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PROSPECTS

Inorganic Phosphate as a Signaling Molecule in Osteoblast Differentiation

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Abstract The spatial and temporal coordination of the many events required for osteogenic cells to create a mineralized matrix are only partially understood. The complexity of this process, and the nature of the final product, demand that these cells have mechanisms to carefully monitor events in the extracellular environment and have the ability to respond through cellular and molecular changes. The generation of inorganic phosphate during the process of differentiation may be one such signal. In addition to the requirement of inorganic phosphate as a component of hydroxyapatite mineral, $Ca_{10}(PO_4)_6(OH)_2$, a number of studies have also suggested it is required in the events preceding mineralization. However, contrasting results, physiological relevance, and the lack of a clear mechanism(s) have created some debate as to the significance of elevated phosphate in the differentiation process. More recently, a number of studies have begun to shed light on possible cellular and molecular consequences of elevated intracellular inorganic phosphate. These results suggest a model in which the generation of inorganic phosphate during osteoblast differentiation may in and of itself represent a signal capable of facilitating the temporal coordination of expression and regulation of multiple factors necessary for mineralization. The regulation of protein function and gene expression by elevated inorganic phosphate during osteoblast differentiation may represent a mechanism by which mineralizing cells monitor and respond to the changing extracellular environment. J. Cell. Biochem. 90: 234–243, 2003. Published 2003 Wiley-Liss, Inc.†

Key words: inorganic phosphate; osteoblast differentiation; calcium; osteopontin; alkaline phosphatase

Biological mineralization, an ongoing process throughout life, is mainly accomplished through the function of two cell types, osteoblasts and chondrocytes [Heinegard and Oldberg, 1989; Karsenty and Wagner, 2002]. The molecular and cellular events required for these two cell types to produce mineralized bone are only partially known, while the temporal and spatial coordination of events are even less understood. When mineralization occurs at inappropriate times and places the consequences can be serious, resulting in conditions such as atherosclerosis and osteoarthritis among others. A more complete knowledge of the cellular and molecular events leading to mineralization will undoubtedly aid in understanding not only disorders of bone metabolism, but also the inappropriate calcification of other tissues.

Osteoblast differentiation is a complex process in which differentiation, in vitro, occurs on the order of weeks. A number of cell culture models have been developed and are thought to reasonably represent the progression of events that occur in vivo. These models, representing both primary cultures and cell lines from various species, follow similar paths to differentiation and mineralization exhibiting a similar pattern and time frame of gene expression [Stein et al., 1990; Aubin and Triffitt, 2002]. As an example, the murine preosteoblast cell line MC3T3-E1 when treated with ascorbic acid and given a source of organic phosphate, beta-glycerophosphate (βGP) will differentiate and mineralize within 21 days [Franceschi and Iyer, 1992; Quarles et al., 1992]. The differentiation process can be generally divided into three distinct stages that are defined by: (1) proliferation, (2) matrix maturation, and

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(3) mineralization (Fig. 1, top panel). A number of genes including alkaline phosphatase, type-I collagen, bone sialoprotein, osteopontin, and osteocalcin have been identified that are expressed at high levels for discrete periods of time during differentiation. In vitro, as differentiation proceeds, the levels of alkaline phosphatase enzyme activity rise and in the presence organic phosphate will generate free inorganic phosphate [Bellows et al., 1992; Chung et al., 1992]. The result of the differentiation process is the formation of hydroxyapatite mineral that is thought to occur through two possible mechanisms, the formation of matrix vesicle, small vesicles that bud from the plasma membrane and accumulate calcium and phosphate [Anderson, 1995], and/or the nucleation of collagen, regulated by associated noncollagenous matrix proteins [Glimcher, 1989; Boskey, 1998].

