Exploring High Dose Effects of a Bisphosphonate (HEBP)
on Osteogenesis in vitro

by

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Exploring High Dose Effects of a Bisphosphonate (HEBP)on Osteogenesis in vitro, by Bruno Girard. Degree: Master of Science, Department of Periodontology, Faculty of Dentistry, University of Toronto, 2000

HEBP induces profound cellular changes in osteoblasts. I sought to investigate the possible mechanisms of action of HEBP on the rat bone marrow cell (RBMC) culture model. I attempted to determine whether there is a receptor for HEBP or whether HEBP induced changes in intracellular calcium. I also analysed HEBP-mediated effects on osteoblastic mRNA production of the following bone related proteins: AP, BSP, Col (I), OPN, and OCN in presence and absence of mineralization. **RESULTS:** HEBP induced significant increases in AP activity and caused inhibition of mineralization in a dose dependent manner. HEBP effects on mineralization appeared to be cumulative and pulse chase experiments demonstrated that the HEBP effect is most profound at periods approaching mineralization and that HEBP has residual inhibitory effects on mineralization even after withdrawal from the cultures. Whole live cell binding studies using radiolabeled HEBP did not produce characteristic binding curves and could not be used to support the concept that there is a receptor for this bisphosphonate on osteoblasts. Further, HEBP did not cause any changes in intracellular calcium concentration suggesting that the observed cellular effects are not calcium mediated which is consistent with the lack of a putative receptor. Northern analysis revealed clear differences for several bone proteins. BSP mRNA was downregulated in non-mineralizing cultures while it was upregulated in mineralizing cultures suggesting an effect *dependent* on mineralization. Col (I) message was upregulated under either condition suggesting an effect *independent* of mineralization. OPN mRNA was downregulated in both cultures conditions suggesting again an effect independent of mineralization. OCN mRNA was increased tremendously in non-mineralizing condition and even further under mineralizing conditions suggesting an effect of HEBP that may be partially dependent on mineralization. **CONCLUSION:** We suggest that HEBP-mediated effects on expression of mRNA for proteins not restricted solely to bone [OPN, Col (I)] might be more *independent* of mineralization while HEBP-mediated regulation of mRNA for proteins that might be more restricted to bone [BSP, OCN] is more *dependent* on mineralization.

À ma famille:

Ce mémoire et dédié à mes parents: Céline et Bruno, mes soeurs: Josée et Sarah ainsi que tous les petits enfants: Justine, Philippe, Alexandra; et a Bruno (medium) pour toutes les années d'encouragement et support inconditionnel sans lequel, ce travail n'aurait jamais pu être accompli.

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List of Abbreviations

AMP or Ap	adenosine monophosphate
AP	alkaline phosphatase
AppCp	adenosine monophosphate with a bisphosphonate
APD	1-hydroxy-pentanylidene-1, 1-bisphosphonate
ATP or Appp	adenosine triphosphate
βGP	beta-glycerophosphate
BSP	bone sialoprotein
cAMP	cyclic adenosine monophosphate
$[Ca^{2+}]$	calcium concentration
col (I)	collagen type I
CPO	chick periosteal osteogenesis culture system
HEBP	1-hydroxyethylidene-1, 1-bisphosphonate
Il-6	interleukin-6
kDa	kilodalton
OPN	Osteopontin
OCN	Osteocalcin
MMP	matrix metalloproteinases
pCCl2p	1,1-dichloro ethyledene- bisphosphonate
pCp	bisphosphonate
RBMC	rat bone marrow cell culture
SBMC	rat bone marrow cell line

Introduction

Bisphosphonates have been established as good therapeutic agents for prevention and treatment of osteoporosis (Fleisch 1997). In fact, etidronate and alendronate have been approved in many countries, and both can increase bone mass and reduce fracture rates at the spine, hip, and other sites in postmenopausal women (Liberman, Weiss et al. 1995; Cummings 1998; Watts and Becker 1999). Bisphosphonates are also used in clinical settings for treatment of Paget's disease, metastatic bone disease and tumor-induced hypercalcaemia. These drugs increase bone mass due in large measure to their ability to inhibit bone resorption and to reduce activation frequency of bone remodeling units, but also may enhance mineralization (Chavassieux, Arlot et al. 1997).

Structurally, bisphosphonates are very similar to inorganic pyrophosphate, and therefore they have the ability to chelate calcium ions and bind avidly to bone mineral. Unlike pyrophosphate, the bisphosphonates are not susceptible to hydrolysis due to the presence of a phosphorus-carbon-phosphorus backbone (P-C-P) (Fleisch, Russell et al. 1970). As noted above, bisphosphonates are known to downregulate bone resorption but also inhibit bone mineralization. One of the first generation bisphosphonates, 1-hydroxyethylidene-1, 1-bisphosphonate (HEBP or etidronate), is known to inhibit bone resorption at doses very close to those that inhibit bone mineralization. For instance, a dose of 5 mg/kg/day, aimed at reducing bone resorption, can induce focal defects in bone mineralization (Weinstein 1982; Boyce, Smith et al. 1984). As well, complete inhibition of mineralization has been demonstrated in humans at a dose of 10 mg/kg/day (Hosking 1990). At such a high dose, many authors have demonstrated accumulation of osteoid

suggestive of osteomalacia (Smith, Russell et al. 1973; De Vries and Bijvoet 1974; Russell, Smith et al. 1974; Boonekamp, van der Wee-Pals et al. 1986). Because of this supposedly untoward side effect, HEBP is now administered in a cyclical regimen instead of continuously (Watts, Harris et al. 1990). This low therapeutic window also prompted researchers to develop bisphosphonates more potent in their capacity to inhibit bone resorption. At the present time, three generations of bisphosphonates are available on the market, classified according to their relative potencies. The antiresorptive potency of bisphosphonates depends mainly on the structure of the two lateral chains (R1 & R2)

$$O=\overset{\overset{\textstyle O^-}{|}}{P}-\overset{\overset{\textstyle R1}{|}}{C}-\overset{\overset{\textstyle O^-}{|}}{P}=O$$
$$\overset{|}{O^-}\quad\overset{|}{R2}\quad\overset{|}{O^-}$$
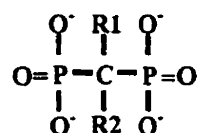
attached to the carbon (see above). In this regard, HEBP has short alkyl side-chains. The second-generation bisphosphonates such as Pamidronate® (APD) are characterized by side chains with amino terminal groups. The complexity of the molecules increases further in the third generation with the placement of heterocyclic structures within the side chains. These can dramatically affect the antiresorptive potency of these bisphosphonates. (Shinoda, Adamek et al. 1983; Sietsema, Ebetino et al. 1989; van Beek, Lowik et al. 1998). In comparison to their predecessors, for every generation of bisphosphonates, potencies have been increased by as much as 10-100 fold (Sietsema, Ebetino et al. 1989). Although the new drugs still inhibit mineralization as HEBP on a mole for mole basis, this increase in anti-resorptive potency allows for a reduction in the dose used clinically to attain a similar inhibition of bone resorption thus avoiding mineralization changes.

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The studies reported above show that bisphosphonates have numerous effects on bone including effects on modeling and remodeling. Therefore, it is important to discuss issues pertaining to bone remodeling as well as the various effects of bisphosphonates on that process and other aspects of bone metabolism.

Section 1.1 Bone remodeling

The adult skeleton is in a dynamic state, being continually broken down and reformed by the coordinated actions of osteoblasts and osteoclasts on trabecular bone and in Haversian systems. This turnover or remodeling of bone occurs in focal and discrete packets throughout the skeleton. The remodeling of each packet takes a finite period but differs in cortical and cancellous bone. Activation of the sequence of cellular events responsible for remodeling is locally controlled, most likely by local mechanisms in the bone microenvironment (Baron 1989). The first event during bone remodeling is osteoclast activation, followed by osteoclast formation, polarization, formation of a ruffled border, resorption and ultimately apoptosis. Apoptosis occurring at the conclusion of the resorbing phase of the bone-remodeling process is a recently observed phenomenon. Recently, it has been suggested that osteoclast apoptosis is a common occurrence at reversal sites and may be precipitated by resorption inhibitors such as estrogen and bisphosphonates (Hughes, Wright et al. 1995; Hughes, Dai et al. 1996).

Based on the aforementioned studies, it is clear that bisphosphonates can interfere with normal bone remodeling as well as osteoclast function. Therefore, it is valuable to discuss in further detail the effects of bisphosphonates on osteoclasts.

Section 1.2 Osteoclasts and bisphosphonates

Classically, bisphosphonates are used to inhibit bone resorption and thus, it is not surprising that the majority of the literature regarding bisphosphonates is focused on their effects on osteoclasts. Several modes of action are being investigated including bisphosphonate-mediated inhibition of the development of osteoclasts, induction of apoptosis, and reduction of activity. For example, it has been demonstrated (Hughes, MacDonald et al. 1989) that bisphosphonates may prevent the development of osteoclasts from hemopoietic precursors. Using long-term human marrow cultures, the authors observed a decreased proportion of multinucleated cells expressing an osteoclast phenotype based on TRAP, calcitonin responsiveness and monoclonal antibodies. Others, (Sato and Grasser 1990) employed osteoclastic cultures derived from the marrow of newborn rats. Cultures were grown on cortical bovine bone slices and quantification of resorption was carried out using a resorption pit assay. Incubation with various bisphosphonates caused a reduction in resorption area, suggesting decreased activity in mature osteoclasts. More recently, it has been shown that a newer bisphosphonate, risedronate, causes apoptosis in cultured osteoclasts derived from murine tibia marrow (Hughes, Wright et al. 1995). Their findings, using five different bisphosphonates, were reproduced *in vivo* (in a murine model) and related strongly with the potency of each agent tested *in vitro*. The investigators also reported that the most potent bisphosphonates *in vivo* (i.e the strongest inhibitors of bone resorption) were also the ones that caused the most apoptosis *in vitro*. Other researchers suggested that ibandronate and alendronate stimulate the production of an osteoclast inhibitory factor of a mass ranging from 1-10 kDa (Vitte, Fleisch et al. 1996). This factor has yet to be identified and could be related to

osteoprotegerin (OPG) which has been deemed responsible for inhibition of osteoclast differentiation and activity (Simonet, Lacey et al. 1997; Sakata, Shiba et al. 1999) However, it is likely not OPG since it has a mass of 55 kDa. Also related, osteoprotegerin ligand (OPG-L), produced by osteoblasts, has been shown to stimulate osteoclastic differentiation (Itonaga, Sabokbar et al. 2000). Other studies have attempted to elucidate the actual mechanisms of action of bisphosphonates on osteoclasts. In a recent study, it was shown that alendronate caused a rise in intracellular calcium levels in an osteoclast-like cell line (Colucci, Minielli et al. 1998). This finding is of great interest since it suggests the presence of a receptor for bisphosphonates on osteoclasts. Other researchers exposed several sarcoma cell lines to various second-generation bisphosphonates and observed a downregulation of bone resorption that correlated with inhibition of matrix metalloproteinases (MMP's) (Teronen, Heikkila et al. 1999). Moreover, it has been shown that low molecular weight bisphosphonates can be metabolized by mammalian cells (Azuma, Sato et al. 1995). The presence of a diphosphate in the bisphosphonate molecule renders a possible substitution with 2 phosphate groups in ATP (Appp) by a series of reversible reactions as follows:

Reaction 1: Enzyme + Amino acid + ATP => Amino acid-AMP + PPi

Reaction 2: Amino acid-AMP + pCp => Amino acid + AppCp (AMP-pCp)

Using mass spectrometric analysis, the presence of a metabolite of clodronate in intact mammalian cells was demonstrated (Azuma, Sato et al. 1995). These observations raise the possibility that osteoclasts could also metabolize bisphosphonates since these cells are most likely to be exposed to relatively high concentrations of bisphosphonate during bone resorption and could conceivably internalize these drugs by endocytosis. Owing to the non-hydrolysable nature of the ATP analogs, their accumulation in the cell could

theoretically inhibit numerous intracellular processes thus having inhibitory effects on cell function, survival, and hence resorption. To explore this hypothesis further, the same investigators used a synthetic analog of the clodronate metabolite (AppCCl$_2$p) encapsulated in a liposome to facilitate internalization by macrophage like cells J774 (Frith, Monkkonen et al. 1997). Interestingly, these findings suggest that the clodronate metabolite leads to inhibition of resorption equal to clodronate alone, and is consistent with the notion that AppCp type bisphosphonate metabolites are detrimental to osteoclast's function and could thereby explain inhibition of osteoclast activity at least for low molecular weight bisphosphonates. More potent nitrogen containing bisphosphonates are not metabolized (Frith, Monkkonen et al. 1997) (Rogers, Brown et al. 1996) (Rogers, Ji et al. 1994) and appear to affect osteoclasts through inhibition of the mevalonate pathway (Luckman, Coxon et al. 1998), which is involved in cholesterol synthesis. The exact enzymes inhibited are not known but incadronate and ibandronate are deemed to inhibit squalene synthase in the mevalonate pathway possible because of the aromatic links present in new generation bisphosphonates (Amin, Cornell et al. 1996). The findings described above clearly separate the less potent bisphosphonates from the more potent nitrogen-containing bisphosphonates with respect to the intrinsic ability to be metabolized thus indicating that osteoclast activity is altered by bisphosphonates through a variety of mechanisms.

It is evident that bisphosphonates affect bone remodeling through a direct action on osteoclasts. Moreover, it has been shown that osteoclast function can be altered by the production of an osteoclast inhibitory factor secreted by osteoblasts following exposure to bisphosphonates (Vitte, Fleisch et al. 1996). Furthermore, abrogation of IL-6

production by bisphosphonates in human osteoblastic cells has been shown (Giuliani, Pedrazzoni et al. 1998). Therefore, these studies demonstrate a potential direct effect of bisphosphonates on osteoblasts, and since osteoblasts are postulated to be involved in osteoclast recruitment and function (Suda, Takahashi et al. 1992), it is important to review osteoblast properties as well as the effects of bisphosphonates on such cells including bone lining cells.

Section 1.3 Osteoblasts, Bone proteins, Glycerophosphate and Bisphosphonates

Section 1.3.1 Osteoblasts

Osteoblasts are mature cells derived from mesenchymal cells. They are cuboidal, alkaline phosphatase (AP) positive cells, lining bone matrix at sites of active osteoid production. Osteoblasts can also be recognized by their ability to synthesize a number of matrix molecules such as collagen I (Col (I)), osteocalcin (OCN), bone sialoprotein (BSP), osteopontin (OPN), proteoglycans, and growth factors (Lian, Stein et al. 1993). The preosteoblasts, considered the immediate precursors of osteoblasts, are identified in part by their localization in the adjacent one or two layers distant from the lining bone cells. Preosteoblasts are similar to osteoblasts histologically and ultrastructurally. They are thought to have a limited capacity to divide (Aubin, Gupta et al. 1996). While preosteoblasts stain for AP, they have not yet acquired many of the other characteristics of osteoblasts (Lian, Stein et al. 1993). Approximately 10-20% of osteoblasts incorporate themselves within the newly formed ECM to become osteocytes and are considered the most mature of the osteoblast lineage.

It has been shown that the stage of differentiation of osteoblasts, can be characterized by identifying when those cells produce specific classes or types of protein (Sodek, Chen et al. 1995). Since the expression/production of those proteins are used to study HEBP effects on osteoblasts, their importance in regard to osteoblast differentiation and function will be reviewed here.

Section 1.3.2 Bone Proteins

Alkaline phosphatase

AP is a non-bone specific membrane phosphohydrolytic enzyme, oriented with the catalytic domain on the external cell surface, and is used as a marker of osteogenic development, but the control of its expression and its true function are still unclear. The best evidence for the involvement of this enzyme in bone mineralization is the human genetic disease hypophosphatasia. As well, knockout mice lacking the tissue non-specific AP have shown profound mineralization defects (Fedde, Blair et al. 1999). *In vitro* studies using levamisole, an inhibitor of AP, have demonstrated an inhibition of matrix mineralization (Tenenbaum 1987; Bellows, Aubin et al. 1991). As well a mutation in the bone/liver/kidney AP gene results in extreme skeletal hypomineralization (Weiss, Cole et al. 1988). Studies suggest that AP expression appears in differentiating osteoblastic cells (Hui, Hu et al. 1993) prior to expression of non-collagenous protein such as OCN. This protein may also play a role in differentiation itself (Hui, Hu et al. 1993) and may even regulate collagen phagocytosis (Hui, Tenenbaum et al. 1997) in connective tissue cells. Hence it is important to study production of this protein in bone cell culture.

