CHAPTER II

LITERATURE REVIEW

2.1 Osteoblasts

Histological morphology of osteoblasts show one nucleus and an extensive network of rough endoplasmic reticulum. Liu and Aubin described the morphological features of osteoblasts in 3 stages (Liu and Aubin, 1994). In early stage, cells are cuboidal cells with refractile matrix. Intermediate stage, cuboidal cells and refractile matrix are present in multilayered cellular system. In mature stage, as intermediated, but matrix being visibly mineralized. Active osteoblasts are plump, cuboidal, mononuclear cells lying on the matrix which they secreted. Actively synthesizing osteoblasts contain an extensive organelle apparatus, a large Golgi complex near the nucleus and a high mitochondrial content. These reflect the capacity of osteoblasts for their primary function of protein synthesis (Doty and Schofield, 1976). Osteoblasts are involved with the synthesis and maintenance of the bone matrix, and play an important role in the bone remodeling control, including the control of osteoclasts.

Many kinds of mediators appear to play regulatory roles in the differentiation of osteoblast, such as, glucocorticoids (Grigoriadis et al., 1988, Grigoriadis et al., 1990, Yamaguchi and Kahn, 1991), molecules of the transforming growth factor-β (TGF-β) superfamily, and also bone morphogenetic proteins (BMPs) (Katagiri et al., 1990, Yamaguchi and Kahn, 1991). Osteoblasts have receptors and response to parathyroid hormone

(PTH), 1.25-dihydroxyvitamin D3 [1,25(OH)2D3], prostaglandins (PGs), epidermal growth factors (EGFs), tumor necrosis factors (TNFs) (Martin et al., 1988; Heath and Reynolds, 1990). Additionally, osteoblasts can express a specific membrane-bound molecule known as receptor activator of NFκB ligand (RANKL), which has an ability to induce nuclear factor κB (Anderson et al., 1997). NFκB plays an important role in osteoclast differentiation and also functions as a dendritic cell survival factor (Anderson et al., 1997, Wong et al., 1997, Lacey et al., 1998, Yasuda et al., 1998).

2.2 Markers of osteoblast differentiation

In cultured osteoblasts, there are 3 stages of osteoblast differentiation: proliferation, extracellular matrix maturation, and matrix mineralization. In fetal rat calvarial-derived osteoblasts, the period of active proliferation is the first 10 to 12 days (Stein and Lian, 1993). This marked by the expression of histone gene, cell cycle regulated gene, and cell growth regulated genes, *c-myc, c-fos, and c-jun*. In addition, several genes associated with ECM formation (COLI, fibronectin, and TGF-β) (Owen et al., 1990) are actively expressed and then gradually down-regulated with collagen mRNA being maintained at a low level during subsequent stage of osteoblast differentiation.

After the down-regulated proliferation, days 12 to 18, the bone cell phenotype-protein are detected immediately. For example, alkaline phosphatase (ALP) enzyme activity and mRNA are increased greater than 10-fold, and histone gene is expressed (Sporn et al., 1983, Shalhoub et al., 1989). Other bone markers such as bone sialoprotein 2 (BSP2) (Nagata et al., 1991),

osteopontin (OPN), and osteocalcin (OCN) (Owen et al., 1990) are induced following the onset of mineralization.

In bone marrow stromal cells, cell differentiation into osteoblast-, chondrocyte-, or adipocyte-like cells, depend on culture conditions (Dennis et al., 1999). Primary marrow stromal cells grown with ascorbate (AA) and beta-glycerophosphate (β-GP), inducing agent for osteoblast differentiation, produce nodules of mineralized matrix (Alford and Hankenson, 2006). In culture conditions with ascorbic acid, the stromal cells will differentiate into preosteoblasts and osteoblasts, respectively. In this stage, cells will express bone markers, namely COLI, ALP, BSP2, OPN, and OCN. These marker levels appear overlap during bone development (Beck, 2003). ALP, COL I, and OPN levels increase prior to the onset of mineralization, while BSP2 and OCN levels peak as nodules appear and mineralization continues (Yao et al., 1994). Osteoblasts will differentiate into osteocytes in the late stage of differentiation, mineralization stage.