Although inorganic phosphate is a necessary component of hydroxyapatite, a number of studies have suggested that it is also integral to bone remodeling in vivo [Baylink et al., 1971] and osteoblast function during the differentiation process in vitro [Bingham and Raisz, 1974; Gerstenfeld et al., 1987; Bellows et al., 1991; Tenenbaum et al., 1992]. The events that define chondrocyte maturation are somewhat different than osteoblasts, however, they do share the common features of increased alkaline phosphatase expression, matrix vesicle formation, hydroxyapatite mineral deposition, and the requirement of inorganic phosphate [Fallon

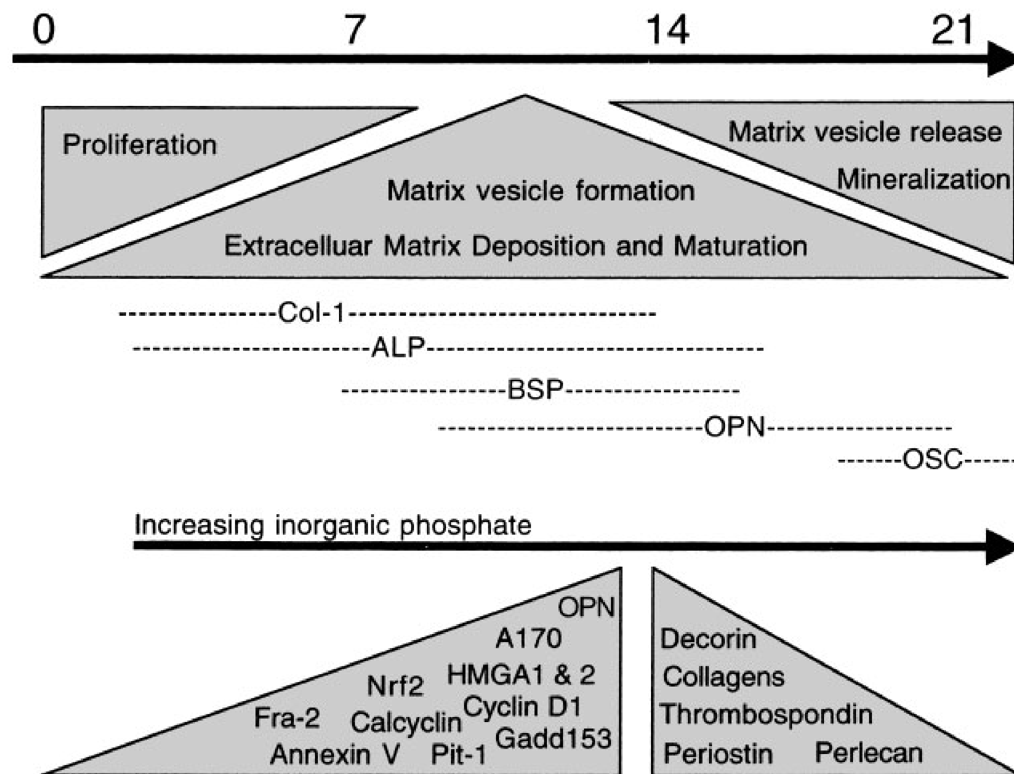

Fig. 1. Temporal coordination of osteoblast differentiation and phosphate regulated genes. **Top panel**: A schematic diagram of the three general stages of osteoblast differentiation. Proliferating osteoblasts, such as the murine preostoblast cell line MC3T3-E1, when treated with ascorbic acid and βGP at confluency (day 0) will go through a limited number of cell divisions and asynchronously exit the cell cycle (approximately day 7). As the cells exit the cell cycle, the presence of ascorbic acid increases both collagen synthesis and alkaline phosphatase gene expression and activity. During the collagen matrix maturation stage collagen accumulates and non-collagenous proteins such as bone sialoprotein (BSP), osteopontin (OPN), and osteocalcin (OSC), among others are deposited within the matrix. This stage also finds the formation of matrix vesicles that fill with a nucleation core of calcium and phosphate before release to the extracellular matrix. The final stage is marked by matrix vesicle release and accumulation of hydroxyapatite nodules within the collagen matrix. **Bottom panel**: Early in the differentiation process alkaline phosphatase levels rise and through the interaction with βGP produce increasing amounts of inorganic phosphate in the extracellular environment. A number of genes have been identified in MC3T3-E1 cells to be both positively or negatively regulated by the increase in phosphate. A general schematic represents the timing of the regulation of these genes by increasing phosphate in the context of the differentiation process.

et al., 1980; Wuthier, 1993]. This article will focus on studies surrounding the significance of inorganic phosphate generation during osteoblast differentiation as a possible signaling mechanism that may temporally coordinate cellular and molecular events preceding mineralization.