Collagen I

Collagen type I is by far the most abundant organic constituent of bone matrix making up approximately 90% of its composition. While it is synthesized in many cell types, it is still considered a characteristic marker of the osteoblast phenotype because col (I) is a primary product of osteoblast. However, it is also clearly expressed at the preosteoblast stage (Rossert and Crombrugghe 1996).

Osteopontin

OPN is a non-bone specific 34-kDa highly phosphorylated glycoprotein with RGD sequences. OPN is produced at late stages of matrix formation just prior to mineralization (Chen, McKee et al. 1994). In vitro, it mediates attachment of many cell types, including osteoclasts. Initial immunolocalization studies showed that OPN was synthesized by preosteoblasts, osteoblasts, and osteocytes and was secreted into the extracellular matrix of bone and osteoid. Localization was especially intense at the mineralization front (Mark, Prince et al. 1987). OPN could play a role in the early formative stages of osteogenesis by mediating attachment of OB to the ECM that they are synthesizing, and could be involved in attachment of OC during bone resorption. It may also regulate crystal size and perhaps habit during mineralization of bone (Butler, Ridall et al. 1996). Ultrastructural studies showed that OPN blocks crystal elongation, thus implicating a regulatory role of OPN in controlling the shape and growth of apatite crystals (Butler, Ridall et al. 1996).

Bone sialoprotein

Bone sialoprotein is a 33-34 kDa phosphorylated and sulphated glycoprotein that is essentially unique to mineralizing connective tissues (Ganss, Kim et al. 1999). Bone sialoprotein (BSP) is highly specific for bone and, like OPN, contains an arginine-glycine-aspartic acid (RGD) cell attachment sequence that may be involved in osteoclast adhesion to bone matrix via the vitronectin receptor (Raynal, Delmas et al. 1996). BSP exhibits a much more limited pattern of expression than OPN. In general, its expression is tightly associated to *de novo* bone formation. In the skeleton, it is found at low levels in chondrocytes, in hypertrophic cartilage and in a subset of osteoblasts at the onset of matrix mineralization. BSP promotes hydroxyapatite nucleation in vitro (Hunter and Goldberg 1993). BSP marks a late stage of OB differentiation and an early stage of matrix mineralization. Interestingly, BSP knockout mice develop only very subtle bone abnormalities (Aubin, Gupta et al. 1996). Consequently, it is highly probable that it plays a role in matrix mineralization because of its relationship to the appearance of mineral, and its Ca2+ binding properties (Ganss, Kim et al. 1999).

Osteocalcin

OCN is a small protein, containing 46-50 amino acid residues and accounts for nearly 10% of the non-collagenous proteins of bone (Gallop, Lian et al. 1980). OCN is possibly the only protein present uniquely in bone and dentin and not in other extracellular matrices. OCN contains gla residues allowing binding to mineral. Of the matrix markers,

OCN is currently considered the latest of expression markers only in postmitotic osteoblasts. Disruption of the 2 genes coding for OCN, promoted an increase in bone mass in OCN-deficient mice (Ducy, Desbois et al. 1996). The increase was postulated to be due to an increase in the rate of cancellous and cortical bone formation. Similar to what is seen in high turnover osteoporosis. No effects on bone resorption or mineralization were seen. These findings suggest that, in vivo, OCN may be an inhibitor of bone formation.

Section 1.3.3 Glycerophosphate and Bisphosphonates

As discussed above, the importance of the osteoblasts in bone remodeling is evident. It has been demonstrated that the presence of osteoblasts in a coculture of osteoblasts and osteoclasts potentiate osteoclast-mediated resorption beyond osteoclasts alone (Jimi, Nakamura et al. 1996). However, their role in mediating the effect of bisphosphonates on osteoclasts is a relatively newly described phenomenon. In fact, only a few studies have suggested a potentiation of the anti-resorptive properties of bisphosphonates by osteoblasts (Sahni, Guenther et al. 1993; Yu, Scholler et al. 1996), possibly through OPG action (Simonet, Lacey et al. 1997). Employing a coculture system in the resorption pit assay the authors were able to demonstrate a much higher inhibition of resorption by bisphosphonates when osteoblasts were present in the culture versus osteoclasts alone. (Sahni, Guenther et al. 1993)

Recently, it was speculated that the enhancing effects of bisphosphonates on AP activity are due to their inhibitory effects on PGE2 production (Igarashi, Hirafuji et al. 1997).

This finding also demonstrated a direct effect of bisphosphonates on osteoblasts. Others have suggested that bisphosphonates are a substrate of AP (Felix and Fleisch 1979). Other putative substrates of AP are phosphoethanolamine, inorganic pyrophosphate, and pyridoxal 5'-phosphate (Fedde, Blair et al. 1999). While these compounds are readily available for mineralization in vivo, they are requisite substrates for mineralization *in vitro* (Tenenbaum 1981; Maniatopoulos, Sodek et al. 1988). The importance of some of these phosphates in mineralized bone forming cultures has been investigated extensively in our laboratory using a variety of model systems. It was demonstrated that alkaline phosphatase (AP) substrates, organic phosphates, such as β-glycerophosphate (β-GP) and phosphoethanolamine (Tenenbaum 1981; Tenenbaum 1987) could induce mineralization of bone formed *in vitro*. In fact β-GP is now used in most bone cell culture systems to induce mineralization *in vitro* with only few exceptions (Abe, Aida et al. 2000). The requirement for β-GP in in vitro systems has been described in over 150 publications e.g (Whitson, Harrison et al. 1984; Robey and Termine 1985; Rattner, Sabido et al. 2000). The mechanisms regulating organic phosphate effects on bone mineralization are unclear but the rationale for their use was originally based on the supposition that the organic phosphates would be metabolized by the enzyme AP, therefore liberating organic phosphate which would then lead to mineral deposition. Subsequent experiments using ultrastructural analysis demonstrated clearly that when the chick periosteal osteogenesis culture (CPO: described in detail previously (Tenenbaum and Heersche 1982)) is treated with βGP, the new bone cells as well as the mineralized bone are indistinguishable from the bone cells and tissues formed *in vivo (Tenenbaum, Palangio et al. 1986)*. The CPO is a unique culture in which 2 layers of osteogenic cells are in apposition. However, βGP treatment of chick periosteal osteogenesis cultures leads to significant and reproducible

reductions in alkaline phosphatase activity (Tenenbaum 1987), (Tenenbaum and Hunter 1987). The change in AP activity was inversely proportional to the dose of βGP and to the levels of both calcium and phosphate in the mineralized bone matrix (Tenenbaum and Palangio 1987). Interestingly, it has been demonstrated that HEBP counteracts the effects on βGP on AP activity (Tenenbaum, Torontali et al. 1992). However, it is unknown if the effects of HEBP (structure similar to inorganic pyrophosphate) are the result of direct or indirect interactions with βGP or through other cellular effects that may be dependent or independent of mineralization. Based on the previous findings, since βGP predictably induced mineralization but also inhibited bone matrix formation, it was hypothesized that there is an inverse relationship between matrix production and mineralization. This intriguing hypothesis led to several experiments, which sought to confirm and further understand this phenomenon. HEBP was used as a biochemical "probe" at a dose sufficient to inhibit mineralization, an effect postulated to produce more osteoid. Subsequent to the putative increase in matrix production, the drug would be removed, thereby allowing mineralization to resume normally, thus ultimately producing a greater amount of bone. This was demonstrated *in vitro* using the CPO model (Goziotis, Sukhu et al. 1995). In fact, HEBP induced a 70% increase in collagen synthesis and a clear increase in bone-osteoid was seen. Cultures exposed to β-GP and HEBP for a period of 6 days had 50% increased osteoid as compared to those treated with β-GP alone. The cultures were then allowed to calcify leading to two-fold increases in mineralized bone. Following these in vitro studies, further experiments were performed in vivo. Using a rat periodontal ligament (PDL) wound model, a 50% decrease in PDL width, suggesting bone ingrowth, was observed along with doubling of osteoid and bone formation around healing wounds and even in non-wounded areas (Lekic, Rubbino et al. 1997). Other

studies using non-critical size circular calvarial defects revealed similar findings. Animal with calvarial defects were exposed to a pulse dose of HEBP followed by drug withdrawal allowing mineralization. An increase in the amount of osteoid was shown as well as a faster closure (about 2-fold) of these calvarial wounds [D'Aoust, McCulloch et al. 2000]. Moreover, the data also suggested that HEBP might stimulate osteodifferentiation (Lekic, Rubbino et al. 1997; Gandolfi, Pugnaloni et al. 1999), a finding which was also suggested in our *in vitro* studies.

Several other effects of HEBP have been shown in bone forming cultures. In this regard, CPO cultures exposed to HEBP have demonstrated elevations in AP activity (Tenenbaum, Torontali et al. 1992). As well, other studies using the CPO have demonstrated an increase in the number of AP positive cells in the osteoblastic layer (Goziotis, Sukhu et al. 1995) suggesting that it stimulates osteodifferentiation as alluded to above. While an increase in collagen production by as much as 70% was reported, collagen degradation in these osteoclast-free cultures was also decreased by about 25% in pulse chase experiments. This decrease may be related to the inhibition of MMP's described recently (Teronen, Heikkila et al. 1999).

The aforementioned studies clearly show that bisphosphonates have effects on osteoblastic function and differentiation. However, whether these effects are related to modulation of mineralization or not, is not fully understood. Since the mineralization marks the final stage of osteoblastic differentiation, and since bisphosphonates do impair mineralization, the known effects of bisphosphonates on mineralization must be explained here.

Section 1.4 Effects on mineralization

Like pyrophosphate. bisphosphonates have high affinity for bone mineral and were found to prevent calcification in vitro and in vivo. (Fleisch, Russell et al. 1970). The ability of bisphosphonates to bind to bone mineral, preventing both crystal growth (Fleisch 1997) and dissolution was enhanced when the R1 side chain (attached to the geminal carbon of the P-C-P group) was a hydroxyl group (as in HEBP) rather than a halogen atom such as chlorine (as in clodronate) (Russell. Muhlbauer et al. 1970). The presence of the hydroxyl group in the R1 position increases the affinity of bisphosphonates to calcium even further since it allows chelation of calcium in tridentate rather than bidentate binding. Another proposed mechanism of inhibition of mineralization would be through the perturbation of osteoblast-derived potential nucleating proteins such as bone sialoprotein (Hunter and Goldberg 1993) and osteopontin (Morris. Randall et al. 1990: Sodek. Chen et al. 1995). Interestingly. other studies suggested that cultures grown in the presence of mineralization-inhibiting doses of HEBP were capable of mineralization again once the drug was withdrawn (Tenenbaum. Torontali et al. 1992) despite residual HEBP in the matrix (Goziotis. Sukhu et al. 1995). As well. regardless of the dose of HEBP used. inhibition of mineralization although profound was not absolute *in vitro* (Tenenbaum. Torontali et al. 1992) or *in vivo* (D'Aoust. McCulloch et al. 2000)

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Statement of the problem

An appreciable amount of evidence suggests that bisphosphonates have a rather notable effect on osteoblast metabolism. HEBP induces profound changes in the osteoblast function such as increases in AP activity (Tenenbaum, Torontali et al. 1992), augmentation in collagen synthesis and decreases in collagen degradation (Goziotis, Sukhu et al. 1995). As well since the presence of βGP is required to induce mineralization in vitro and since HEBP blocks the latter, we have performed experiments in presence or absence of βGP to determine if the effects of HEBP are largely fortuitous. Bisphosphonates may also induce rises in intracellular calcium in osteoclasts (Colucci, Minielli et al. 1998) and one may speculate similar effects on osteoblasts. Additionally it is reasonable to suggest that HEBP effects are modulated through activation or repression of one or several genes affecting specific bone protein RNA levels and that the activation of these genes is mediated through a receptor. This study aims to elucidate or clarify some of these mechanisms or pathways. One is justified in asking the following questions that are centered largely on the notion that the bisphosphonates, and HEBP in particular, have direct effects on osteoblasts. Hence I wished to determine the following:

1)   Is there a bisphosphonate receptor on the osteoblast?

2)   Does HEBP induce production of second messengers (Ca2+)?

3)   Does HEBP modulate gene expression for important bone proteins?

Objectives

1) Determine if HEBP effects on osteoblasts are receptor mediated.

2) Study HEBP effects on second messenger production (Ca2+).

3) Assess effects of HEBP on the levels of mRNA of various bone related proteins.

4) Evaluate expression of diverse bone related proteins in response to HEBP treatment.

Hypotheses

1) HEBP will bind to a receptor on osteoblasts.

2) HEBP triggers (directly or indirectly) the release of intracellular calcium.

3) HEBP will regulate the levels of mRNA of bone related proteins, that modify mineralization.

4) HEBP will regulate the levels of bone related proteins (BSP, AP, OPN) that are deposited in bone matrix.

Material and Methods

2.- Culture systems used

2.1-Rat Bone Marrow Cell culture (RBMC)

The RBMC model has been characterized by Maniatopoulos in 1988 (Maniatopoulos, Sodek et al. 1988). These cells predictably form bone-like nodules after a period of approximately 10 days in presence of Dex, βGP and ascorbic acid (Maniatopoulos, Sodek et al. 1988). Ultrastructurally, these nodules have similar appearance to that of bone. Mineralization in this culture system, has been shown to be visible microscopically as early as 3 days (Yao, Todescan et al. 1994). RBMC osteoblasts produce AP, collagen type I and III and express a variety of non-collagenous proteins such as OCN, OPN, BSP, and osteonectin (Maniatopoulos, Sodek et al. 1988). Therefore the RBMC system is a good model for studying osteogenesis because it reliably forms bone nodules and allows for assessment of mineralization as well as stages of osteoblasts differentiation. Moreover, unlike the CPO, it is mammalian system, for which numerous probes and antibodies are widely available.

2.2-Stromal bone marrow cell system (SBMC)

The SBMC is an osteoblast-like cell line generously provided by Dr Sandu Pitaru (Tel Aviv University, Israel). SBMC cells constitute a much more homogeneous population than that seen in RMBC since the former is a cell line. These cells also form mineralized nodules after a period of approximately 20 days in the presence of fibroblast growth factor, dexamethasone and βGP (Pitaru, Kotev-Emeth et al. 1993). The cells have been

shown to express AP and OCN (Pitaru, Kotev-Emeth et al. 1993). This cell line was used only in experiments requiring a more homogeneous cell population such as for receptor binding studies and calcium signalling.

3.-Culture Methods

3.1-Rat RBMC primary culture

Primary cultures of rat bone marrow were obtained by explantation of young adult Wistar rat strain femurs using a sterile technique. The explants were then sectioned at the distal segments to gain access to the marrow, from which mesenchymal cells were explanted by lavage of the medullar cavity using 10 ml of culture media. The cells were then aliquoted in T_{25} tissue culture flasks (Sarstedt, Newton, NC, USA) in a medium composed of αMEM+RNA+DNA+antibiotics (100ug/ml Penicillin G [Sigma], 50ug/ml Gentamycin Sulfate [Sigma]). The media were also supplemented with 10% fetal calf serum, 10mM βGP [Sigma], 10^{-8}M Dex [Sigma], and 50μg/ml ascorbic acid [Sigma], as these factors are required for osteodifferentiation, matrix formation and mineralization (Tenenbaum 1981) of nodules (Maniatopoulos, Sodek et al. 1988; Bellows, Aubin et al. 1991). The media were changed every 48 hours and the cells subcultured when almost confluent (approximately every 6 days). Using 0.01% trypsin at 37°C for about 5 minutes to release the cells from their substrate, the cells were subcultured at a ratio of 1:10 (~0.4 x 10^6 cells/cm^2) in T_{25} tissue culture flasks.

