Research Article

Biological Effects of Phosphate on Fibroblast-Like Synoviocytes

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This study sought to examine the expression of genes implicated in phosphate transport and pathological calcification in osteoarthritis (OA) and rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) and investigate the biological effects of phosphate. Results revealed that several genes, which were implicated in phosphate transport and pathological calcification, were differentially expressed in OA FLS and RA FLS. Phosphate stimulated the expression of matrix metalloproteinase-1, matrix metalloproteinase-3, cyclooxygenase-2, and interleukin-1β in a dose-dependent manner. Phosphate also induced OA FLS cell death but not RA FLS cell death at higher concentration. Calcification inhibitors, phosphocitrate (PC), and ethane-1-hydroxy-1,1-diphosphonate (EHDP), effectively inhibited these detrimental biological effects of phosphate. These findings suggest that abnormal expression of genes implicated in phosphate transport and pathological calcification may contribute to the progression of OA through the induction of extracellular matrix-degrading enzymes, proinflammatory cytokines, cell death, and calcium deposits. Calcification inhibitors such as PC and EHDP are potent inhibitors of these detrimental biological effects of phosphate.

1. Introduction

Osteoarthritis (OA) is characterized by the degeneration of articular cartilage. Although the precise biochemical events that initiates OA are not well understood, many risk or contributory factors have been identified including aging, obesity, and pathological calcification. Basic calcium phosphate (BCP) and calcium pyrophosphate dihydrate (CPPD) crystals are the two most common forms of articular calcium phosphate crystals. These crystals are found in the synovial fluid of the patients with OA, and their presence in the synovial fluid correlates with the radiographic evidence of cartilaginous degeneration [1–3]. Calcium phosphate crystals were also found in the synovial fluid of up to 26% of the patients with rheumatoid arthritis (RA), and a worse clinical outcome was associated with the presence of these crystals [4].

BCP and CPPD crystals stimulate the expression of matrix metalloproteinases (MMPs), mitogenesis, and endocytotic activity of cells in monolayer culture [5, 6]. These crystals may also alter the biomechanical properties of menisci and articular cartilage [7]. Recently, Cheung et al. demonstrated that calcium phosphocitrate (CaPC), a potent calcification inhibitor, reduced the degeneration of articular cartilage in Hartley guinea pig [8]. Their study has provided support for the hypothesis that pathological calcification is a therapeutic target for the treatment of crystal-associated OA.

Treatment of human fibroblasts with BCP crystals induced a transient 10-fold rise of intracellular calcium within seconds and a second sustained rise starting at 60 minutes, which resulted from crystal dissolution [9]. It is conceivable that the intracellular concentration of phosphate could also rise due to crystal dissolution. To reduce the elevated intracellular concentration of phosphate, phosphate ions transporters or other mechanisms must be activated to pump out the extra intracellular phosphate, which, in turn, may lead to a transient rise of the concentration of local extracellular phosphate.

Phosphate plays a critical role in skeletal development, mineral metabolism, and diverse cellular functions. Besides being a key component of biomineral, it is a vital component of DNA, RNA, ATP, phospholipids, and phosphorylated
proteins. It has been shown that phosphate plays an important role in chondrocyte differentiation, chondrocyte death, and biomimeralization within the growth plate cartilage [10–12]. Since 100% of OA patients have synovial crystals, phosphate ions in the synovial fluid before crystal being formed or after crystal dissolution may potentially have some effects on fibroblast-like synoviocyte (FLS). The biological effects of calcium phosphate crystals on FLS have been examined extensively [5, 6, 13]; however, the biological effect of phosphate on FLS has not been studied. We carried out this study to investigate the expression of several genes implicated in phosphate transport and pathological calcification in OA FLS and examine the biological effects of phosphate.

2. Methods

Dulbecco’s minimum essential medium (DMEM), fetal bovine serum (FBS), and stock antibiotic/antimycotic mixture were products of Invitrogen (Carlsbad, CA, USA). OA and RA FLS cell lines, hTERT-OA 13A FLS and hTERT-RA 516 FLS, have been described previously [14, 15].