GENERATION AND TIMING OF ELEVATED INORGANIC PHOSPHATE: ALKALINE PHOSPHATASE

Alkaline phosphatase is a membrane bound enzyme situated so that the catalytic subunit is extracellular. Although originally identified in 1923 and theorized to be responsible for the production of inorganic phosphate during skeletal mineralization [Robison, 1923], the function of this enzyme in osteoblast differentiation remains a source of some debate. The importance of alkaline phosphatase in the process of mineralization is suggested by the human genetic disease hypophosphatasia [Rathburn, 1948; Whyte, 1994] that arises from mutations in the alkaline phosphatase gene and is characterized by varying degrees of bone defects. The phenotype is recapitulated in the alkaline phosphatase knock out mouse [Fedde et al., 1999]. Other evidence for the role of this enzyme in mineralization comes from the ability to transfect alkaline phosphatase cDNA into alkaline phosphatase negative cells and promote mineralization [Yoon et al., 1989] and to inhibit mineralization by inhibiting alkaline phosphatase activity [Tenenbaum, 1987]. Although numerous functions have been proposed for the enzyme [Whyte, 1994], the ability of inorganic phosphate to substitute for both alkaline phosphatase activity and βGP supplementation [Bellows et al., 1992; Boskey et al., 1992] in the mineralization process argues that one critical function is to locally increase inorganic phosphate levels.

Early in the differentiation process (days 1–4) as osteoblasts become confluent, exit the cell cycle, and respond to ascorbic acid with deposition of a collagen matrix, the levels of alkaline phosphatase RNA and activity rise (Fig. 1, top panel). As the activity of the enzyme increases in the presence of βGP, the amount of inorganic phosphate also rises. Studies investigating the requirement and timing of alkaline phosphatase and βGP in the process of mineralization have revealed that βGP, and hence elevated levels of inorganic phosphate, are required for the initiation of mineralization but, once the process is initiated, mineralization will continue at non-elevated levels in both osteoblasts [Tenenbaum, 1987; Bellows et al., 1991; Fratzl-Zelman et al., 1998] and chondrocytes [Zimmermann et al., 1992]. The study by Fratzl-Zelman et al., also investigated the length of time required for the initial exposure to 10 mM βGP to result in mineralization. These authors found a pulse of 10 mM βGP for 24 h but not 12 h, followed by exposure to low levels of βGP (2 mM) still resulted in mineralization. This agrees with the Bellows et al. [1991] study which found mineralization was blocked when alkaline phosphatase was inhibited within 8 h after the addition of 10 mM βGP but was not blocked when alkaline phosphatase was inhibited 24 h after the addition of βGP. There also seems to be a critical time point during the differentiation process, likely a stage of matrix maturation, at which the generation of phosphate promotes mineralization after which no mineralization occurs regardless of amount of phosphate added [Tenenbaum et al., 1992; Zimmermann et al., 1992]. Taken together, the above studies support the notion that the generation of inorganic phosphate may be more important to the differentiation process than the actual hydroxyapatite formation, and the ability of phosphate to affect cell function may be dependent on a particular stage of maturation.