3.2-Rat SBMC line culture

Cells for the SBMC model were maintained in T_{25} tissue culture flasks (Sarstedt, Newton, NC, USA) in a medium composed of αMEM+RNA+DNA+antibiotic supplemented with 10% fetal calf serum, 10mM β-gp, 10^{-8}M Dex, 50μg/ml vitamin C and 3ng/ml fibroblast growth factor [Sigma]. The media were changed every 48 hours and the cells subcultured when almost confluent (approximately every 72hours) as described for RBMC.

3.3-Determination of AP activity in RBMC

The assay for AP activity is based on the enzymatic cleavage of phosphate from para-nitrophenol phosphate (pN-p) to produce the yellow reaction product para-nitrophenol. Para-nitrophenol levels, and hence AP activity, was evaluated via colorimetric analysis using a Titertek® Multiskan® MCC/340 Spectrophotometer (Flow Laboratories, Mississauga, ON, Canada) and compared to a standard curve obtained from a serial dilution of 10mM pN with bicarbonate buffer (pH 7.4) as described previously (Tenenbaum, Torontali et al. 1992). Using a flat bottomed Titertek 96-well plate, 50μl of sample and 100μl of reagent were added per well. The reagent was prepared in advance and was composed of a 3:7 ratio of 0.02M pN-p: 0.1M NaBarbital, pH9.3. Each sample was done in quadruplicate as a minimum. A single well blank was also prepared using

50μl of bicarbonate buffer. pH 7.4 instead of sample. The plate was mixed well and then incubated at 37°C with 5% CO_2 for 30 – 60 minutes. The reaction was stopped with 100μl of 0.2N NaOH. The absorption was then read at 405nm on the Titertek.

3.4-Soluble protein determination in RBMC

Soluble protein content was assayed using cultured RBMC cells. The assay for soluble protein determination was accomplished by using the Bio-Rad Protein Assay® (Bio-Rad Laboratories. CA. USA) based on the Coomassie dye-binding procedure as described previously (Bradford 1976). Using a flat bottomed Titertek 96-well plate. 80μl of sample and 20μl of reagent were added per well. Each sample was done in quadruplicate as a minimum. A single well blank was also prepared using 80μl of bicarbonate buffer. pH 7.4 instead of sample. The plate was mixed well and the reaction was complete in 5 minutes. The absorption was read at 620nm on the Titertek.

3.5-Whole cell competition binding assay in the RBMC line

Replicates of four were used in the whole cell competition binding assay as described previously (Tenenbaum. Kamalia et al. 1995:   Sukhu. Rotenberg et al. 1997). The cells were seeded at a density of 10,000 cells/well in Titertek 96-well plates and allowed to attach overnight. The media (see section RAT RBMC PRIMARY CULTURE) were removed by aspiration and replaced by media containing C^{14} HEBP at 12,000 cpm/well.

An increasing concentration of HEBP was added in the wells ranging from 0.008 μM to 500 μM. One row was not supplemented with cold HEBP to represent total binding. A binding period of 4 hours was used after which the media were removed and cells were washed 4 times with 100 μl of PBS. The PBS was replaced with Sulphuric acid (100 μl. 0.1N) and left at 37 °C overnight to break cells. Radioactivity was assessed in a beta counter. The experiment was replicated twice.

3.6-Intracellular calcium measurements

Measurement of intracellular calcium ion concentration ($[Ca^{2+}]$) has been carried out using the modified method described by Grynkiewicz (Grynkiewicz, Poenie et al. 1985). Briefly. SBMC cells on coverslips in media (see above) were incubated at 37°C with 3μM of fura-2 AM (Molecular Probes. Eugene. OR) for 30 minutes. Whole cell $[Ca^{2+}]$ measurements were obtained with a dual excitation. microscope-based spectroflorimeter (Photon Technology Int.. London. ON). A variable aperture. intrabeam mask is used to restrict measurements to a single cell. Estimates of $[Ca^{2+}]$ are calculated from dual-excitation emitted fluorescence. HEBP 50-100 μM has been added and the changes in $[Ca^{2+}]$ have been estimated.

3.7-RNA extraction and Northern Blot Analysis in RBMC

Total RNA was extracted using the guanidinium thyocyanate method [Current Protocols].

The concentration of RNA extracted was determined by measuring the absorbance at

260nm (A_{260}) in a spectrophotometer. Ten µg of total RNA were dissolved in 10 µl H_2O,

and precipitated with 20 µl of 2% KOAc in absolute EtOH R -20°C for 20 minutes. After

centrifugation for 10 minutes (4°C and 14,000 g), the pellet was dissolved in 1x MOPS

running buffer containing 50% formamide and 2.5M formaldehyde. RNA was denatured

by heating for 10-15 min at 60°C. RNA running buffer, containing bromophenol blue

was added, and the sample were ran on an 1% agarose gel at 60V until the bromophenol

blue migrated 2/3 the length of the gel (approx 4 hours). The gel was examined on a UV

transilluminator at 254 nm to visualize the RNA and photographed with a ruler laid along

one side. The gel was then transferred by capillary blot overnight to a Hybond™-NX

nylon membrane optimized for nucleic acid transfer (Amersham LIFE SCIENCE,

Buckinghamshire, England). Finally, a 4-minute UV exposure at 254nm was used to bind

the RNA covalently to the membrane. Northern blot analysis began with prehybridization

of the nylon membrane in 15ml of ExpressHyb™ Hybridization solution overnight at

68°C. Probe to be used was prepared the next morning using 50-75ng of double strand

cDNA template, 10µl of a mix of complementary nucleic acids (Reagent mix-Amersham

pharmacia biotech, Buckinghamshire, England), 5µl of 0.25mM dCTP-^{32}P (Amersham

pharmacia biotech, Buckinghamshire, England) and 1µl of T7 polymerase (Amersham

pharmacia biotech, Buckinghamshire, England) which were added together. The sample

was then incubated at 37°C for ½ hour. Product was then loaded on a Probe Quant™ G-

50 spin column (Micro Columns, Amersham pharmacia biotech, Buckinghamshire,

England) and spun at 3,000g for 2 min. Following this, 2µl of the solution was added into

5ml of scintillation fluid (Ecolume-ICN) and counted in a scintillation counter

(Beckmann) to determine the activity of the probe. The probe was boiled for 5 minutes, cooled on ice for 2 minutes and then appropriate volume of probe was added to attain 1×10^6 counts per ml of hybridizing solution. This was added into 15ml of hybridization solution and hybridized to the membrane for 2 hours at 65°C. The membrane was then washed in 1L of 2x SSC and 0.05% SDS for 1 hour at 58°C and was then exposed on BioMax™ Film (Eastman Kodak, Rochester, New York) overnight at -70°C. The film was developed in an automatic processor (Kodak, Rochester, New York) and scanned on an Epson Expression 636 flatbed scanner. The bands were quantified using ImageQuant™ version 1.2 for Macintosh (Molecular Dynamics, Sunnyvale, California).

3.8-Comparison of HEBP and dexamethasone effects on AP in RBMC primary cultures

Cells were seeded at a density of 3,500 cells/well in 96-well plates (Titertek). Media containing decreasing concentrations of Dex ranging from 10^{-6} to 10^{-12}M were added. On another plate, Dex was added to the cells in a similar fashion and supplemented with HEBP 50μM. The media were changed every second day and the cultures were allowed to grow for 4 days then were assessed for AP activity and protein content.

3.9-Mineralization of Rat RBMC primary cultures

RBMC cultures were seeded at a density of 100,000 cells/well in 6 well plates. Cultures were allowed to grow for 8 days in media containing 25μM, 50μM, 75μM, or no HEBP. The media were replaced every 2 days. At the end of the 8-day culture period, the media

were removed from the wells and the cells were washed with PBS once. The cells were then fixed with 10% formalin for a period of 5 minutes. After washing three times with 1 ml of H2O, 500 µl of 2% Alizarin Red-S (Sigma) was added, again for 5 minutes. Final washing with water was done and photographs of the cultures were taken.

Similarly, 96 well plates were seeded at a density of 5000 cells/well. HEBP at a concentration of 50µM was added for various periods into the media according to the timeline diagram shown below. After 6 days, The media were removed from the wells and the cells were washed with PBS once. The cells were then fixed with 10% formalin for a period of 5 minutes. After washing three times with 1 ml of H2O, 500 µl of 2% Alizarin Red-S (Sigma) was added, again for 5 minutes. Final washing with water was done and quantification was done in the Titertek plate reader at 525nm.

Table 1: Timeline diagram of temporal exposure of RBMC to 50µM HEBP
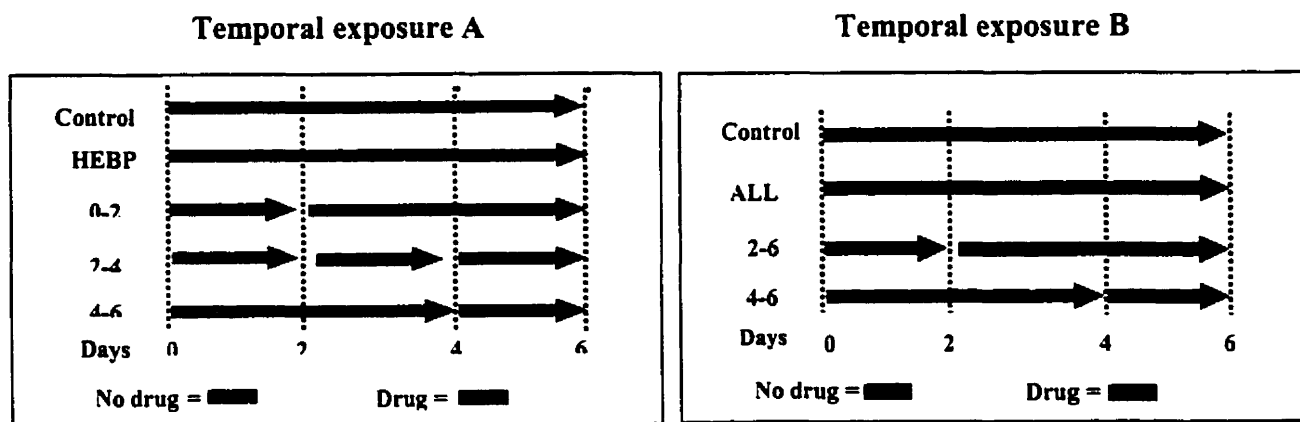
3.10-Immunohistochemistry of non-collagenous protein in the RBMC model

Cells were plated at a density of 3,500 cells per well in a 16 well chamber slide. Cells were allowed to grow for 4 days in media containing 25µM, 50µM, 75µM, or no HEBP.

The media were replaced every 2 days. The media were removed from the wells and the cells were washed with PBS once. The cells were then fixed with 2% paraformaldehyde for a period of 15 minutes then washed with PBS 3 three times. Cells were then exposed to Tween 0.5% for 10 minutes followed by a rinse with PBS three times. To block endogenous peroxidase activity, cells were incubated with 3% H_2O_2 and 30% methanol for 15 minutes. Blocking of non-specific background was achieved by incubating cells 20 minutes with casein containing 0.1% sera of the animals in which the primary and secondary antibodies were produced. Cells were then incubated with primary antibody for one hour then washed 5 times with PBS. Background measurements were obtained by exposing to casein buffer only. Secondary antibody was left for 15 minutes. Cells were washed 3 times and incubated 15 minutes with avidin-biotin complex conjugated to horseradish peroxidase enzyme (ABC-HRP Vector Laboratories, Calif) which contains 0.1M Tris buffer with 2% reagent A and 2% reagent B (Vector Laboratories, Calif). Cells were washed three times and then incubated with 3,3'-Diaminobenzidine (DAB substrate for peroxidase) for 10 minutes

RESULTS

AP activity in RBMC at various time points

AP activity in 2-day cultures continuously treated with 25, 50, or 75 μM HEBP was no different from control (fig. 1,▨). At 4 days, several differences were found between groups. Primarily, longer exposure to HEBP alone led to an increase in AP activity for all the groups beyond the 2-day results. Specifically for the 4 days exposure, the addition of 25, 50, and 75 μM HEBP induced significant (116%, 88%, and 62% respectively; $p < 0.05$) increases beyond control (fig. 1,▣). For 7 day cultures, AP activity in the control groups increased even further as compared to 2 and 4 days (i.e. the expected increase over time). Total AP activity for HEBP treated groups appeared to plateau beyond 25 μM. Even in this case, there was an average 19% increase above control level (fig. 1,▮).

Immunostaining for AP was evaluated following continuous exposure to either no, 25 μM, 50 μM, or 75 μM HEBP at 6 days (fig. 2). Qualitative assessment of the cultures suggested higher staining than control especially at higher concentrations of HEBP (50 and 75 μM) as shown biochemically. Densitometric quantification with Macintosh ImageQuant revealed some staining differences between groups however these were not significant (Data not shown) due to variation in staining intensity.

Dex versus Dex + HEBP 50 mM in RBMC

AP activity in RBMC cultures was measured in response to various Dex concentrations

in the presence or absence of 50 μM HEBP. AP activity in RBMC with Dex alone

essentially followed the trend of a bell shape curve following exposure to the various Dex

concentrations. (fig. 3, ▬). The addition of HEBP in the media (fig. 3, ▨) followed

a similar pattern. The addition of HEBP to the media (Dex-HEBP) appeared to slightly

increase AP activity beyond dex only cultures. However the differences were not

significant. The AP levels found for the HEBP alone groups (no Dex) were comparable

to the levels observed for 10^{-6} M, 10^{-9} M, 10^{-10} M Dex.

Effect of continuous exposure of HEBP on mineralization in RBMC

Alizarin red staining was used to assess mineralization in RBMC. A dose dependent

effect on inhibition of mineralization was observed following continuous exposure to

increasing doses of HEBP for 10 days (Fig. 4a). Decreased staining was observed with

increasing concentrations of HEBP. Indeed, at 75 μM HEBP, almost complete inhibition

of mineralization was observed.

Temporal effects of exposure to HEBP on mineralization

To probe the phase of culture maturation during which HEBP has greater effects on mineralization, this agent was added according to time line diagram shown in figure 4B. Control cultures mineralized normally. Continuous exposure to HEBP led to a significant ($p<0.001$) reduction in mineralization, as much as 93% less than control. Early exposure to HEBP (0-2 days) altered the mineralization potential of the culture by 5%, which was statistically insignificant. However at later time points (2-4 and 4-6), significant decreases ($p<0.05$) in mineralization were observed (26% and 63% less than control respectively). As well, a significant difference in alizarin red staining was found between groups treated with HEBP between day 4-6 and continuous exposure to HEBP.

When HEBP was used to probe other phases of culture (fig. 4C), mineralization was reduced in all groups ($p<0.001$) to 4%, 5%, and 12% of control respectively.

Competitive Binding of HEBP vs C14 labelled HEBP

Because HEBP's known effects on AP activity and osteoid production, I sought to investigate one of the possible mechanisms of its action on osteoblasts in which HEBP might bind to a putative surface receptor on osteoblasts. The competitive binding assay using radiolabelled HEBP did not demonstrate any counts beyond background. Despite numerous attempts using variable conditions, it was not possible to demonstrate competitive binding of radiolabelled HEBP to RBMC cells. Therefore, this experiment

did not support hypothesis that osteoblasts possess a receptor to HEBP (DATA NOT SHOWN).

Intracellular calcium experiments

Intracellular calcium measurements using a calcium containing buffer

Despite the findings using radiolabelled HEBP, I still chose to evaluate whether or not the phenotypic changes induced by HEBP in osteoblasts could be tied to calcium signalling. Viability and responsiveness of cells was assessed by addition of ionomycin (I) in presence of a calcium buffer (fig. 5a). The intracellular calcium concentration increased by about 12 times and diminished over a period of few minutes, reaching a plateau located about two times above the original levels. This suggested that the cells were both viable and that appropriate dye loading had occurred. Therefore, if HEBP would perturb calcium levels, it should have been detected, but this did not occur.

Intracellular calcium measurements using calcium free buffer

The measured intracellular concentration changed from 100 nM to 400 nM immediately subsequent to the addition of ionomycin (I) (fig. 5b). Addition of EGTA (E) caused a diminution of intracellular calcium by about 50 nm, which rapidly went back to levels observed prior to addition of EGTA. Intracellular calcium slowly decreased with time although it appeared to remain higher than baseline concentrations. Subsequent

experiments examined addition of 100 μM HEBP in the culture, for which single cell

fluorescence was investigated (fig. 5c). The addition of HEBP did not appear to alter the

intracellular calcium levels. Cell responsiveness was then assessed using ionomycin,

which increased the intracellular calcium levels by about 4 times.