2.1. Cell Culture and Phosphate Treatment. hTERT-OA 13A FLS and hTERT-RA 516 FLS were plated at 90% confluence in 100 mm plates. On the second day, DMEM containing 10% FBS was added. Twenty-four hours later, RNA was extracted. For phosphate treatment experiments, hTERT-OA 13A FLS and hTERT-RA 516 FLS were plated at 85% confluence in 100 mm plates. On the second day, DMEM containing 0.5% FBS was added. Twenty-four hours later, cells were treated with increasing amounts of NaH2PO4 in DMEM containing 0.5% serum. Twenty-four hours posttreatment, total RNA was extracted.

2.2. Semiquantitative RT-PCR. Briefly, 1 μg of RNA sample was reverse transcribed at 50°C for 60 minutes, followed by enzyme inactivation at 85°C for 5 minutes. RT-PCR was carried out using gene-specific primers and a ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA, USA). Amplifications were carried out for 28–40 cycles by denaturing at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extending at 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and photographed using a low light image system (ChemilImage 4000, Alpha Innotech Corporation, San Leandro, CA, USA). Each RT-PCR experiment was repeated twice using two different batches of RNA samples.

2.3. Cell Morphology and Viability. The effects of phosphate on cell morphology and viability were examined. Briefly, hTERT-OA 13A FLS and hTERT-RA 516 FLS were plated in six-well plates at a density of 1.8 × 105/well. Twenty-four hours later, the media were changed to DMEM media with 0.5% FBS and cultured for forty-eight hours. The serum-starved cells were then treated with increasing amounts of NaH2PO4 in serum-free DMEM. Twenty-four hours later, the cells were examined and photographed under a microscope. The cells were then harvested, and number of cells was counted using a hemocytometer.

2.4. Inhibition by PC. To investigate the inhibitory activity of calcification inhibitors on the biological effects of phosphate, hTERT-OA 13A FLS were treated with NaH2PO4 at the presence or absence of PC or EHDP at a final concentration of 20 μM.

3. Results

3.1. Expression of Genes Implicated in Phosphate Transport and Pathological Calcification. We have recently carried out a microarray analysis of hTERT-OA 13A FLS and hTERT-RA 516 FLS and found that several genes implicated in lipid transfer, phosphate transport, or pathological calcification were differentially expressed in OA and RA FLS cell lines hTERT-OA 13A FLS and hTERT-RA 516 FLS [16]. These genes included macrophage scavenger receptor type 1 (MSR1), scavenger receptor class A, member 5 (SCARA5), progressive ankylosis homolog (ANKH), ectonucleotide pyrophosphatase-phosphodiesterase 2 (ENPP2), adenyl cyclase 3 (ADCY3), and ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1). To confirm these differential expressions, we decided to examine the expression of these genes using semiquantitative RT-PCR. Besides these genes, we also examined the expression of ectonucleotide pyrophosphatase-phosphodiesterase 1 (ENPP1) and tissue nonspecific alkaline phosphatase (TNSAP) using semiquantitative RT-PCR.

MSR1 and SCARA5 are involved in both lipid and phosphate transport [17, 18]. ANKH is a multipass transmembrane protein, which regulates transport of pyrophosphate ions across the plasma membrane [19]. ENTPD1 is an integral membrane ectoapyrase that hydrolyzes extracellular ATP/ADP. ADCY3 is a membrane-associated enzyme that converts ATP to cAMP. ENPP1 and ENPP2 are phosphodiesterase and nucleotide pyrophosphatase that hydrolyzes nucleotides to pyrophosphate/phosphate [20, 21]. TNSAP is a pyrophosphatase that hydrolyzes pyrophosphate. ENPP1, TNSAP, and ANKH have been previously implicated in pathological calcification in OA [22–25].

As shown in Figure 1, the expression of MSR1, SCARA5, ENTPD1, ENPP1, ENPP2, TNSAP, and ADCY3 were upregulated in hTERT-OA 13A FLS compared to hTERT-RA 516 FLS. These results are consistent with the previous findings that elevated activity of ENPP1 and TNSAP was detected in OA cartilage and OA-derived chondrocytes [22–24].

