivered from cells mediating inflammation and/or via blood vessels into ligament tissues. Further study from this point of view may be required.

**P2 family subtypes expression**

P2 purinoceptor subtypes are classified into two major subtypes, P2X (ligand-gated ion channel) and P2Y (G-protein-coupled). These receptors induce an intracellular Ca$^{2+}$ response and the activities of mediators such as PKC and ERKs, which generate transcriptional factors activities (31, 32). P2 purinoceptors are detected in osteoblasts, osteoclasts, and osteosarcoma. These receptors are also found in human spinal ligament cells (OPLL and non-OPLL cells), but the expression profiles of these receptors in OPLL cells were quite different from those in non-OPLL cells (normal ligament cells) and rather similar to those in an osteosarcoma cell line, SaOS2. In these subtypes, P2Y1 was expressed intensively in both OPLL cells and SaOS2 cells, but only weakly in non-OPLL cells. Extracellular ATP is a high affinity agonist for P2Y1 in osteoblasts and SaOS2
increased the release of ATP in both cell types. P2Y1
Cyclic stretch as a mechanical stress dramatically
affected the presence of MRS2179, suggesting that ATP may be
involved in the ossification process of OPLL and SaOS2 cells. However, the expression of the P2Y6 subtype seems to
be very low compared with that of P2Y1 in OPLL
cells (Fig. 3B), indicating another possibility that lower
expression of P2Y2 permits ectopic ossification. The expression of P2Y6 subtype in OPLL cells was relatively higher than that in non-OPLL and SaOS2 cells. However, its role in physiological and pathophysiological ossification has been unknown and remains to be investigated.

Various genes have been enumerated as candidates underlying the disease state of OPLL because inhibition of the function of each gene resulted in lowering the expression of several osteogenic marker genes. However, OPLL is thought to be a multifactorial disorder (1, 43). It may be important to confirm whether normal spinal ligament cells will be transformed into OPLL cells only by the expression of these genes. In this context, experiments on the overexpression of P2Y1 in non-OPLL cells are now in progress.

Effect of ATP on the osteogenic genes in spinal ligament cells

Several studies have reported that ATP and ADP are
high affinity agonists of P2Y1 compared with other
nucleotides in osteoblasts and SaOS2 cells (31 – 33, 36). MRS2179 has been thought to be a selective P2Y1
antagonist (26). Addition of ATP evoked increases in the
mRNA expression levels of osteogenic marker genes,
such as ALP and osteopontin, in OPLL and SaOS2 cells,
but not in non-OPLL cells. ADP was a relatively weak
agonist for the stimulation of osteogenic genes in
ligament cells (T. Sawada et al., unpublished observation). The increase in ALP expression was abolished in the presence of MRS2179, suggesting that ATP may be a potent agonist of P2Y1 in human ligament cells and that it might commit ectopic ossification of ligament tissue.

In summary, the basal extracellular ATP level in
OPLL cells was higher than that in non-OPLL cells. Cyclic stretch as a mechanical stress dramatically increased the release of ATP in both cell types. P2Y1
purinoceptor subtypes were intensively expressed in
OPLL cells, but only weakly expressed in non-OPLL
cells. ATP stimulated the expression of osteogenic
genes, and this expression became diminished in the
presence of a specific P2Y1 antagonist. Therefore, it is
suggested that as one of the extracellular factors influencing the process of OPLL, a pathophysiological action of ATP via P2Y1 and the synergistic effects of a high basal concentration of extracellular ATP and mechanical stress are important. P2Y1 may become a target for drug therapy to control the ectopic ossification of spinal ligaments.

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