Analysis of HEBP effects on mRNA expression in RBMC cultures

Although my initial studies failed to elucidate the signal transduction mechanisms

governing HEBP effects on osteoblasts, it is still clear that due to the observed

phenotypic changes in osteoblasts, induced by HEBP, there must be perturbations in gene

expression. Thus mRNA expression for various important bone proteins was assessed.

The quantification of the band density on Northern blots was made using Imagequant®

for Macintosh®. The interpretation of the results is based on the mathematical results

obtained from the computerized scans. In some cases, the message or the gapdh levels

were too low, leading to an unacceptable noise to message ratio, thus magnifying the

error. When such a case occurred, the mathematical data were not shown on the graphs.

Two RBMC explant cultures were pooled and then plated in 100 mm dishes at a density

of 700,000 cells/dish, for a total of 18 dishes + 6 as backup. It was decided that using

these dishes rather than working with seventy-two 35mm dishes would simplify analysis

and reduce error in pooled samples. However using only one culture may not be

characteristic of the general culture behavior. Yet, similar results were obtained from

replicate blots of pooled cultures grown for other experiments (+βGP or -βGP) focused

on mRNA for BSP and OCN suggesting that pooling cultures provided reliable results. It

should also be pointed out that gapdh message appears quite uneven in some culture groups suggesting uneven loading of lanes. However, since mRNA readings were also normalized against gapdh, the variation in loading was controlled for.

BSP expression

As shown in figure 6, 50 μM HEBP caused an approximate 50% reduction in message for BSP in cultures grown without βGP. βGP treatment alone also reduced BSP message by almost 60%. This βGP-mediated reduction in BSP message was attenuated by increasing concentrations of HEBP such that at 75 μM HEBP, BSP message was almost 2-fold higher than that noted in cultures treated with βGP alone. This message level was still lower than that seen in cultures grown without βGP. Therefore, it appears that without βGP, HEBP downregulates BSP expression while in the presence of βGP, HEBP may actually upregulate BSP at day 5, which are apparently opposite effects. At 7.5 days (fig. 7), HEBP (50m μM) appears to upregulate BSP expression in the presence and absence of βGP. By 10 days in culture (fig.8) the effects of HEBP and βGP expression are modest at best and not as noteworthy as the effects at earlier stages.

Col (I) expression

In relation to mRNA for type I collagen, the following findings were obtained. As suggested in earlier investigations, βGP treatment reduced col (I) expression relative to control. There were no measurable levels for col (I) in 5-day cultures suggesting that

there were little or no matrix synthesis occurring. By 7.5 days (fig .9), it was shown that

HEBP stimulated col I mRNA synthesis by at least 2-fold (at 50 μM HEBP) whether or

not βGP was present in the medium. However, by day 10 (fig. 10) HEBP alone had no

stimulatory effect on col (I) message in cultures not treated with βGP whereas the

stimulatory (approximately 2-fold) effects of HEBP were still present in cultures also

treated concurrently with βGP.

OPN expression

As shown in figure 11, HEBP 50μM caused an approximate 20% reduction in message

for OPN mRNA in cultures grown without βGP. Addition of βGP had no effect on

mRNA levels for OPN. In βGP treated cultures, the addition of HEBP led to a trend

showing decreased mRNA levels by 20% to 45% as compared to βGP alone groups.

Therefore, it appears that HEBP decreases OPN mRNA levels with or without βGP but

with greater effects in βGP treated cultures. However this also suggests an effect that is

independent of βGP-induced mineralization or at least βGP treatment. As the culture

matured, at day 7.5 (figure 12), it appeared that the addition of HEBP upregulated OPN

mRNA in cultures grown without βGP while it downregulated the mRNA in presence of

βGP. At day 10 (fig. 13), addition of 50 μM HEBP resulted in a 20% decrease in OPN

mRNA in cultures grown without βGP. The addition of βGP caused an approximate 20%

decrease in OPN mRNA compared to cultures grown with βGP. Again the addition of

HEBP in cultures grown in presence of βGP led to an approximate 50% reduction in OPN mRNA levels.

Immunostaining for OPN

Immunochemical staining for OPN was carried out under similar conditions as described for AP. Visual assessment of the staining showed no significant differences between groups (DATA NOT SHOWN). Slightly higher staining was observed of the 50 and 75 μM HEBP treated groups. The areas where the staining was increased were very localized around nodules.

OCN expression

Unlike BSP and OPN message, OCN mRNA was not detectable at five and 7.5 days (DATA NOT SHOWN). In mature osteoblasts (day 10, fig. 15), the addition of 50 μM HEBP resulted in a greater than 4-fold increase in cultures grown without βGP. Addition of βGP resulted in an almost 20-fold increase compared to cultures grown without βGP. In cultures treated with βGP, the addition of HEBP 25 μM resulted in a 150% increase while addition of 75 μM HEBP led to a decrease of approximately 60 % compared to plus βGP alone however still higher than minus βGP alone by over 700%. Therefore, it appears that HEBP upregulates OCN mRNA in cultures grown with or without βGP. However, the addition of βGP alone also has a profound stimulatory effect on OCN mRNA production.

Fig. 1 - Alkaline phosphatase activity relative to soluble protein at various time points in RBMC culture treated with 0, 25, 50, or 75 μM HEBP. Treatment with HEBP significantly increased alkaline phosphatase activity beyond control (p<0.05) except for 2 days exposure. Each bar represents the mean of 9 cultures (N=9) and the vertical bar represents the standard error of the mean.
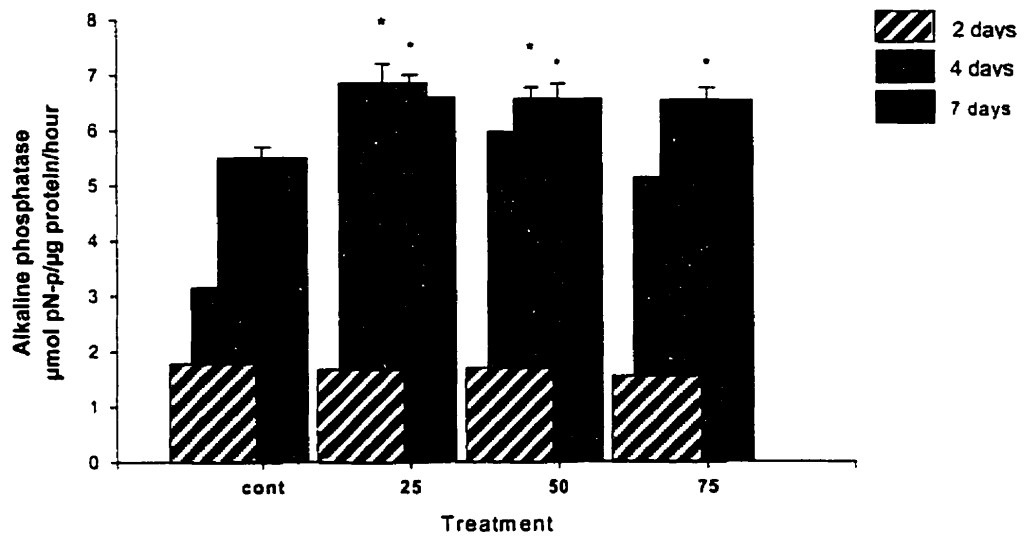
Fig. 2 – Photomicrographs of 6 day RBMC cultures stained immunohistochemically for AP. The staining for control group (C= no drug) was more elevated than background (B=no primary antibody). The addition of 25, 50, and 75 μM HEBP to the cultures resulted in a modest increase in staining for alkaline phosphatase versus control(N=3).
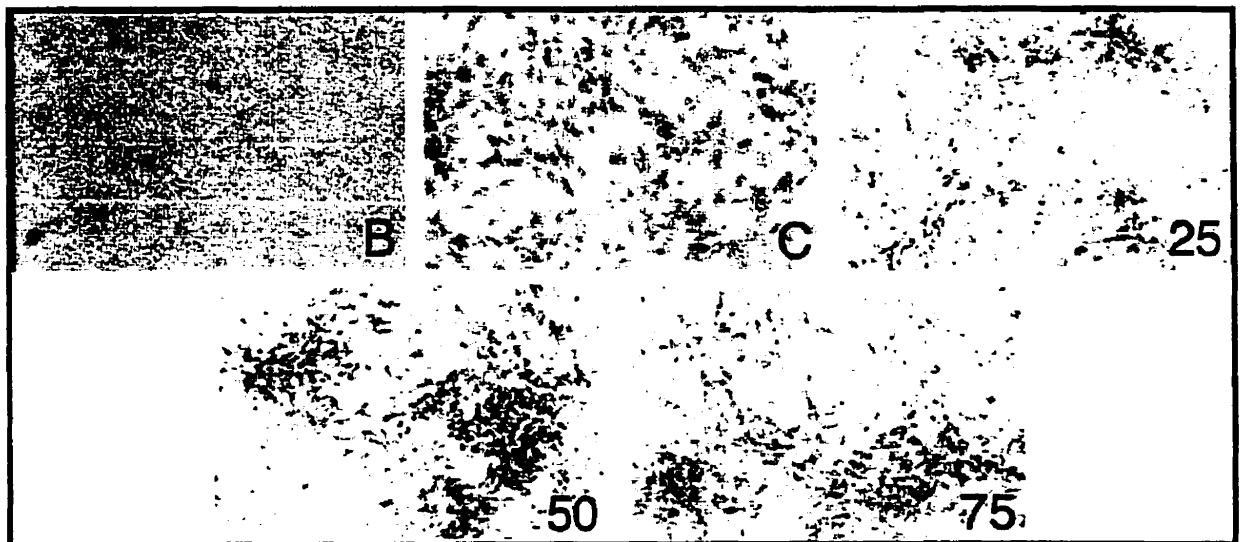
Fig. 3 - AP relative to soluble protein in RBMC at 4 days. Culture were treated with either no or 50 μM HEBP combined with variable Dex concentrations. Treatment with HEBP did not increase AP activity of the cultures beyond Dex alone, except when suboptimal doses (10^{-9}, and 10^{-10}M) of Dex were used.)
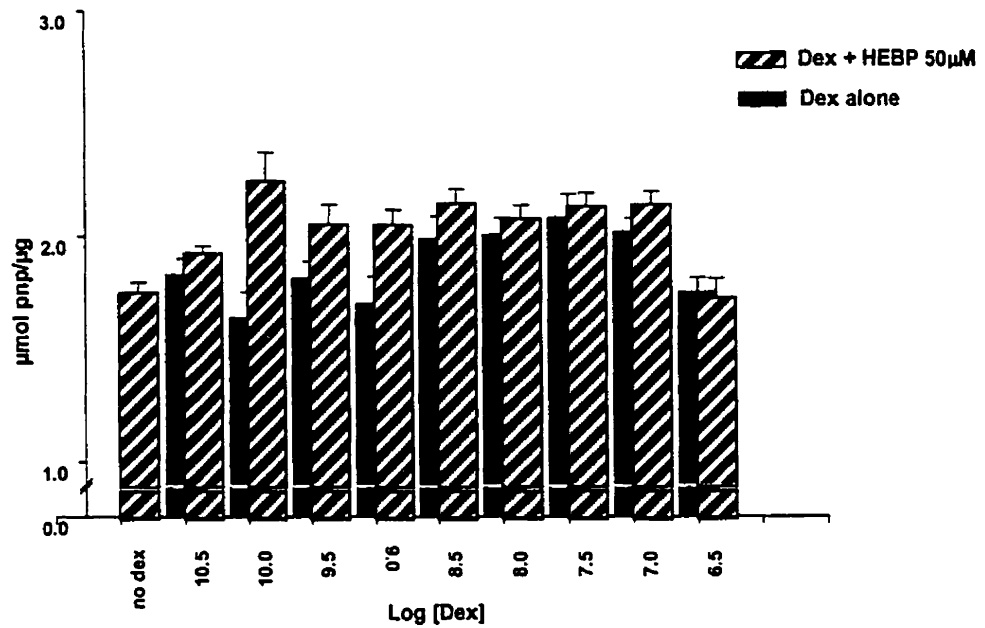
Fig. 4A – Alizarin red stained (appearing black in this figure) RBMC culture showing mineralization following continuous exposure to either no, 25, 50, 75 μM HEBP for 10 days. A dose dependent reduction in staining and hence mineralization is shown with virtually complete inhibition at 75 μM (N=3).

CONTROL   25   50   75

Fig. 4B - Calcification of RBMC cultures pulse-treated with 50 μM HEBP for specific time periods (see timeline diagram below). Absorbance (525 nm) was measured for alizarin red staining (appearing black in this figure). Pulse treatment with HEBP over days 0-2 did not affect the mineralization potential of the cultures while other exposure periods lead to a significant decrease in mineralization compared to control (p<0.05). Each bar represents the mean of 9 cultures (N=9) and the vertical bar shows the standard error of the mean