3.2. Effects of Phosphate on the Expressions of MMPs, IL-1β, and Cox-2. To investigate the biological effect of phosphate and its potential role in disease progression of OA, we treated hTERT-OA 13A FLS with increasing amounts of NaH2PO4 and analyzed the expression of matrix metalloproteinase-1 (MMP1), matrix metalloproteinase-3 (MMP3), and interleukin-1β (IL-1β). As shown in Figure 2(a), NaH2PO4, similar to BCP crystals [5, 26, 27], stimulated the expression
of MMP1, MMP3, and IL-1β in a dose-dependent manner. However, unlike BCP crystals [28], NaH₂PO₄ had no effect on the expression of early growth response-2 (egr-2). We also examined the expression of MMP1, cyclooxygenase-2 (Cox-2), and early growth response-1 (egr-1) in both hTERT-OA 13A FLS and hTERT-RA 516 FLS. As shown in Figure 2(b), NaH₂PO₄ stimulated the expression of MMP1 and Cox-2 in both hTERT-OA 13A FLS and hTERT-RA 516 FLS. Interestingly, NaH₂PO₄ stimulated the expression of egr-1 in hTERT-RA 516 FLS but suppressed it in hTERT-OA 13A FLS.

3.3. Phosphate Induces Cell Death of OA FLS. It has been shown that phosphate plays an important role in chondrocyte differentiation and death at a higher concentration [10–12]. Therefore, we decided to examine the effect of phosphate on FLS at a higher concentration. As shown in Figure 3, NaH₂PO₄ treatment at a concentration 3.2 mM

Figure 1: Differential expressions of genes implicated in lipid transfer, phosphate transport, and pathological calcification. hTERT-OA 13A FLS and hTERT-RA 516 FLS were plated in 100 mm plates at 90% confluence. On the second day, medium containing 10% serum was added. Twenty-four hours later, RNA was extracted and examined with semiquantitative RT-PCR for the expression of MSR1, SCARA5, ANKH, ENTPD1, ENPP1, ENPP2, TNSAP, and ADCY3.

Figure 2: The effect of phosphate on the expression of MMPs, IL-1β, and Cox-2. (a) hTERT-OA 13A FLS were treated with increasing amounts of NaH₂PO₄. The expression of MMP1, MMP3, IL-1β, and egr-2 was examined. (b) hTERT-OA 13A FLS and hTERT-RA 516 FLS were treated with NaH₂PO₄ at a final concentration of 2.6 mM for twenty-four hours. The expression of MMP1, Cox-2, and egr-1 was examined.
caused severe cell shrinkage and cell death in serum-starved hTERT-OA 13A FLS. More than 80% of the phosphate-treated hTERT-OA 13A FLS were shrunken or dead after a twenty-four-hour phosphate treatment. Interestingly, the same treatment had much less effect on the viability of hTERT-RA 516 FLS (Figure 3). It is worth noting that calcium phosphate deposits were formed and deposited on these FLS cells, especially on the OA FLS.

To further investigate the effect of phosphate on the cell viability, we treated serum-starved hTERT-OA 13A FLS and hTERT-RA 516 with increasing amounts of NaH₂PO₄. Twenty-four hours later, the numbers of cells were determined by cell count using a hemocytometer. As shown in Table 1, phosphate reduced the viability of hTERT-OA 13A FLS in a dose-dependent manner, while had no significant effect on the viability of hTERT-RA 516 FLS.

3.4. PC and EHDP Inhibited the Detrimental Biological Effects of Phosphate. We have previously shown that calcification inhibitors PC and ethane-1-hydroxy-1,1-diphosphonate, (EHDP) inhibit crystals-induced endocytotic activity and calcium-phosphate-DNA aggregates induced cell death [6, 29]. We thought that the inhibitors might also inhibit the detrimental biological effects of phosphate. To test this, we treated hTERT-OA 13A FLS with NaH₂PO₄ at the presence or absence of PC or EHDP. As shown in Figure 4, PC and EHDP effectively inhibited phosphate-induced cell death and the expression of MMP1 and IL-1β.

4. Discussion

Studies have found that excess intake of phosphate for long periods is an important factor in bone impairment and aging [30]. In addition, elevation of extracellular phosphate increases the production of reactive oxygen species [31]. In the present study, we demonstrate that several genes implicated in phosphate transport and pathological calcifications are differentially expressed in OA and RA FLS cell lines hTERT-OA 13A FLS and hTERT-RA 516 FLS. Significantly, TNSAP and ENPP1 are expressed at higher levels in hTERT-OA 13A FLS compared to hTERT-RA 516 FLS. TNSAP and ENPP1 are known for their role in pathological calcification. The differential expression of these genes suggests that OA FLS may not be passive bystanders in OA.