COLI, secreted from osteoblasts, is a major component in bone matrix, which comprises 80-90% of the organic component in mineralized bone tissues. It is composed of two α1 chains and one α2 chain, to form a heterotrimer triple helix. Collagen fibrils act as a scaffold for the extracellular matrix, provide the sites for nucleation of bone (Kadler, 1994). Genetic study has revealed that a mutation in either the COLIA1 gene or the COLIA2 gene cause in osteogenesis imperfecta (Kuivaniemi et al., 1991). Osteogenesis imperfecta (*oim*), brittle bone disease, is a genetic disease characterized by an extreme fragility of bones. Most severe cases of osteogenesis imperfecta result from mutations that lead to the synthesis of normal amounts of an

abnormal chain. Bone marrow stromal cells from *oim* mice synthesize and deposit α(I) collagen homotrimers in the extracellular matrix instead of the normal α1(I)2α2(I) collagen heterotrimers. These α(I) collagen homotrimers deposited in the extracellular matrix of *oim* stromal cells may have an influence on the differentiation of these cells toward osteoblastic lineage (Balk et al., 1997). Furthermore, it has been demonstrated that osteoblast precursor cells cultured without ascorbate that is necessary for collagen synthesis and deposition failed to differentiate into osteoblasts (Franceschi et al., 1994). It has been suggested that extracellular matrix rich in normal COLI may be required for the optimal expression of osteoblast-specific markers (Brenner et al., 1989). The expression of ALP activity was lower in the stromal cells of the *oim* mice than normal mice (Balk et al., 1997).

ALP is an early marker expressed by preosteoblasts and osteoblasts and also to a significant extent in lining cells (Ducy et al., 1996), but failed to be found in osteocytes (Doty and Schofield, 1976) and bone matrix (Ducy et al., 1996). In the mineralization stage, all cells expressed alkaline phosphatase histochemically. In heavily mineralized cultures, the expression of mRNA alkaline phosphatase decrease. In the absence of mineralization, osteopontin and osteocalcin are not induced to high levels, while ALP does not decline (Stein and Lian, 1993). ALP is associated with the plasma membrane of cells. Although the exact function of ALP is unknown, it appears to play role in the transport of substances from the intracellular compartment across the membrane to extracellular region. ALP may also be involved in the breakdown of inorganic pyrophosphate (PPi), a potent inhibitor of calcium phosphate deposition at the extracellular level (Risteli and Risteli, 1993). Plasma cell membrane glycoprotein-1 (PC-1) has a reverse effect of ALP by increase in

PPi. ALP knockout mice were able to form cellular nodules as well as wild type mice do, but they lacked the ability to initiate the mineralization these nodule *in vitro* (Wennberg et al., 2000). Tissue-nonspecific alkaline phosphatase (TNAP/*Akp2*) knockout mice osteoblasts cultured for 21 days precipitated significantly less mineral than wild type osteoblasts, and increased amounts of PPi. Conversly, PC-1 (*Enpp1*) knockout mice osteoblasts precipitated significantly more mineral than control cells (Hessle et al., 2002).

BSP2 is acidic, phosphorylated glycoproteins which are highly expressed in bone matrix (Ganss et al., 1999). BSP contains the Arg-Gly-Asp (RGD) sequence that act as an adhesive domain, and poly-glutamic acid residues (Alford and Hankenson, 2006). The RGD domain can bind both extracellular matrix proteins and integrins on cell surface. The poly-glutamic acid residues have negative charge that can bind to calcium ion molecules. Therefore, BSP has properities to bind hydroxyapatite, extracellular matrix and cells. Otherwise, BSP2 was reported to promote osteoclast adhesion, differentiation and function (Giachelli and Steitz, 2000, Ganss et al., 1999), and also promotes angiogenesis (Bellahcene et al., 2000). BSP is first detected in differentiated osteoblasts forming bone. BSP mRNA levels peak just prior to the onset of mineralization, and the protein is secreted by osteoblasts which are actively synthesizing matrix (Cowles et al., 1998). One immunocytochemical study revealed that BSP can be detected mostly in epiphyseal-metaphyseal border in endochondral ossification. Thus, it appears that BSP2 has a specific role during the initial phases of bone formation at the cartilage/bone interface (Hultenby et al., 1994). Another study showed negative expression of BSP in preosteoblasts, osteoid, bone-lining cells and

osteoclasts, but can be found in bone matrix near the ruffled border of osteoclasts (Ducy et al., 1996).

OPN is expressed during the period of active proliferation (at 25% of maximal levels), decreases post-proliferatively and then is induced again at the onset of mineralization achieving peak levels of expression (Stein and Lian, 1993). OPN can express in oncogene transformation, after serum stimulation of quiescent fibroblasts, or phorbol ester treatment of fibroblasts (Craig et al., 1989). OPN is also acidic, phosphorylated glycoproteins (Giachelli and Steitz, 2000). It has a similar structure to BSP2, the RGD sequence and poly-acid residuals, which is poly-aspartic acid residual. Thus, OPN has abilities to bind hydroxyapatite, extracellular matrix and cells as BSP2 does. One study suggests that tumorigenic related functions of osteopontin may be related to Arg-Gly-Asp containing sequence that mediates cell attachment between cells and extracellular matrix (Glimcher, 1989). This protein is present as a major component of cell-matrix and matrix-matrix interfacial structures called cement lines and laminae limitantes. It has been suggested that OPN in cement lines and laminae limitantes may participate in initial and late extracellular matrix organization and mineralization, matrix-matrix/mineral adhesion and/or cell adhesion at bone interfaces (McKee and Nanci, 1995).