* = p<0.05

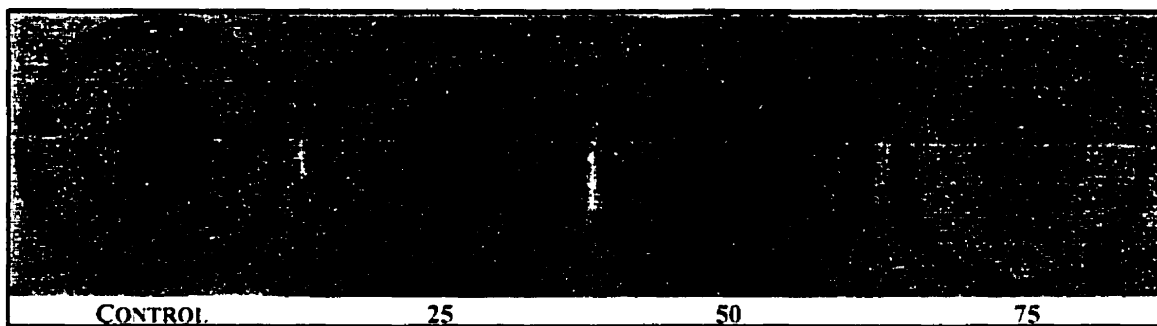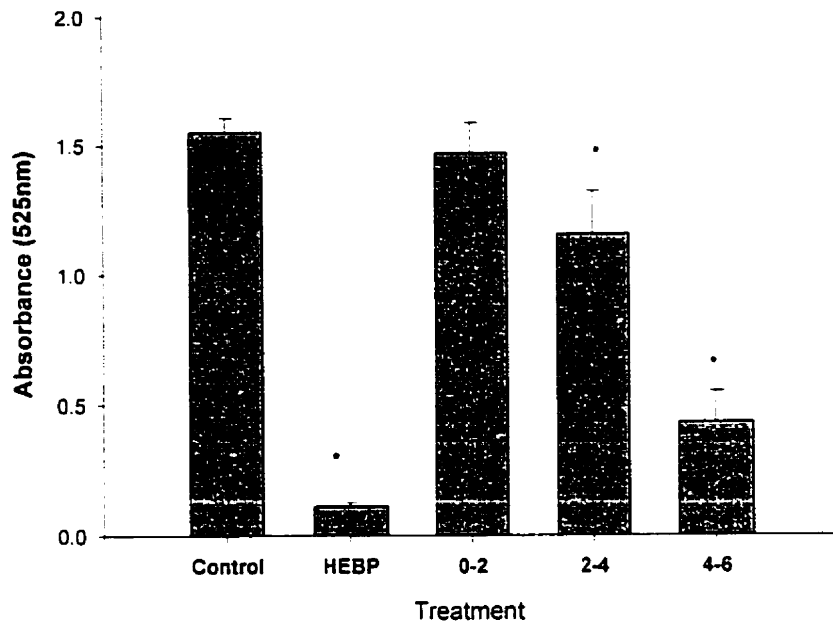**Timeline diagram of drug exposure**
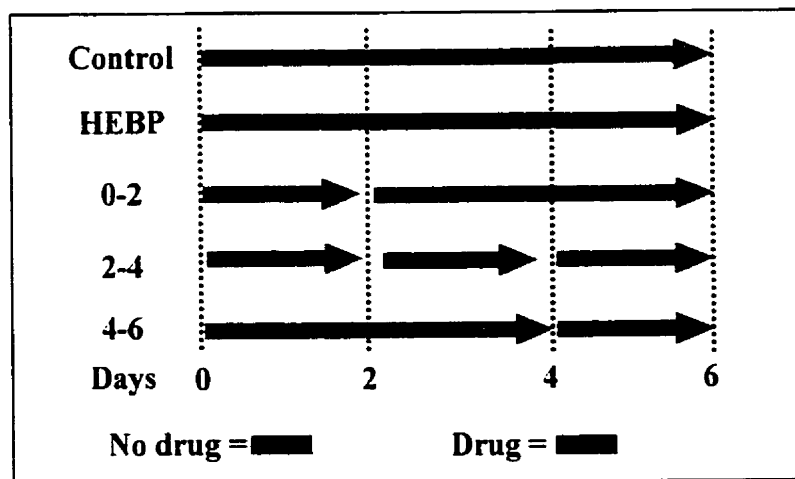
Fig. 4C - Calcification of RBMC cultures pulse-treated with 50 μM HEBP for specific time periods (see timeline diagram below). Absorbance (525 nm) was measured for alizarin red staining (appearing black in this figure). Treatment with HEBP consistently caused a decrease in mineralization of the cultures in comparison to control ($p<0.05$). Each bar represents the mean of 9 cultures (N=9) and the vertical bar shows the standard error of the mean
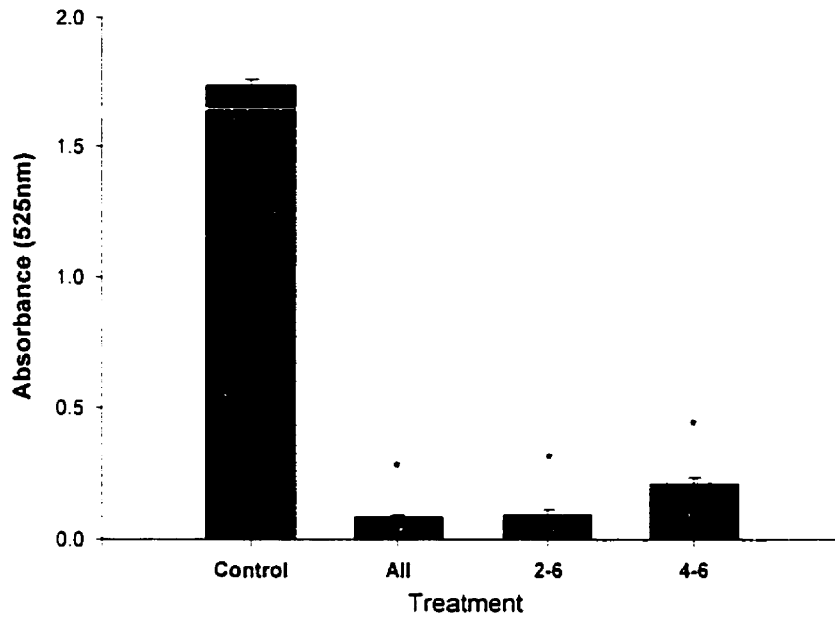
* = $p<0.05$

Timeline diagram of drug exposure
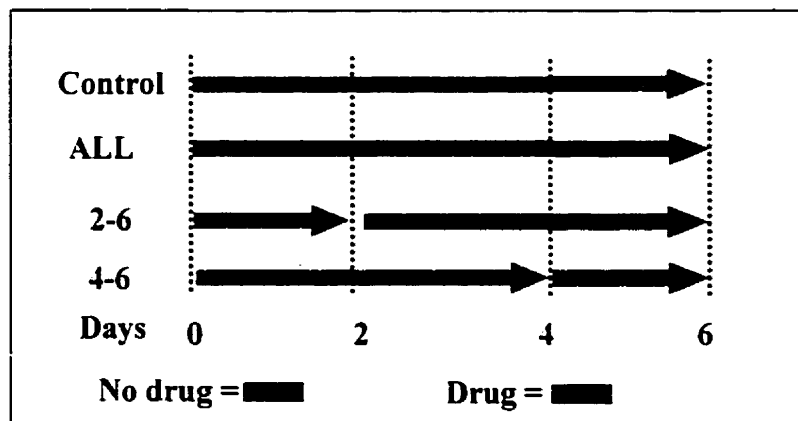
Fig. 5A – Single cell (using SBMC model) intracellular calcium concentration in the presence of a calcium-containing buffer following addition of ionomycin (I) alone. The addition of ionomycin to the cells resulted in a dramatic increase in intracellular calcium, which decreased with time. Typical results from 3 different trials (N=3).
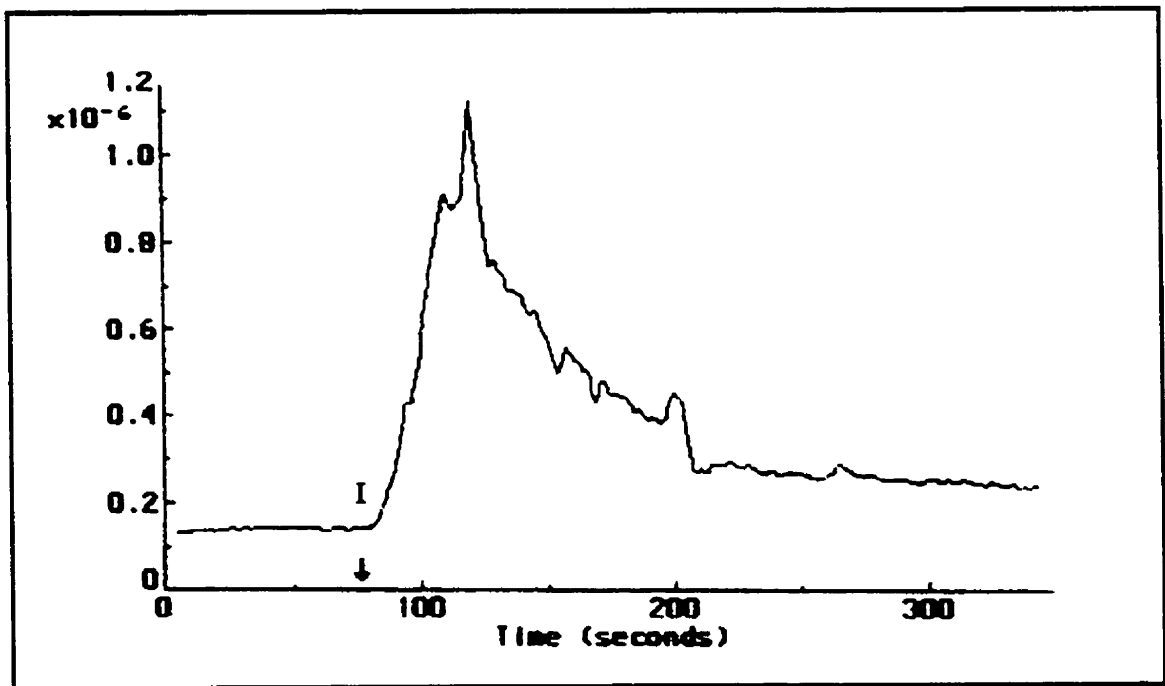
Fig. 5B - Intracellular calcium concentration (single cell SBMC) in calcium-free buffer following addition of ionomycin (I) and EGTA (E). The addition of ionomycin resulted in an increase in intracellular concentration, which was briefly reduced by the addition of EGTA. Typical results from 3 different trials (N=3).
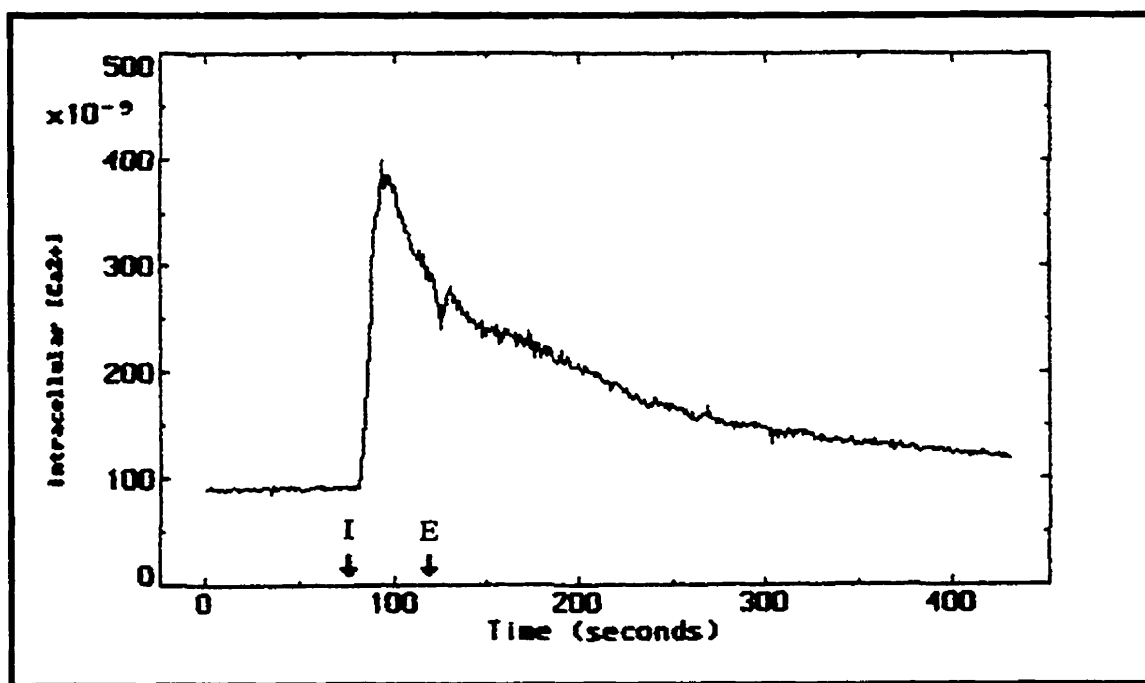
Fig. 5C - Intracellular calcium concentration (single cell SBMC) in calcium-free buffer following addition of HEBP (H) and ionomycin (I). The addition of HEBP did not alter intracellular calcium concentration while the addition of ionomycin resulted in an increase in intracellular calcium. Typical results from 3 different trials (N=3).

Fig. 6 - Levels of mRNA for BSP in RBMC cultures at 5 days (normalized against gapdh). Levels in cultures grown without βGP were taken as 100% (control). Treatment with HEBP, in absence of βGP, appeared to cause of reduction in BSP mRNA levels. However in the presence of βGP, there appears to be a dose dependent stimulatory effect of HEBP on the expression of BSP. The total RNA was extracted from 1 culture per group ($n=1$).
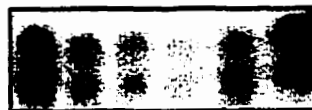
Fig. 7 - Levels of mRNA for BSP in RBMC cultures at 7.5 days (normalized against gapdh). Levels in –βGP treated cultures were taken as 100% (control). The presence of HEBP in the minus βGP group appeared to cause an increase in BSP mRNA levels. The presence of βGP alone in the media resulted in higher mRNA levels than the minus β-gp groups. The presence of HEBP in the βGP-treated group resulted in increased levels of BSP mRNA. Total RNA was extracted from 1 culture per group (N=1).

-gp	= no βGP, no HEBP
-gp50	= no βGP + HEBP 50μM
gp	= βGP 10 mM, no HEBP
gp25	= βGP 10 mM +HEBP 25μM
gp50	= βGP 10 mM +HEBP 50μM
gp75	= βGP 10 mM +HEBP 75μM

BSP

gapdh

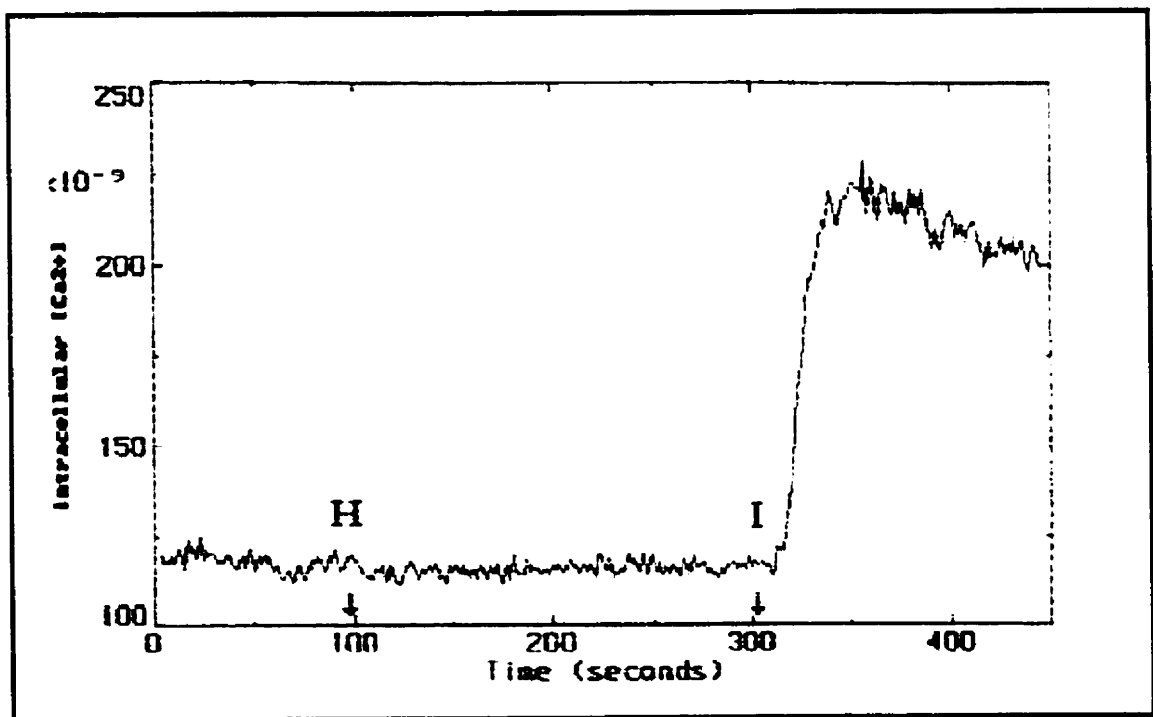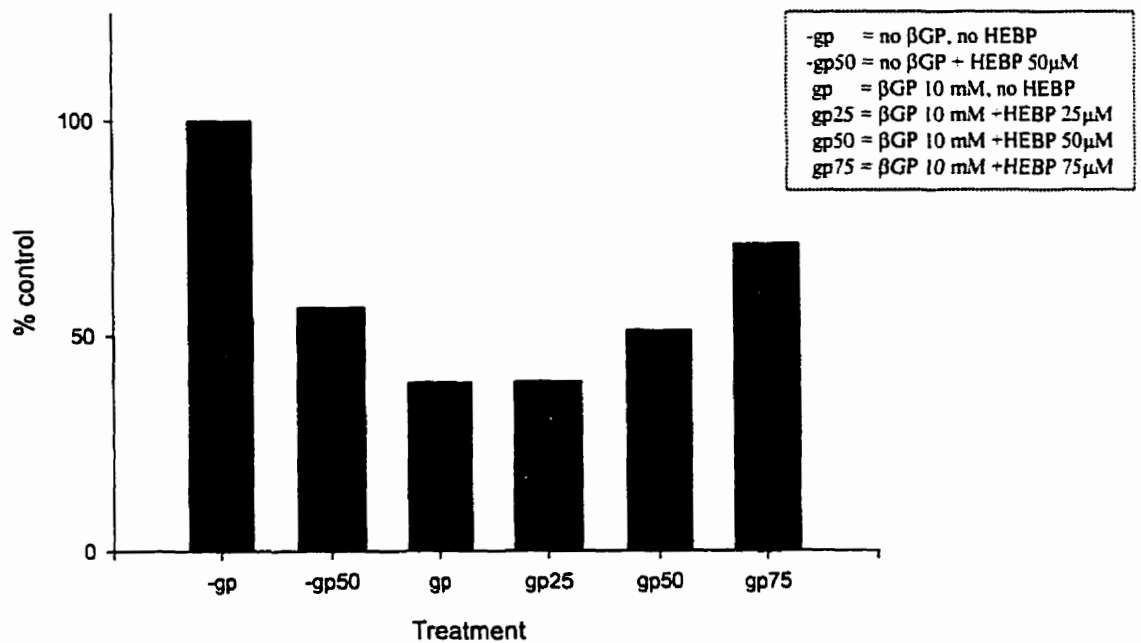**Fig. 8** - Levels of mRNA for BSP in RBMC cultures at 10 days (normalized against gapdh). Levels in cultures grown without βGP were taken as 100% (control). The addition of HEBP in the minus βGP group resulted in a slight decrease in levels of mRNA. The addition of βGP alone to the media resulted in a more profound decrease in BSP mRNA levels while the addition of HEBP in βGP-treated cultures caused a clear increase in BSP mRNA relative to βGP alone. The total RNA was extracted for 1 culture per group (n=1).