Both MMPs and some proinflammatory cytokines have been implicated in OA. The finding that phosphate stimulated the expression of MMP1, MMP3, IL-1β, and Cox-2 suggests that phosphate, similar to calcium phosphate crystals, may play a role in the progression of OA. Previous studies found that calcium phosphate crystals stimulated the expression of egr-1 and egr-2 [28, 32]. In this study, we found that phosphate had no effect on the expression of egr-2 and suppressed the expression of egr-1 in hTERT-OA 13A FLS. In contrast, phosphate stimulated the expression of egr-1 in hTERT-RA 516 FLS. We also found that phosphate induced cell death of hTERT-OA 13A FLS but not hTERT-RA 516 FLS. These findings are consistent with a previous report that egr-1 was expressed at lower level in OA articular cartilage [33] and that RA synovium displays severe synovial hyperplasia.

PC inhibits crystals-stimulated production of MMPs and the development of OA in Hartley guinea pigs [8, 34]. In this study, we demonstrate that PC is a potent inhibitor of phosphate-mediated induction of MMPs and IL-1β. PC at a final concentration 20 μM, which was fivefold less than the concentration of PC required to inhibit crystal-mediated induction of MMPs [29], inhibited phosphate-mediated induction of MMPs completely. This finding indicates that phosphate, similar to crystals, is a molecular target of PC.

We also demonstrate that EHDP, a stable analogue of pyrophosphate, inhibits the detrimental biological effect of phosphate. This observation suggests that pyrophosphate, an endogenous calcification inhibitor, may protect articular from degeneration. Taken together, our findings provide a support for the hypothesis that small molecule calcification

### Table 1: Cell number.

<table>
<thead>
<tr>
<th>Phosphate (mM)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>hTERT-OA 13A FLS</td>
<td>1.8 × 10⁵</td>
<td>1.3 × 10⁵</td>
<td>0.9 × 10⁵</td>
<td>0.3 × 10⁵</td>
</tr>
<tr>
<td>hTERT-RA 516 FLS</td>
<td>1.8 × 10⁵</td>
<td>1.9 × 10⁵</td>
<td>1.9 × 10⁵</td>
<td>1.6 × 10⁵</td>
</tr>
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hTERT-OA 13A FLS and hTERT-RA 516 FLS were plated in six-well plates at a density of 1.8 × 10⁵/well. Twenty-four hours later, the media were changed to serum-free DMEM media and cultured for forty-hours. The cells were then treated with increasing amounts of NaH₂PO₄. Twenty-four hours later, the cells were harvested, and cells numbers were counted using a hemocytometer.

Figure 3: Phosphate induces cell death of hTERT-OA 13A FLS. Serum-starved hTERT-OA 13A FLS and hTERT-RA 516 FLS were treated with NaH₂PO₄ at a final concentration of 3.2 mM for twenty-four hours. The cells were photographed under microscope. Results demonstrated that NaH₂PO₄ at a final concentration of 3.2 mM caused cell shrinkage and induced cell death of hTERT-OA 13A FLS but had less effect on the morphology and viability of hTERT-RA 516 FLS.
inhibitors are potential disease-modifying drugs for OA therapy. Our study had limitations. Although phosphate-containing crystals exist in the joint fluid of patients with OA, the phosphate level in the synovial fluid may change during disease process due to disease flare, new crystal formation, and crystal dissolution. Therefore, the actual biological effect of phosphate in clinical setting requires further study. Hartley guinea pigs subjecting to a high-phosphate diet may be a suitable model for the study of the in vivo effect of phosphate and its role in the disease progression of OA.

References


Figure 4: PC and EHDP inhibit the detrimental biological effects of phosphate. (a) Serum-starved hTERT-OA 13A FLS were treated with NaH₂PO₄ at a final concentration of 3.2 mM for twenty-four hours at the presence or absence of 20 μM of PC or 20 μM of EHDP. PC and EHDP inhibited cell death induced by phosphate. (b) hTERT-OA 13A FLS were treated with NaH₂PO₄ at a final concentration of 2.6 mM for twenty-four hours at the presence or absence of 20 μM of PC. The expression of MMP1 and IL-1β was examined with semiquantitative RT-PCR.