Both BSP2 and OPN have also been shown to modulate the structure of mineralized matrix *in vitro*. Explicitly, while BSP2 appears to be required for nucleation of hydroxyapatite crystal formation, OPN inhibits mineralization by physically blocking crytal growth (Giachelli and Steitz, 2000, Ganss et al., 1999). Both of their levels increase in inflammation and tissue remodeling, as well as ectopic mineralization, that is associated with multiple pathologies

(Ganss et al., 1999, Giachelli and Steitz, 2000). Recently, one study suggests that BSP and OPN specifically bind to matrix metalloproteinases (MMP)-2 and promote activation of MMP-3, even in the presence of MMP-specific tissue inhibitors of metalloproteinases (TIMP) (Fedarko et al., 2004). These observations imply that increased OPN or BSP2 levels might contribute to increased MMP activity associated with tumor metastasis to bone (Alford and Hankenson, 2006).

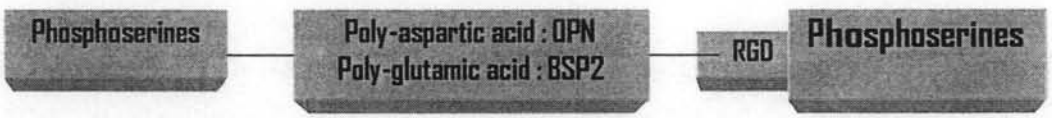

Figure 3. Structures of OPN and BSP2 (modified from Alford and Hankenson, 2006)

In contrast to OPN, OCN is expressed only post-proliferatively with the onset of nodule formation, mineralization stage. OCN is a relatively small protein, produced by osteoblasts during the mineralization stage (Power and Fottrell, 1991). It is the most abundant noncollagenous protein in bone matrix (Power and Fottrell, 1991). Synthesis of OCN is dependent on vitamin K. This protein contains 3-gamma-carboxyglutamic acid (Gla) residuals, it is also called bone Gla protein (Christenson, 1997). Late expression in the osteoblast development sequence suggests that OCN is a marker of the mature osteoblast. OCN is both released into circulation and incorporated into the bone matrix where it is the most abundant noncollagenous protein (Power and Fottrell, 1991). The expression of marker genes demonstrates in Figure 4.