TRANSPORT OF INORGANIC PHOSPHATE: SODIUM DEPENDENT PHOSPHATE TRANSPORTERS

In addition to the previously mentioned studies other lines of research, including the regulation of phosphate transport, have also suggested the importance of inorganic phosphate in the differentiation process. The primary mechanism for inorganic phosphate entry through the cell membrane is via a family of sodium dependent phosphate transporters. This family of transporters is subdivided into three groups, based in part on tissue specificity [Takeda et al., 2000]. Osteoblasts and chondrocytes express mainly the type III (NPT3) transporters [Caverzasio and Bonjour, 1996] which were first identified as receptors for the gibbons ape leukemia virus (Glvr-1, Pit-1) and amphotropic murine retrovirus (RAM, Pit-2) [Kavanaugh and Kabat, 1996]. These

transporters regulate phosphate transport not only through the cell membrane but also through the membrane of matrix vesicles. A number of agents have been identified, including parathyroid hormone, insulin like growth factor-1, platelet derived growth factor, fluoride [Caverzasio and Bonjour, 1996], and calcium [Schmid et al., 1998] that promote or enhance inorganic phosphate entry into the cell or matrix vesicles through these transporters. The consequences following phosphate entry into the cell are only beginning to be understood.

CELLULAR AND MOLECULAR CONSEQUENCES OF INCREASED INTRACELLULAR PHOSPHATE: POSITIVE REGULATION

An early demonstration that an increase in inorganic phosphate during bone development plays an important role in addition to mineral deposit formation came from a study by Bingham and Raisz [1974]. Using fetal rat long bones in organ cultures, this study examined the effect of increasing phosphate (1.5–4.5 mM) on bone growth and mineralization. Increasing amounts of phosphate resulted in increased collagen content, synthesis of labeled hydroxyproline, and calcification. The increase in collagen content and hydroxyproline synthesis suggested that increased phosphate regulates aspects of cell function in addition to mineralization. Although this study, and those previously mentioned in this article point to the importance of elevated inorganic phosphate in the differentiation process, the functional significance of increased intracellular phosphate has been elusive.

Over the past few years, a number of studies have begun to examine the significance of increased inorganic phosphate on osteoblast function at the cellular and molecular level. One of the first suggestions that increased inorganic phosphate may participate in directly regulating gene expression important in osteoblast function came from the study of a cell line with repressed alkaline phosphatase activity [Beck et al., 1998]. In addition to showing repressed alkaline phosphatase activity, this cell line also failed to induce expression of osteopontin as differentiation proceeded. Through a series of experiments using exogenously added alkaline phosphatase it was determined that osteopontin expression was regulated by increased inorganic phosphate [Beck et al., 2000]. Use of the phosphate transport inhibitor, foscarnet (phosphonoformic acid or PFA) established that phosphate must enter the cell to produce changes in gene expression. The sequencing of the human and mouse genomes and proliferation of microarray technology has made it possible to analyze thousands of genes instead of the limited set of osteoblast marker genes discussed thus far. A recent microarray study has identified a discrete set of genes up and downregulated in MC3T3-E1 osteoblasts by treatment with 10 mM phosphate for 72 h [Beck et al., 2003]. A number of these genes and their protein products have been identified as regulated during osteoblast differentiation or the mineralization process and are shown in (Fig. 1, bottom panel). The response of these genes to increased inorganic phosphate may provide insight into the temporal coordination of the differentiation process.

The identification of two important matrix vesicle proteins, the calcium channel, annexin V, and the phosphate transporter, Pit-1, as genes regulated by increased inorganic phosphate suggests a potentially exciting mechanism by which phosphate may help temporally coordinate differentiation and mineralization [Beck et al., 2003]. The expression of these two genes, and an additional, non-Na-dependent phosphate transporter have been previously associated with either βGP or inorganic phosphate induced differentiation and mineralization [Kirsch et al., 1997; Nielsen et al., 2001; Wang et al., 2001; Garcia et al., 2002; Wu et al., 2002]. Prior to budding and release from the plasma membrane, matrix vesicles are supplied with proteins from the cell including among others, annexin V, phosphate transporters, and alkaline phosphatase. These proteins are thought to be necessary for the eventual accumulation of calcium and phosphate within the vesicle [Anderson, 1995; Caverzasio and Bonjour, 1996]. The increased expression of both a phosphate and calcium transporter to increased inorganic phosphate suggests a mechanism by which osteoblasts and chondrocytes might coordinate the initiation of matrix vesicle formation with the generation of phosphate early in the differentiation process.