-gp   = no βGP, no HEBP
-gp50 = no βGP + HEBP 50μM
gp   = βGP 10 mM, no HEBP
gp25 = βGP 10 mM +HEBP 25μM
gp50 = βGP 10 mM +HEBP 50μM
gp75 = βGP 10 mM +HEBP 75μM

* signal to noise values did not allow relative expression to be quantified adequately

BSP
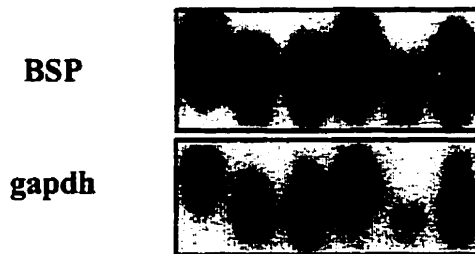
gapdh

Fig. 9 - Levels of mRNA for col (I) in RBMC cultures at 7.5 days (normalized against gapdh). Levels in minus βGP treated cultures were taken as 100% (control). For the minus βGP groups the addition of HEBP lead to an increase in col (I) mRNA levels. The addition of βGP alone to the media resulted in a decrease compared to minus βGP alone. However the addition of HEBP caused an increase in mRNA levels. The total RNA was extracted from 1 culture per group (N=1).

-gp   = no βGP, no HEBP
-gp50 = no βGP + HEBP 50μM
gp   = βGP 10 mM, no HEBP
gp25 = βGP 10 mM +HEBP 25μM
gp50 = βGP 10 mM +HEBP 50μM
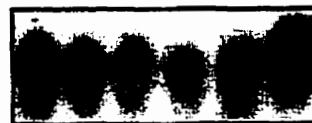gp75 = βGP 10 mM +HEBP 75μM
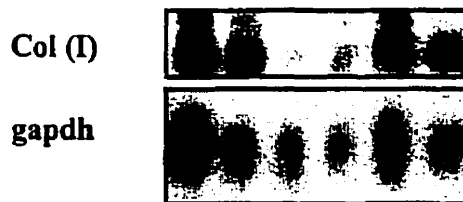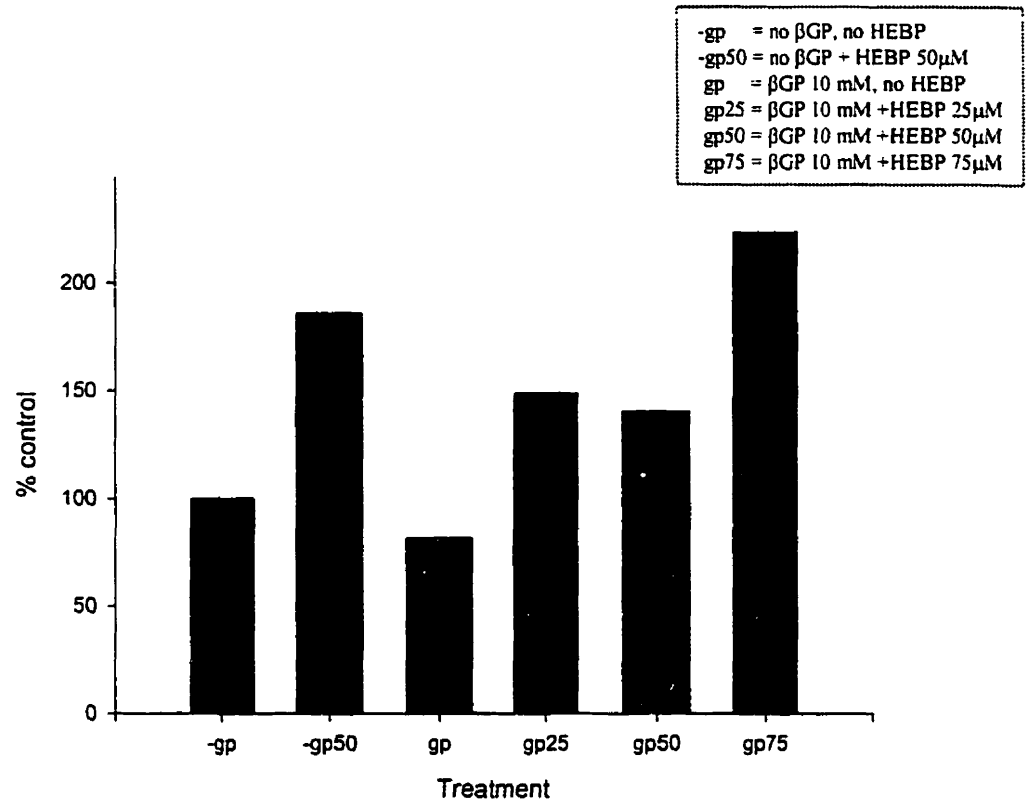
Col (I)

gapdh

Fig. 10 - Levels of mRNA for col (I) in RBMC cultures at 10 days (normalized against gapdh). Levels in minus βGP treated cultures were taken as 100% (control). The addition of HEBP in the minus βGP group resulted in a slight increase in col (I) mRNA levels. The βGP alone treated group expressed half of the minus βGP alone group. The addition of HEBP in the βGP treated group resulted in an increase in the levels of col (I) mRNA. The total RNA was extracted from 1 culture per group (N=1).

-gp   = no βGP, no HEBP
-gp50 = no βGP + HEBP 50μM
gp   = βGP 10 mM, no HEBP
gp25 = βGP 10 mM +HEBP 25μM
gp50 = βGP 10 mM +HEBP 50μM
gp75 = βGP 10 mM +HEBP 75μM
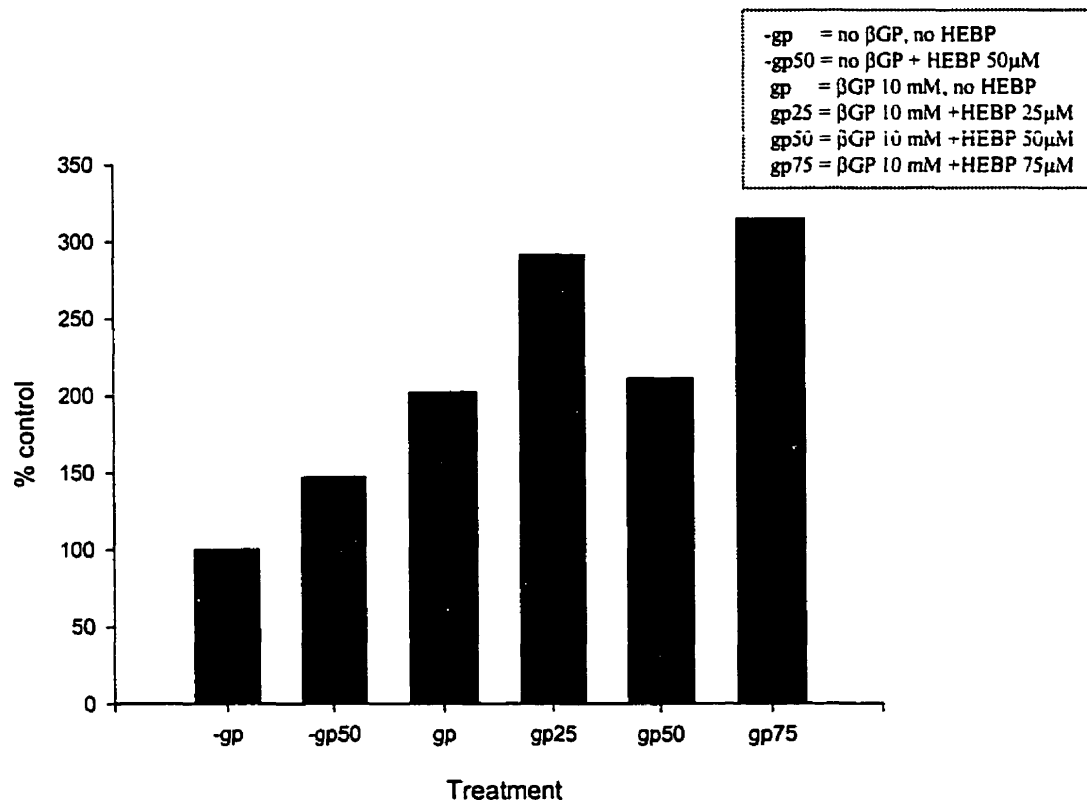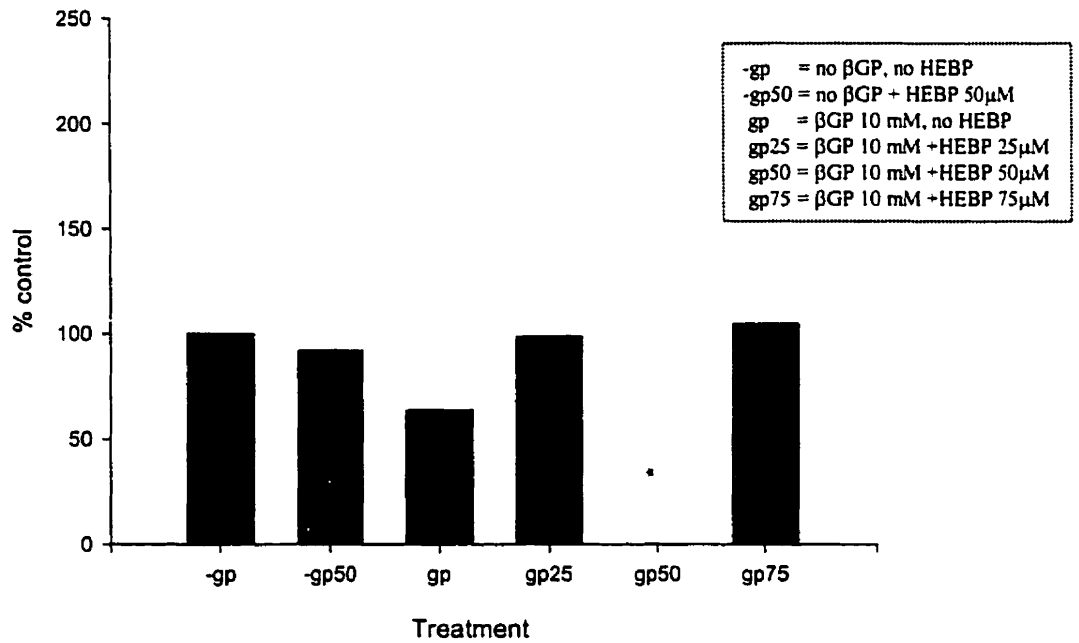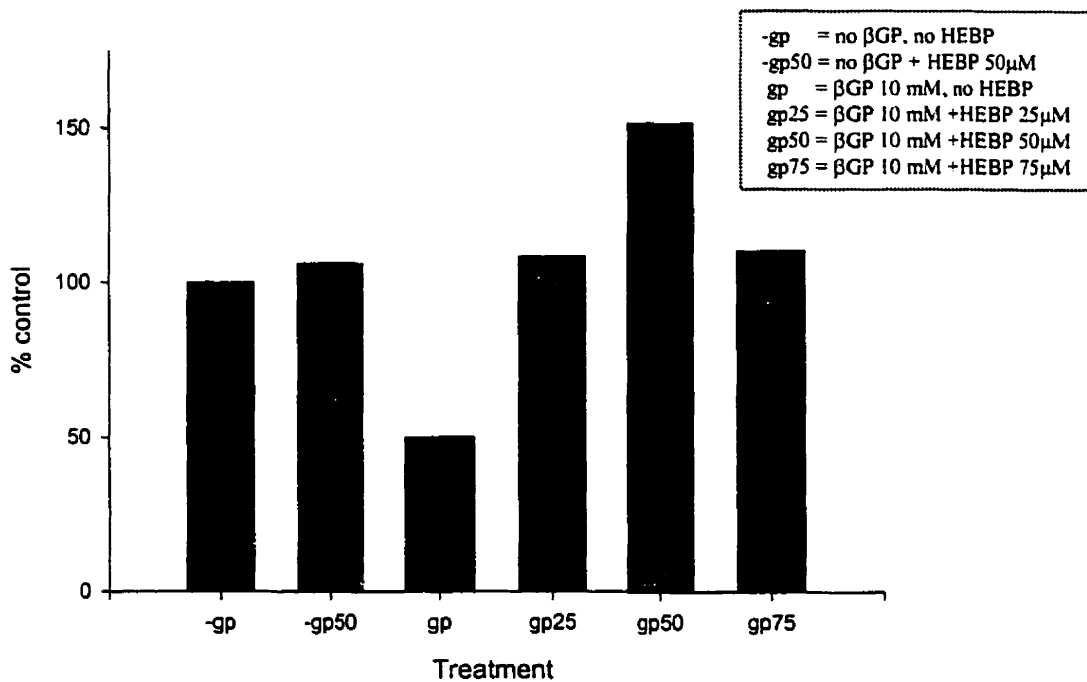
Col (I)

gapdh

Fig. 11 - Levels of mRNA for OPN in RBMC cultures at 5 days (normalized against gapdh). Levels in cultures grown without βGP were taken as 100% (control). Generally the presence of HEBP in the media resulted in a downregulation of the OPN mRNA levels. The total RNA was extracted for 1 culture per group (N=1).

-gp	= no βGP, no HEBP
-gp50	= no βGP + HEBP 50μM
gp	= βGP 10 mM, no HEBP
gp25	= βGP 10 mM +HEBP 25μM
gp50	= βGP 10 mM +HEBP 50μM
gp75	= βGP 10 mM +HEBP 75μM
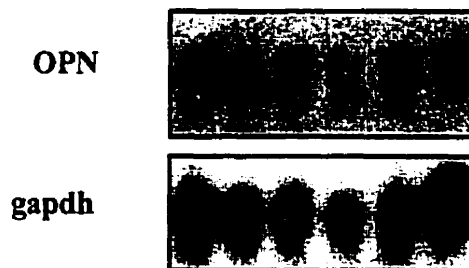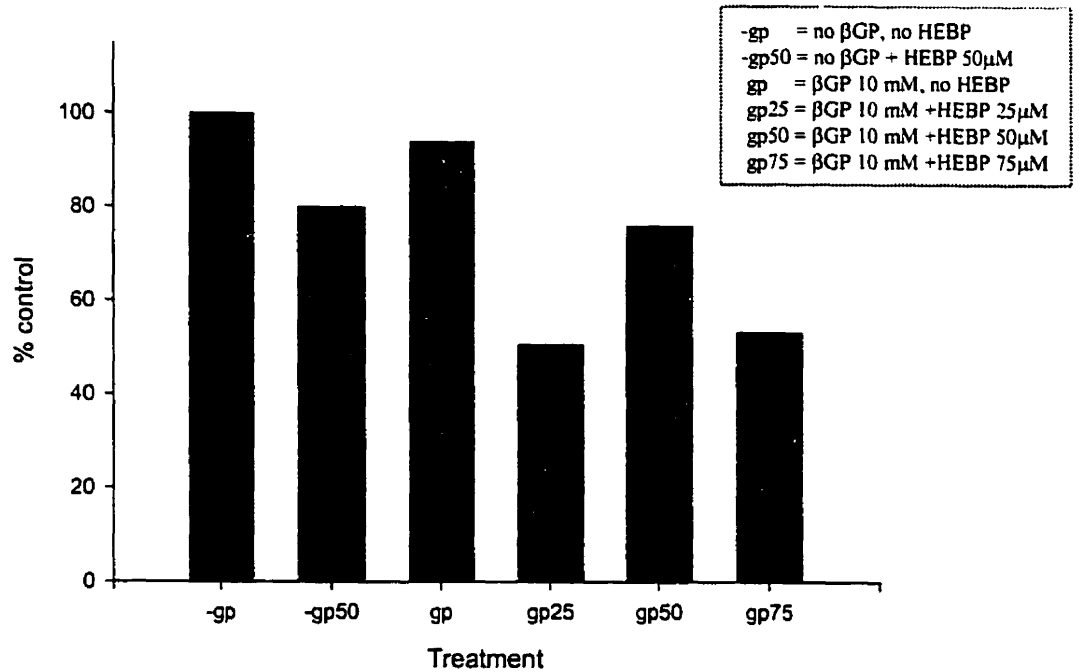
Fig. 12 - Levels of mRNA for OPN in RBMC cultures at 7.5 days (normalized against gapdh). Levels in cultures grown without βGP were taken as 100% (control). For the minus βGP groups the presence of HEBP lead to an increase in the OPN mRNA levels. The addition of βGP alone in the media resulted in a tremendous increase in mRNA levels. In general, the addition of HEBP to the plus β-gp group resulted in a decrease in mRNA levels.