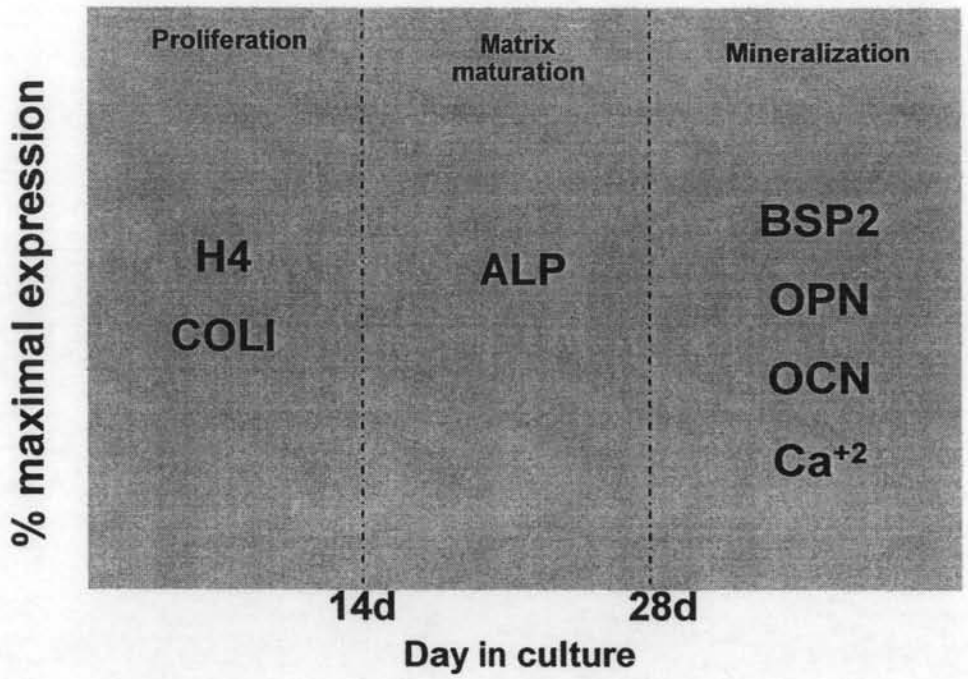

Figure 4. The expression of osteoblast marker genes in cultured rat calvarial-derived osteoblasts. In the proliferation stage, H4 histone reflects DNA synthesis, *c-fos* and *c-jun* (ALP), and type I collagen (COLI) are expressed. In maturation stage, alkaline phosphatase (ALP) is expressed postproliferatively. Osteopontin (OPN), osteocalcin (OCN) are highly expressed in mineralization stage. Calcium (Ca^{2+}) accumulation indicated. From the study of Stein and Lian, 1993.

2.3 Bacterial infection and periapical lesions

Periapical lesions begin as bacterial infection from the dental pulp that subsequently lead to inflammatory bone resorption. Black-pigmented *Bacteroides* such as *Prevotella* and *Porphyromonas* have been found in pulpal lesions (van Winkelhoff et al., 1985, Jin et al., 1989, Sundqvist et al., 1989). These bacterial products, including fimbriae, proteases and LPS endotoxin, play an significant role in the development of clinical symptoms through the induction of inflammatory mediators (Chang et al., 2003, Yang et al., 2003). LPS can stimulate a variety of host cells to release inflammatory mediators,

such as interleukin-1 (IL-1), TNF-α, PGE_2, and MMPs (Robertson et al., 1982). Several reports showed that LPS from *P. gingivalis* inhibits osteoblast differentiation and bone formation (Loomer et al., 1995, Kadono et al., 1999). Treatment with *P. intermedia* LPS in fetal mouse calvarial cells inhibited bone formation by reducing ALP activity and mineralization in dose-dependent manner, and induced the release of nitric oxide, IL-6 and the latent proforms of MMP-2 and MMP-9 (Pelt et al., 2002). One study revealed that LPS had no direct effect in apoptosis on MC3T3-E1, mouse-osteoblastic cell lines. However, there could be indirect effect via TNF-α which released from LPS-stimulated macrophage (Thammasitboon et al., 2005).

2.4 Osteoblasts-osteoclasts coupling function

Toll-like receptors (TLRs) are members of a family of mammalian proteins homologous to *Drosophila* Toll. At present, 10 TLRs have been documented in humans (Beutler, 2004). Each TLRs recognizes the different microbial structures. Asai and coworkers found the expressions of TLR1, TLR4, TLR5, TLR6 and TLR9 on the human osteoblastic cell lines, SaOS-2 cells, but TLR2, TLR3, TLR7, TLR8 and TLR10 failed to be found on them (Asai et al., 2003). Some types of TLRs have been reported that they can response to LPS and other products from gram negative bacteria (Asai et al., 2003, Kikuchi et al., 2001, Yang et al., 1999, Schwandner et al., 1999). Osteoblasts derived from human alveolar bone may play an important role in periapical lesion by reponse to LPS.

Tani-Ishii and associates demonstrated that IL-1α and TNF- α produced primarily from macrophages, are related with the progression of

bone loss (Tani-Ishii et al., 1995). LPS from gram-negative pathogens of endodontic lesion, elicited the secretion of IL-1α and TNF-α from J774 (a mouse monocyte/macrophage cell line) in a time-dependent manner (Hong et al., 2004). These pro-inflammatory cytokines modulate the subsequent production of MMP-1 from macrophages to promote periapical bone resorption. One study has recently failed to find a direct activation of osteoclasts with the international LPS standard, a potent and highly purified LPS from *E. coli* (Ralston et al., 1995). Ueda and cowokers reported that the polysaccharide part of LPS from *Actinobacillus actinomycetemcomitans* failed to stimulate osteoclast formation in mouse bone marrow (Ueda et al., 1995). In addition, recombinant (r)TNF-α and rTNF-β do not stimulate resorption by osteoclasts from neonatal rat long bone, but do so if the osteoclasts are incubated together with calvarial cells or cloned osteoblast-like osteosarcoma cells (Thomson et al., 1987, Ueda et al., 1995). Osteoclasts from mouse bone marrow cells were shown to express TLR4 and CD14 mRNA (Itoh et al., 2003). LPS stimulated the survival of osteoclasts, but did not stimulate the production of proinflammatory cytokines such as IL-1β, TNF-α, and IL-6 (Itoh et al., 2003). These lead us to believe that the osteoclasts function in response to LPS in endodontic lesion depends on osteoblasts.

Altogether, osteoblasts and osteoclasts coupling action should involve in periapical diseases.