A recent study investigating the effects of phosphate and calcium on mineralization noted that both transcription and translation were required for mineralization induced by either βGP or inorganic phosphate [Chang et al.,

2000]. The microarray study by Beck et al. [2003] identified a number of transcriptional regulators that were upregulated in response to phosphate and may provide a clue to the regulation of phosphate responsive genes. One such gene was *Nrf2*, a basic leucine zipper transcription factor that functions in the regulation of phase II detoxifying enzymes [Itoh et al., 1997], and has previously been identified as a gene upregulated during osteoblast differentiation [Beck et al., 2001]. Both the demonstration that elevated phosphate will increase expression of Nrf2 in the presence of the translation inhibitor cycloheximide and the analysis of the Nrf2 promoter suggest that it is directly regulated by increased phosphate and may be considered a primary response gene [Beck et al., 2003]. The role of Nrf2 in osteoblast differentiation remains to be elucidated, although the knockout mice are viable and have not been reported to have any obvious bone defects [Chan et al., 1996]. Since Nrf2 is a member of a family of proteins and only functions as a heterodimer, it is possible that there are compensatory mechanisms involved. A number of other transcription factors were identified to be upregulated by increased phosphate including two members of the high-mobility group proteins, HMGA1 and HMGA2, the growth arrest and DNA damage inducible gene, *Gadd153* and *Fra-2*, a member of the AP-1 family of proteins. Although Gadd153, HMGA1, and HMGA2 have not been previously associated with osteoblast differentiation, the AP-1 family has been linked to bone development both in vivo [Wagner, 2002] and in vitro [McCabe et al., 1996]. Of course, as many transcription factors are regulated post-translationally, more work is required to determine the transcriptional complexes responsible for regulating the phosphate-induced response.

Another possible transcriptional mediator of the phosphate response is Cbfa1/RUNX2, a transcription factor of the RUNT family critical for the formation and function of osteoblasts [Ducy, 2000]. Cbfa1 does not appear to be highly regulated at the RNA level in response to increased phosphate in osteoblasts [Beck et al., 2003]. However, Fujita et al. [2001b] using MC3T3-E1 osteoblasts and ATDC5 chondrocyte cells identified the nuclear export of the Cbfa1 in response to the addition of 3–10 mM inorganic phosphate. The negative regulation of Cbfa1 by elevated phosphate would seem to be contradictory to the requirement of Cbfa1 in the expression of osteocalcin that occurs later in the differentiation process when phosphate levels are high. It is possible that elevated phosphate induces post-translational modifications of Cbfa1 and that these modifications are transient. Clearly, more work will be required to determine the nature of this regulation in the context of differentiation. Although the role of Cbfa1 in phosphate induced gene expression remains to be determined in osteoblasts, Cecilia Giachelli et al. have identified the elevation of inorganic phosphate and subsequent upregulation of Cbfa1 and osteocalcin expression in human smooth muscle cells (HSMC) as a key factor in ectopic vascular calcification [Jono et al., 2000]. This response is also dependent on the presence and function of the phosphate transporter Pit-1. The regulation of Cbfa1 at the RNA level differs from the osteoblast model. This may be explained by cell type specific mechanisms and/or the possibility that basal levels of Cbfa1 are much lower in HSMC, making an increase more detectable. Interestingly, in this model osteopontin also plays a key role in the response to elevated phosphate and is thought to function as an inhibitor of ectopic calcification [Giachelli, 2001].

Most of the mineralization studies mentioned so far involve in vitro culture of either primary or immortalized cells and use 4–10 mM βGP or inorganic phosphate. The use of higher levels of phosphate such as 10 mM βGP in these systems has been questioned as to its physiological relevance [Gronowicz et al., 1989; Khouja et al., 1990; Chung et al., 1992]. The findings of Beck et al. [2003] suggest that the dose of phosphate required to affect gene expression is related to the amount of time the cell is exposed to phosphate. A general curve can be constructed illustrating the time/dose relationship related to the ability of phosphate to upregulate gene expression (Fig. 2). The similar changes in gene expression in response to low doses of phosphate at longer time points relative to those at higher doses at shorter time points suggest the events occurring at the 10 mM dose are likely representative of the events that occur at lower doses but longer exposure times. Furthermore, the demonstration that cellular exposure to lower doses of phosphate requires longer times to result in gene changes, and that elevated inorganic phosphate must enter the cell to affect gene expression, suggest that it is the