-gp	= no βGP, no HEBP
-gp50	= no βGP + HEBP 50μM
gp	= βGP 10 mM, no HEBP
gp25	= βGP 10 mM +HEBP 25μM
gp50	= βGP 10 mM +HEBP 50μM
gp75	= βGP 10 mM +HEBP 75μM
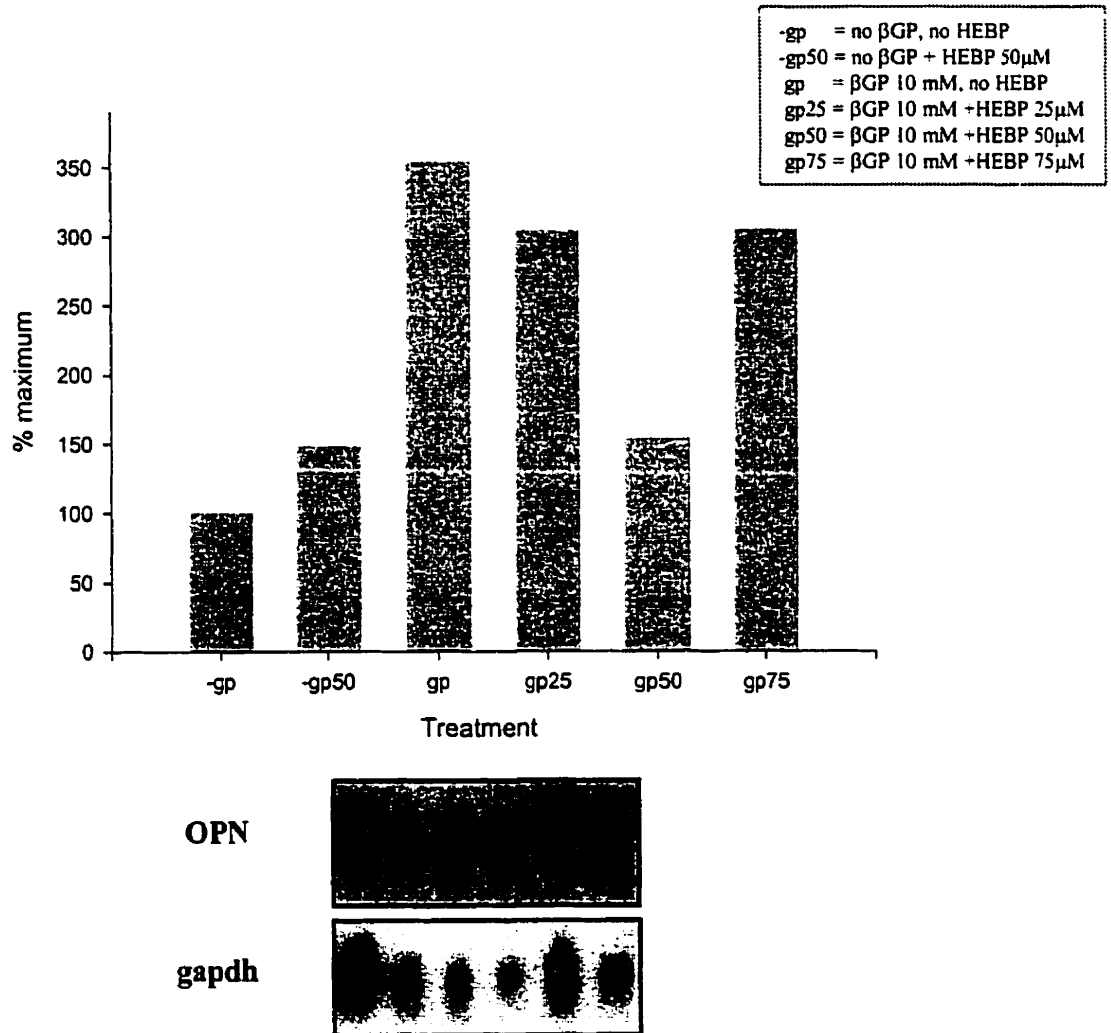
Fig. 13 - Levels of mRNA for OPN in RBMC cultures at 10 days (normalized against gapdh). Levels in cultures grown without βGP were taken as 100% (control). For the minus βGP group, the addition of HEBP resulted in a decrease in mRNA levels resulting in levels comparable to βGP-treated cultures alone. For the βGP-treated groups, the addition of HEBP caused a decrease in OPN mRNA levels except for the 50 μM group.
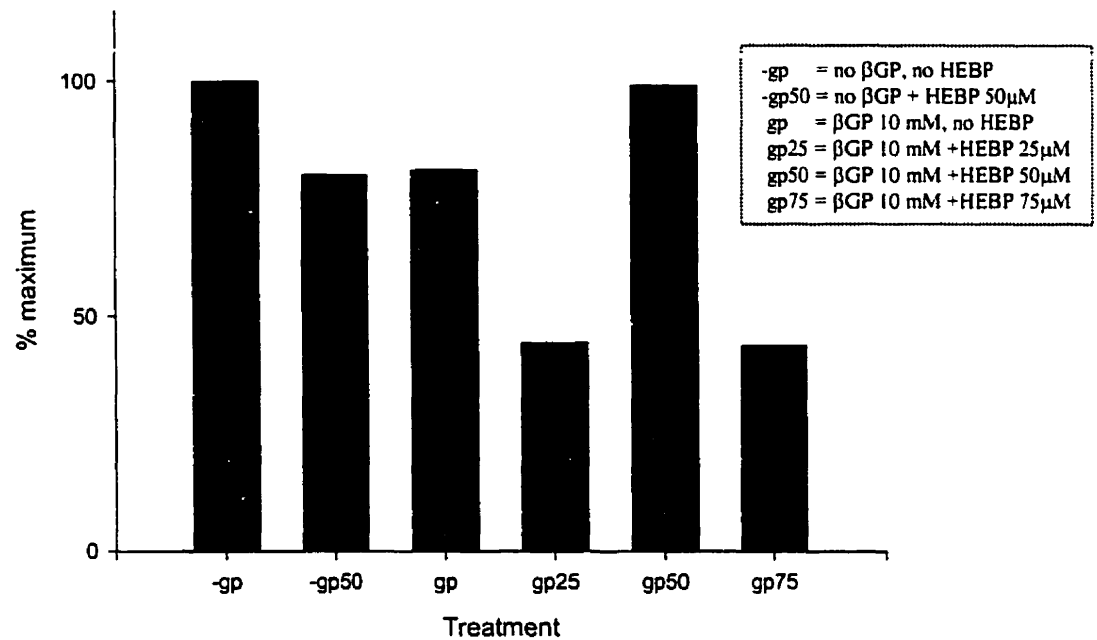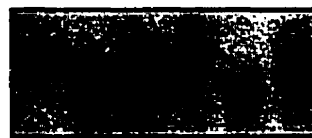
Fig. 14 - Levels of mRNA for OCN in RBMC cultures at 10 days (normalized against gapdh). No detectable message was found in earlier cultures. mRNA Levels in cultures grown without βGP were taken as 100% (control). For the minus βGP groups, the addition of HEBP resulted in an increase in OCN mRNA. The addition of βGP to the media caused a further increase in mRNA levels even more magnified with the addition of HEBP for 25 μM and 50 μM. However, the mRNA levels for the βGP plus 75 mM HEBP were found to be decreased compared to βGP alone. The total RNA was extracted from 1 culture per group (N=1).

-gp	= no βGP, no HEBP
-gp50	= no βGP + HEBP 50μM
gp	= βGP 10 mM, no HEBP
gp25	= βGP 10 mM +HEBP 25μM
gp50	= βGP 10 mM +HEBP 50μM
gp75	= βGP 10 mM +HEBP 75μM

OCN

gapdh
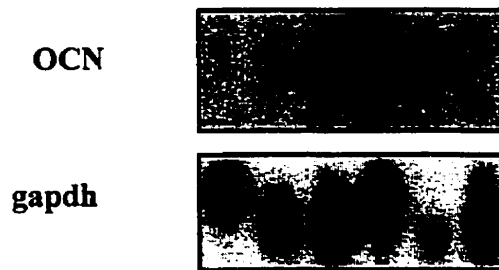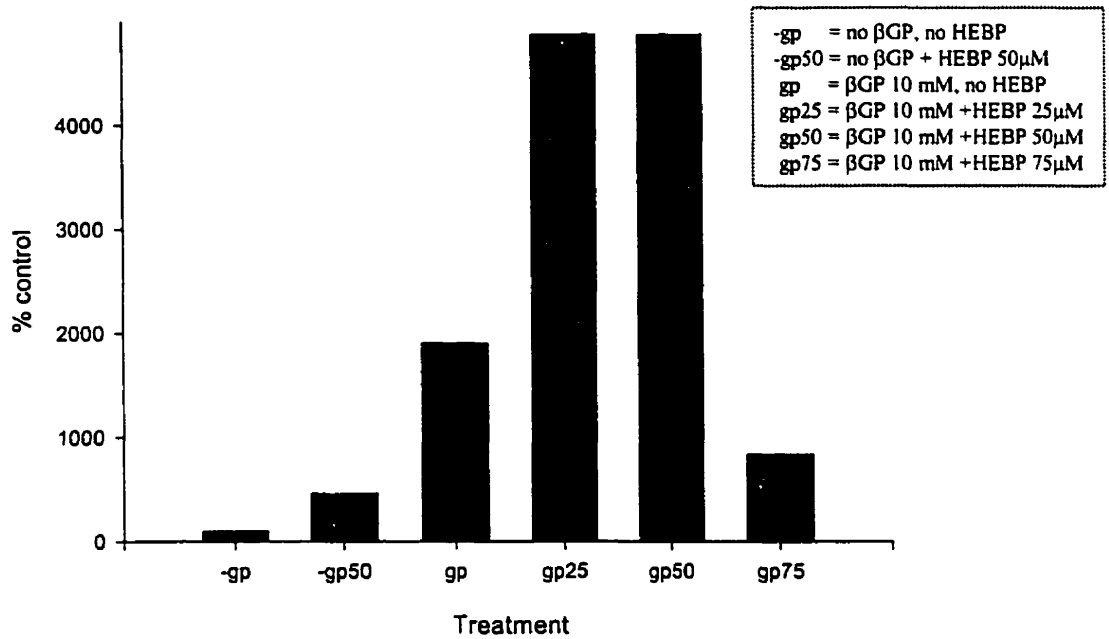
Discussion

The studies I employed were designed to elucidate the mechanisms of action of

bisphosphonates on osteoblasts, and to investigate the underlying events taking place

during the process of mineralization by studying in more detail the effects of HEBP using

both the RBMC and SBMC models. It was demonstrated that HEBP increases AP

activity and modulates mRNA levels for bone specific proteins. HEBP did not appear to

bind to osteoblasts, nor did it trigger changes in intracellular calcium concentration

suggesting that HEBP's actions are not receptor-mediated. Nonetheless, in light of

HEBP's effects on protein synthesis and gene expression, a direct effect is suggested.

Alkaline phosphatase activity

Using the RBMC model, administration of increasing doses of HEBP over 2, 4 and 7

days caused an increase in AP activity, but only at days 4 and 7. AP is known as an

inorganic phosphate provider (Golub 1996). Generally, synthesis of AP is accepted as a

hallmark of an osteoblastic phenotype and bone formation (Lian, Stein et al. 1993). The

doses of HEBP used were consistent with doses used in previous investigations

(Tenenbaum, Torontali et al. 1992; Goziotis, Sukhu et al. 1995). Increases in AP

following bisphosphonate administration have been shown in other models such as the

MC3T3-EI murine osteoblast cell line (Igarashi, Hirafuji et al. 1997), the CPO model

(Tenenbaum, Torontali et al. 1992), and rat calvarial cells (Felix and Fleisch 1979).

Although there was a dose dependent increase in AP, a decrease was also noted at the

higher levels of HEBP on day 4, which might be suggestive of toxicity. However, such reductions were not observed in 7-day cultures. This could suggest that higher doses of HEBP are toxic to less well-differentiated osteoblasts. Toxicity has been reported in other studies while using higher concentration bisphosphonates (up to 75 μM HEBP)(Tenenbaum, Torontali et al. 1992; Igarashi, Hirafuji et al. 1997). Overall though, one may conclude that HEBP increases AP activity and that our experiments support the notion that HEBP promotes osteoblast function and enhances differentiation of pre-osteoblastic cells into osteoblasts.(Lekic, Rubbino et al. 1997; Gandolfi, Pugnaloni et al. 1999; D'Aoust, McCulloch et al. 2000).

We further examined the effects of HEBP on AP to determine whether the increase is the result of higher AP activity or by an increase in the amount of protein itself. Our experiments show that AP immunostaining was elevated at 4 days for the higher doses of HEBP. The staining appeared to correlate with the results seen for AP activity suggesting the increased AP activity measurements are related at least in part to increases in the amount of enzyme produced. These results also agree with findings reported in previous studies showing an increase in the number of AP positive cells in the CPO model after treatment with HEBP (Goziotis, Sukhu et al. 1995) and in a murine model (Gandolfi, Pugnaloni et al. 1999). This experiment further supports the notion that HEBP has a direct cellular effect on osteoblasts. However, it must be recognized that HEBP alters mineralization meaning that HEBP effects could be mediated indirectly secondary to perturbation of (βGP-induced) mineralization. These issues will be addressed further below.

Dex versus Dex+HEBP studies

To further investigate the effects of HEBP on RBMC osteoblasts we sought to evaluate

its effects in the presence or absence of the glucocorticoid Dex. Dex has been shown to

be a requirement for differentiation of various cell types such as adipocytes (Bennett,

Joyner et al. 1991) and chondrocytes (Grigoriadis, Heersche et al. 1988). Dex has been

shown to stimulate cell proliferation (McCulloch and Tenenbaum 1986) and osteogenesis

in vitro (Tenenbaum and Heersche 1985) and that its presence is required for bone nodule

formation *in vitro* (Maniatopoulos, Sodek et al. 1988). Given the obligate requirement for

Dex to induce nodule formation (osteodifferentiation) and the profound stimulatory effect

of Dex on AP (Tenenbaum and Heersche 1985), it was possible that some HEBP effects

on AP might be masked or otherwise modified in the presence of the steroid.

Interestingly, the AP levels found for cultures treated with HEBP alone were practically

equivalent to those observed for cultures treated with Dex (10^{-6} M, 10^{-9} M, and 10^{-10} M).