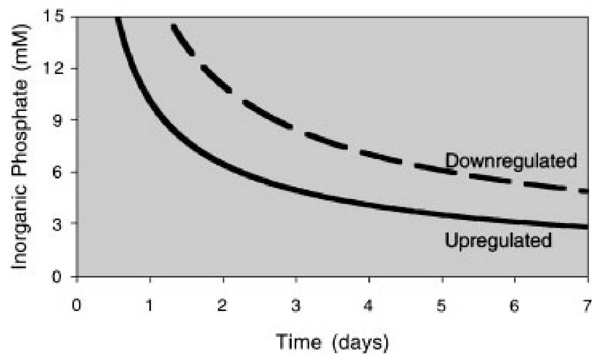

Fig. 2. Dose/time relationship of inorganic phosphate regulation of gene expression. Based on Northern blots described in Beck et al. [2003] a general curve representing the dose/time relationship of phosphate-induced changes in gene expression can be generated. The longer the cells are exposed to elevated phosphate the lower the dose required to produce changes in gene expression. For example, the dose of phosphate required for increased OPN expression within 24 h is approximately 10 mM, but if the cells are exposed for 96 h a dose of only 2.5–5.0 mM is required. Genes with increased expression (solid line) respond to lower doses of phosphate at shorter time points than the genes that are downregulated by phosphate (dashed line).

accumulated level of intracellular inorganic phosphate that is critical not necessarily the amount of extracellular phosphate added. In light of the fact that various hormones and growth factors may enhance phosphate transport and that these may differ in vitro relative to in vivo, comparing the intracellular levels of phosphate may be the most physiologically relevant determinant. Additionally, hormones, growth factors, and extracellular signals are capable of regulating many of the phosphate responsive genes mentioned in this article and therefore, in vivo, lower doses of inorganic phosphate may act in synergy with these other factors to enhance gene expression. In this case, relatively small changes in inorganic phosphate may result in elevated gene expression, although this has yet to be demonstrated.

CELLULAR AND MOLECULAR CONSEQUENCES OF INCREASED INTRACELLULAR PHOSPHATE: NEGATIVE REGULATION

Multiple studies have suggested the possibility that increased inorganic phosphate may represent a negative feedback loop capable of downregulating alkaline phosphatase activity in both chondrocytes [Genge et al., 1988] and osteoblasts [Gerstenfeld et al., 1987; Tenenbaum, 1987; Aronow et al., 1990]. However, the results from other studies found no decrease in the level or function of the enzyme in the presence of βGP [Lee et al., 1992; Chak et al., 1995; Anagnostou et al., 1996]. Yet another study found an increase in enzyme activity but a decrease in mRNA levels [Kyeyune-Nyombi et al., 1995]. The conflicting results on the response of alkaline phosphatase to inorganic phosphate suggest the likelihood that phosphate acts in synergy with other signals generated during the differentiation process and therefore the timing and amount of phosphate present in relation to stage of differentiation may be critical to the final response. For example, Farley et al. [1994] demonstrate that the level of enzyme activity is inversely proportional to calcium levels, as mineralization proceeds, the increased amount of localized calcium and phosphate may lead to the downregulation of alkaline phosphatase enzyme activity, as suggested in Genge et al. [1988].

Data generated from microarray studies on MC3T3-E1 cells also identified a number of genes downregulated in response to treatment with 10 mM phosphate for 72 h [Beck et al., 2003]. The products of these genes represent almost exclusively extracellular matrix proteins. Many of them have been previously implicated in osteoblast differentiation and include; collagens type I and III, decorin, perlecan (heparan sulfate proteoglycan 2), thrombospondin, and periostin (Fig. 1, bottom panel). The downregulation of both type-I and II collagens in response to mineralization [Gerstenfeld et al., 1987; Aronow et al., 1990; Thomas et al., 1990; Tenenbaum et al., 1992; Garcia et al., 2002] and inorganic phosphate [Boskey et al., 1992; Fujita et al., 2001b] has previously been noted in osteoblasts and chondrocytes. However, Lee et al. [1992] found no difference in the expression of matrix-associated proteins in osteoblasts treated with differentiation medium for 72 h, although this may be the result of different cell culture protocols. The downregulation of matrix proteins later in the differentiation process may serve two purposes. Since expression and translation require energy the downregulation of proteins no longer required may conserve energy stores. Additionally, the expression of genes such as decorin, periostin, and thrombospondin are more closely associated with the earlier stages of differentiation and may represent inhibitors of mineralization. In fact the requirement for the downregulation of decorin

protein levels in the mineralization process has previously been described [Hoshi et al., 1999].

Analysis of collagen type-I and periostin expression revealed that downregulation occurs only in the presence of higher levels of phosphate ($\geq$4 mM) relative to upregulated genes, at least at times tested [Beck et al., 2003]. Based on those results a general schematic of the dose/time relationship can be constructed (Fig. 2). The regulation of various extracellular matrix associated genes at higher phosphate levels and the requirement for longer exposure times agree with the hypothesis of a negative feedback mechanism that would only occur at the later stages of differentiation once matrix maturation is complete. This again emphasizes the possible role of elevated inorganic phosphate in the temporal coordination of the differentiation process.

As osteoblasts and chondrocytes are responsible for the creation of bone, osteoclasts are responsible for bone resorption. Studies suggest that inorganic phosphate may also influence bone formation by the inhibition of mineral resorption [Brand and Raisz, 1972] and osteoclast differentiation [Takeyama et al., 2001; Kanatani et al., 2003]. The Kanatani et al. [2003] study found that increasing inorganic phosphate concentrations (2.5–4.0 mM) inhibited osteoclast differentiation and the bone resorbing activity of mature osteoclasts. In this way, inorganic phosphate may not only promote bone formation but may simultaneously block bone loss.

CALCIUM TO PHOSPHATE RATIO

The localized concentration of both calcium and phosphate during differentiation and the nature of calcium and phosphate to spontaneously precipitate suggests that osteoblasts and chondrocytes must perform a delicate balancing act to create proper hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). Non-physiological precipitation must be avoided but so also must the negative effects of the simultaneous increase in both ions. Adams et al. [2001] investigated the consequences of an increase of both inorganic phosphate and calcium. The increase of relatively small amounts of calcium (0.1–1 mM), above the 1.8 mM in the medium, in the presence of elevated inorganic phosphate caused rapid apoptosis in both chondrocytes and osteoblasts. Similar studies were conducted using phosphate alone and also found significant cell death in osteoblasts [Meleti et al., 2000] and chondrocytes [Mansfield et al., 2001]. However, Wu et al. [2002] using chondrocyte cultures at a different stage of development and grown in different culture medium than the study by Mansfield et al., did not find significant apoptosis in response to elevated phosphate. These studies highlight the fact that a number of factors may influence the effect of phosphate on a given cell including, the stage of differentiation and cell type, the amount of fetal bovine serum (FBS) present (FBS is likely to contain factors that buffer calcium precipitation), and the pH of inorganic phosphate used (usually a 4:1 ratio of Na_2HPO_4 and NaH_2PO_4 resulting in a pH of 7.4). Differences such as these may significantly alter the cell response to phosphate and may be at least partially responsible for the conflicting results discussed throughout this article.