Yet HEBP on its own did not induce nodule formation showing that AP expression alone

cannot be used to gauge whether or not osteodifferentiation has occurred. Nonetheless,

AP upregulation is at least a hallmark of some degree of osteodifferentiation further

confirming, as noted above, that HEBP on its own, has some positive effects on

differentiation of osteoblasts as shown in vivo(Lekic, Rubbino et al. 1997; D'Aoust,

McCulloch et al. 2000). Additional longer-term studies could be carried to assess whether

nodule formation in HEBP (no Dex) treated cultures would occur over time. One may

speculate that although the HEBP-only treated cultures would not mineralize, they could

still form nodules even without the presence of Dex stimulatory effect if HEBP does

induce osteodifferentiation. It is also conceivable that inasmuch as HEBP may not block

mineralization absolutely (Torontali. Holmyard et al. 1994; Goziotis. Sukhu et al. 1995;

Lekic. Rubbino et al. 1997; D'Aoust. McCulloch et al. 2000), some mineralized nodules

might even be found in long-term cultures grown in the presence of HEBP but without

Dex.

Effects of HEBP exposure on mineralization in RBMC

Because AP activity is intimately related to initiation of mineralization (Tenenbaum and

Palangio 1987; Bellows. Aubin et al. 1991). I attempted to further characterize the effects

of HEBP on mineralization. Inhibition of mineralization with HEBP was found to be

dose-dependent as shown in figure 4A. The control cultures mineralized normally in a

way similar to previous reports with RBMC at various time points (Yao. Todescan et al.

1994). This finding thus agreed with comparable studies with the CPO model showing

decreasing calcium levels in presence of increasing doses of HEBP (Tenenbaum.

Torontali et al. 1992) as well as in human osteoblasts (Tsuchimoto. Azuma et al. 1994).

Possible mechanisms for this inhibition of mineralization include the fact that HEBP may

act as crystal poison preventing further crystal growth (Fleisch 1997) (Grynpas 1993).

Some authors have shown that that bisphosphonates exert morphological changes in

hydroxyapatite crystals, in a dose dependent manner. at least when high concentrations

are used (Hein. Grassi et al. 1997). HEBP might also prevent AP-mediated hydrolysis of

βGP, an effect that would also interfere with mineralization. Temporal exposure to HEBP demonstrated that the cultures could recover, at least partially, from exposure to HEBP as demonstrated in other models (Tenenbaum, Torontali et al. 1992). As well, the data demonstrated that the effect of HEBP on mineralization is cumulative (fig 4C.), finding which underscores why older generation bisphosphonates are used in cyclical regimen in clinical settings (Watts, Harris et al. 1990) so as to prevent excessive osteoid formation. However, it is important to reiterate that the doses employed in this study, which inhibit mineralization, are significantly higher than those used clinically, since I wanted to gain more insight into osteoblast behavior under matrix-stimulatory conditions (i.e. when mineralization is inhibited). These studies suggest that although AP activity is increased in presence of HEBP, mineralization does not occur normally at high doses, suggesting that although AP is important for mineralization it does not appear to be directly correlated with mineralization. Interestingly, previous studies have suggested that there was an inverse relationship between AP activity and calcification in bone formed *in vitro* (Tenenbaum, Torontali et al. 1992) and this relationship appears to hold in the presence of HEBP. It is also important to note that even at high doses (75μM), mineralization still occurs (fig 4A), showing that the cultures can circumvent HEBP-inhibited mineralization by other mechanisms. Previous studies in our laboratory have shown that while mineralization is inhibited by HEBP, osteoid synthesis is increased and upon drug removal, mineralization would proceed normally, resulting in a higher production of bone overall (Goziotis, Sukhu et al. 1995; D'Aoust, McCulloch et al. 2000)

HEBP signalling and receptor binding in osteoblasts

Because the of the known direct effects of bisphosphonates on osteoblasts such as the

production of an osteoclast inhibitory factor (Vitte, Fleisch et al. 1996), decreased IL-6

production (Giuliani, Pedrazzoni et al. 1998), decreased PGE2 production (Igarashi,

Hirafuji et al. 1997), increased osteoid production (Goziotis, Sukhu et al. 1995), and

increases in the amount of AP protein, we hypothesized that these changes were

dependent on regulation of certain genes that could possibly be activated through a

second messenger calcium signaling pathway. Generally, the activation of cell surface

receptors results in increases in cytoplasmic $Ca2+$ concentration due to the release or

mobilization of $Ca2+$ from intracellular stores and to the entry of $Ca2+$ from the

extracellular space (Hughes and Putney 1990). Calcium ionophores, such as ionomycin,

facilitate the entrance of calcium ions in the cells and indirectly induce the release of

calcium from internal stores through the IP3 pathway (Dedkova, Sigova et al. 2000).

Ionomycin is used to confirm responsiveness of the cells as well as determine maximal

fluorescence under appropriate dye loading and experimental conditions. Cells were

deemed to be responsive to ionomycin, in calcium containing and calcium free buffer.

Tests with HEBP had to be performed in a calcium free buffer because of the avidity of

the drug for calcium. Despite numerous attempts, no changes were seen in intracellular

calcium concentration following exposure to HEBP, unlike osteoclasts, in which

increases in calcium may occur (Colucci, Minielli et al. 1998). It has been suggested that

osteoclasts may take up the drug most likely through resorption of bone mineral

containing bisphosphonates (Rogers, Frith et al. 1999). This phenomenon does not likely

occur in osteoblasts. In fact, the intrinsic negative charge of the HEBP molecule may prevent the drug's passage through the biphospholipid layer. Therefore HEBP action in osteoblasts may be triggered through external or surface disturbances, but these do not appear to be mediated via second messenger calcium signalling.

Future studies pursuing the possibility of a cell signaling mechanism would require investigation of other types of messengers such as cAMP, IP3, MAPKKK and should be done.

Based on the speculation that HEBP would act on osteoblast through surface stimuli, we also sought to investigate further the possible presence of a receptor for HEBP on osteoblasts. Despite a number of attempts, characteristic competitive binding curves were not generated, refuting the speculation of the presence of a receptor for HEBP on RBMC cells. However, if HEBP does indeed bind to osteoblasts with low affinity or only few receptors it may not be possible to detect binding using C^{14} labelled drug because of its low energy emission. Using a higher energy radionuclide such as P^{32}, subtle differences might have been disclosed, but it was not possible to obtain P^{32}-labelled HEBP. In any event, the lack of calcium signaling also argues against the presence of an HEBP receptor.

HEBP effects on gene expression

As noted above, HEBP has clear effects on phenotypic behavior of osteoblasts. Despite

the fact that neither cell surface binding nor second messenger signalling could be shown,

I wanted to investigate further the underlying mechanisms whereby mineralization

inhibiting doses of HEBP might affect bone cell function. Therefore, I chose to assess

HEBP effects on gene expression for a variety of bone proteins. Moreover, because of the

important influence of HEBP on mineralization, we performed the analysis using

mineralizing (+βGP) and non-mineralizing (-βGP) cultures to verify whether the

observed effects on osteoblasts were dependent or independent of HEBP-mediated

pertubation of βGP-dependent mineralization (section 1.3.3).

BSP

At early time points (5 days, fig. 6) it appeared that HEBP reduced BSP expression in

-βGP cultures while an increased trend was observed in +βGP groups. While BSP

function is not known, its expression generally corresponds to *de novo* bone formation

(Chen, Shapiro et al. 1992) and my results are consistent with previous observations

using RBMC (Yao, Todescan et al. 1994). In immature osteoblasts it appears that HEBP

effects are more dependent on the presence of βGP and thus by extension, mineralization

or at least the mineralization process. Because RBMC has a finite period of maturation

and well defined periods of mineralization (Maniatopoulos, Sodek et al. 1988), perhaps

the BSP genes are upregulated in an attempt to stimulate mineralization as shown in vitro

by Hunter (Hunter and Goldberg 1993). This would explain the higher mRNA levels

observed for –βGP and. +βGP+HEBP cultures. In non-mineralizing cultures (-βGP),

HEBP appears to downregulate BSP mRNA. While no study has described the effect of

βGP on BSP message or protein, the effects have been described for AP protein, which

have shown a decrease in protein levels in the presence of βGP (Tenenbaum, Torontali et

al. 1992). It is reasonable that BSP expression, which correlates with the onset of bone

formation and mineralization, might behave in a way similar to AP. Unlike the effects

observed at 5 days, it appears that HEBP has no clear effect on BSP mRNA levels (fig. 8)

in more mature osteoblasts. Interestingly, BSP protein has been shown to decrease in

presence of bisphosphonates in vivo in humans (Woitge, Oberwittler et al. 2000), and in

rodents (Lekic, Rubbino et al. 1997). While mRNA levels do not always correlate with

protein levels, the behavior of these proteins in vivo is more in line with what we would

expect since BSP is thought to be a stimulator of mineralization at least in vitro (Hunter

and Goldberg 1993) and that HEBP has clear inhibition of mineralization as shown here.

These results reiterate the importance of correlating *in vitro* studies with *in vivo*. Based

on the observations in mineralizing and non-mineralizing cultures, it appears that BSP

expression is, at least partially, dependent of βGP-induced mineralization.

Col (I)

Collagen mRNA was detected at day 7.5 and 10 (figs. 9 and 10) and was only barely

detectable at day 5 thus not allowing quantification at the earliest time point tested

(suggesting little matrix formation at that point). The onset of expression of col (I)

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mRNA observed in our experiments agrees with findings reported in previous studies in RBMC (Yao, Todescan et al. 1994). In my experiments, message levels were upregulated by HEBP in cultures grown with or without βGP. This increase in col (I) mRNA is consistent with the observation that bisphosphonates increase cell proliferation and collagen protein in the CPO (Goziotis, Sukhu et al. 1995) as well as in other culture systems (Tsuchimoto, Azuma et al. 1994). The increase in col (I) mRNA, coupled with our studies on mineralization, further support the hypothesis suggesting that osteoid matrix production is inversely related to mineralization. Moreover, these results can be correlated with recent in vivo studies demonstrating faster closure of calvarial wounds subsequent to a pulse dose of HEBP (D'Aoust, McCulloch et al. 2000). Yet my findings also suggest that the HEBP effects on col (I) mRNA are independent of βGP-induced mineralization since they were also observed in the absence of βGP and hence mineralization. Thus, the findings also suggest a direct stimulatory effect of HEBP on osteoblastic matrix production, which may be unrelated of its effects on mineralization. Perhaps the inverse relationship between mineralization and matrix formation as well as HEBP's effects thereon are largely fortuitous.

OPN

Generally, HEBP downregulated OPN mRNA levels (figs 11-13) in cultures grown with and without βGP (i.e mineralizing and non-mineralizing conditions). OPN is not bone specific (Sodek, Chen et al. 1995) and while its function is not clear, it appears to have a

multiplicity of functions since it may likely play a role in bone apposition (Butler 1989) as well as resorption (Reinholt, Hultenby et al. 1990). Its expression in developing osteoblasts corresponds to onset of *de novo* bone formation (Nomura, Wills et al. 1988). The expression of OPN mRNA in our cultures is consistent with previous studies in RBMC (Yao, Todescan et al. 1994). OPN mRNA has been reported to decrease following exposure to lower doses of bisphosphonates in RBMC (Sodek, Chen et al. 1995), as well as in other culture systems (Yasui, Fujita et al. 1998). The downregulation of OPN mRNA would signify that that if OPN plays a supportive or stimulatory role in bone resorption, bisphosphonates would impair OPN effects in mature osteoblasts, thereby inhibiting resorption. Based on the expression of mRNA in + and − βGP cultures, our studies suggest that the effects of HEBP on OPN mRNA are independent of βGP-induced mineralization, as seen with other markers of bone formation.

OCN

OCN mRNA was only clearly observed at 10 days, which is consistent with it being a late marker in post-mitotic osteoblasts (Ducy and Karsenty 1996). HEBP, under both mineralizing (+βGP) and non-mineralizing (-βGP) conditions, caused profound increases in OCN mRNA levels. While the true function of OCN is not known, it is considered as a serum marker of bone formation in humans (Marc, Prezelj et al. 1999). However, in OCN knockout mice (Ducy, Desbois et al. 1996) higher bone mass was found and moreover these bones had improved functional quality, suggesting that OCN could even be an inhibitor of bone formation. As well other studies have shown that glucocorticoids known

to stimulate osteoblast differentiation also downregulate OCN mRNA (Stromstedt, Poellinger et al. 1991). Interestingly, glucocorticoids are known to deplete bone mass in vivo, but this effect is due to proliferation of non-bone forming cells causing a net decrease in bone. The observation that OCN mRNA is upregulated both in mineralizing and non-mineralizing cultures suggest that HEBP may act on OCN independently of mineralization to a certain extent. However, the tremendous increase observed between cultures grown with and without βGP cultures does not rule out some mineralization mediated effects on OCN expression. Because of its potential function as a nucleator of hydroxyapatite, such an increase in message could perhaps be the result of the feedback related to inhibition of mineralization caused by HEBP. In other words, HEBP-induced inhibition of mineralization could lead to a compensatory over-production of OCN, a phenomenon that may also explain why, even the presence of high dose of HEBP, some mineralization is still evident (D'Aoust, McCulloch et al. 2000). These findings are also in agreement with studies suggesting that OCN protein levels increase subsequent to bisphosphonate exposure (Tsuchimoto, Azuma et al. 1994). However other studies with HEBP did not show any changes in OCN mRNA (Kung and Ng 1994: Sodek, Chen et al. 1995) following exposure to HEBP. This is probably due to the higher doses of HEBP used in my study. Based on these findings, one may speculate that the effects of HEBP on OCN expression are regulated partially by mineralization but are also independent of mineralization to some extent.

To express more clearly HEBP effects on osteoblastic mRNA production, a summary

table is shown below:

Table 2: Summary of HEBP effects on osteoblastic mRNA expression.
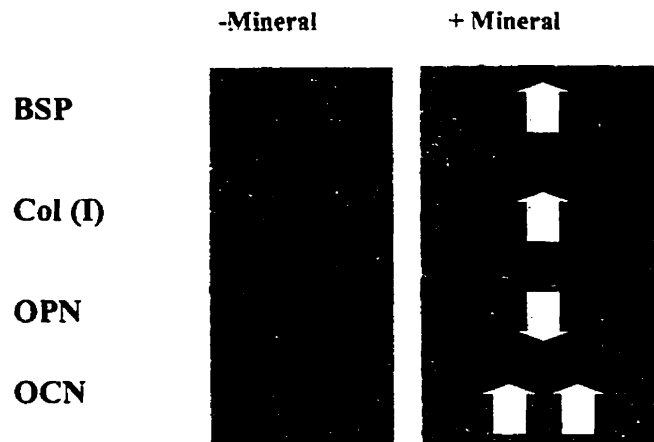
Based on these results, we suggest that HEBP-mediated effects on expression of mRNA

for proteins not restricted solely to bone [OPN, Col (I)] might be more *independent* on

mineralization while HEBP-mediated regulation of mRNA for proteins that might be

more restricted to bone [BSP, OCN] is more *dependent* on mineralization

Conclusion

My studies suggest a direct effect of high dose bisphosphonate (i.e. HEBP), on osteoblasts. Increases in AP activity levels, as well as AP protein levels were shown, suggesting a stimulatory effect on osteoblastic differentiation and metabolism. The data also suggested that the effects of HEBP on mineralization are not only dose dependent but also cumulative. As well, collagen mRNA upregulation findings, coupled with the mineralization data support the hypothesis suggesting an inverse relationship between matrix production and mineralization but also suggest a more refined control, in which HEBP may disturb these processes. Studies of intracellular calcium have shown that HEBP did not appear to cause any changes in calcium levels. Despite numerous attempts, it was not possible to demonstrate competitive binding to HEBP on osteoblasts in RBMC. Yet, while my data did not demonstrate a possible receptor, they did not refute its presence, since other cellular communication mechanisms may be involved. The latter may be true since several differences were found in expression of mRNA levels. I suggest that in general, proteins whose expression is not restricted to bone (i.e OPN, col (I) appear to be regulated by HEBP through mechanisms that could be independent of mineralization. The effects of HEBP on BSP, a protein that is more restricted to bone tend to be more mineralization dependent. Striking differences in mRNA levels were observed for OCN. While this protein has been closely associated with mineralization processes, these results appear to show that its expression, and HEBP-mediated changes are to an extent, independent of mineralization. My studies further suggest that control of

mineralization is a closely regulated phenomenon and imply that other studies will be

necessary to investigate other possible sites of action of HEBP.

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