Although the results in chondrocyte cultures may still be a matter of some debate, many of the osteoblast studies discussed thus far use 10 mM βGP and 10% FBS and do not report significant apoptosis during the differentiation stage. The upregulation by inorganic phosphate of stress related factors such as Nrf2, A170, and Gadd153 and calcium binding proteins such as osteopontin, annexin V, and calcyclin may help protect the cell from the possible negative effects of calcium phosphate precipitation. Furthermore, calcium binding proteins, by balancing the calcium to phosphate ratio in the extracellular space or matrix vesicles, may aid in the formation of proper hydroxyapatite crystal as opposed to non-physiological mineral deposition. The subsequent release of bound calcium at an appropriate stage of differentiation may be another mechanism to facilitate proper hydroxyapatite formation as proposed by Wuthier [1977]. It is possible that once differentiation and mineralization is complete the lack of continued expression of calcium/phosphate regulated genes results in the cells becoming more susceptible to various bone remodeling processes that may generate unregulated increases in both ions, eventually leading to apoptosis.

SIGNALING MECHANISMS

How can an increase in intracellular phosphate produce changes at both the transcriptional and posttranslational level? Although

studies on the intracellular signaling mechanisms of increased phosphate have just begun, some understanding is emerging. Studies using calcium chelators and calcium channel blockers suggest that phosphate is neither acting by sequestering available calcium pools [Beck et al., 2003] nor by producing an influx of calcium through traditional calcium channels [Adams et al., 2001; Beck et al., 2003]. However, intracellular calcium may play a functionally significant role in mediating phosphate-induced changes. Adams et al. [2001] identified an increase in intracellular calcium following treatment of osteoblast cells with elevated calcium and phosphate prior to apoptosis. Additionally, Narayanan et al. [2003] demonstrated that the nuclear export of the calcium binding protein dentin matrix protein 1 (DMP-1) requires intracellular calcium and these authors speculate that the events are triggered by an influx of inorganic phosphate.

We have recently found that phosphate selectively activates the extracellular signal-regulated kinase (ERK1/2) signaling pathway [Beck and Knecht, submitted]. Treatment of MC3T3-E1 cells with elevated phosphate caused phosphorylation of ERK1/2 but did not activate the other mitogen activated protein kinase (MAPK) signaling proteins, p38 or the c-jun N-terminal kinase (JNK). In response to addition of 10 mM inorganic phosphate, phosphorylated ERK1/2 levels rise within 10–15 min followed by a second and more sustained phosphorylation of ERK1/2 occurring after 10–12 h of treatment. The timing of the second activation closely precedes the increased transcription of osteopontin. Inhibitors of a number of other pathways including PI3-kinase, protein kinase A, and protein kinase G do not inhibit phosphate induced osteopontin expression suggesting a high degree of specificity in the signaling mechanism induced by increased inorganic phosphate. These observations agree with a recent study demonstrating an increase in ERK1/2 phosphorylation in response to bisphosphonates that is further induced by addition of 3 mM inorganic phosphate [Fujita et al., 2001a]. These authors also did not detect activation of either p38 or JNK in response to phosphate. Although further investigation is needed to fully understand the mechanism by which increased intracellular inorganic phosphate might regulate gene expression and ultimately cell function, it does appear that specific signaling pathways exist resulting in the possibility of manipulating these pathways in the treatment of bone related diseases.

SUMMARY AND FUTURE

The studies discussed in this article have begun to shed light on the significance of inorganic phosphate in osteoblast differentiation and mineralization. The increase in inorganic phosphate may not only represent an important constituent of the mineral itself but also an important signaling molecule. The elevation of intracellular inorganic phosphate triggers a series of cellular and molecular changes that may transition the cell, matrix vesicles, and the extracellular matrix to a mineralization competent state. The role of inorganic phosphate as a signaling molecule in osteoblasts and chondrocytes is just beginning to be understood and many challenges lie ahead. The regulation of gene expression or protein function is usually the sum of multiple effectors. Therefore, it will be important to determine how signals generated by elevated inorganic phosphate are integrated with other signals generated during the differentiation process including ascorbic acid treatment, collagen matrix formation, cell to cell contact, cell cycle exit, and perhaps most importantly the accumulation of calcium. The complexity of these interactions will first require in vitro experimentation but ultimately will require confirmation in vivo. In the short term, it will likely be important to establish the mechanisms by which an increase in intracellular phosphate produces changes in gene transcription and protein function, identifying signaling pathways either stimulated or inhibited and the transcriptional complexes responsible for these responses.

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