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MINIREVIEW

Bacterially Induced Bone Destruction: Mechanisms and Misconceptions

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INTRODUCTION

Bone research languished until the 1980s when the sociomedical importance of idiopathic bone diseases such as osteoporosis (60, 92), rheumatoid arthritis, and osteoarthritis (75) became apparent. For example, it is estimated that there are 275,000 new osteoporotic hip fractures each year in the United States (60). With this increasing focus on bone, it is often forgotten that the most prevalent diseases of the skeleton are the periodontal diseases (113). These diseases and conditions such as dental cysts, bacterial arthritis, osteitis, osteomyelitis, Pott's disease, and infected orthopedic implant failure are all due to the actions of bacteria on bone (51). Many of these conditions were common in the preantibiotic age, and with the rapid increase in antibiotic resistance, particularly with staphylococcal and mycobacterial species, the prevalence of bacterially induced skeletal pathology is destined to increase. The development of effective therapies for bacterially induced bone pathology will require an understanding of the cellular and molecular mechanisms involved. It is only within the last decade that such mechanisms have begun to be investigated, and some fascinating results are emerging.

BONE REMODELLING

Bone is a fiber-reinforced calcified tissue which is perpetually remodelling; this is a process controlled by the joint, but opposing, actions of the two major bone cells—the bone matrix-forming osteoblast and the bone matrix-resorbing osteoclast (5, 8, 90, 99, 122, 129) (Fig. 1). The osteoblast derives from an undifferentiated bone marrow mesenchymal precursor cell population and exists in bone in three forms—the preosteoblast, the mature osteoblast, and the osteocyte, which is trapped within the bone. One major function of the osteoblast is to produce the components of the bone matrix—largely type I collagen with lesser amounts of proteoglycans and glycoproteins—and to catalyze the calcification of the matrix (71, 94, 99). The other major function of this cell is to control the activity of the osteoclast (72, 99). The osteoclast is a multinucleated cell which is believed to derive from the same myeloid precursor cells (CFU of granulocytes-monocytes) which give rise to monocytes, although this is not a universally accepted view (Fig. 2). These cells proliferate and differentiate into mononuclear preosteoclasts which fuse with each other (9, 25, 90). The process of bone remodelling requires the coupled activity of these two cell populations (72). The resorption of bone first requires the osteoblasts to release collagenase to remove the nonmineralized organic matrix which covers bone surfaces. The osteoclast is then attracted to this site, seals itself onto the calcified matrix, and acidifies it by pumping protons outwards, thus solubilizing the calcium salts. The osteoclast then releases various lysosomal enzymes to remove the exposed organic matrix (9, 25, 90). The solubilization of the matrix is believed to release bound growth factors (produced by the osteoblasts) which stimulate mesenchymal cells to proliferate and differentiate into preosteoblasts and osteoblasts which can then replace the previously removed bone matrix (72, 99). Thus there is a perpetual process of removal and replacement of the bone matrix which requires the mutual recruitment (a process involving the proliferation and differentiation of precursor cells into mature osteoblasts or osteoclasts) and activation of osteoblasts and osteoclasts.

Bone remodelling is controlled by a wide variety of systemic factors including hormones and steroids and local factors such as prostaglandins, leukotrienes, cytokines, and growth factors (8, 91) (Table 1). The majority of these bone-modulating factors act on the osteoblast, and it is this mesenchymal cell which transduces the actions of these factors onto the osteoclast (72, 99).

Major insights into the cell biology of bone remodelling have come from the study of osteopenias (conditions of lowered bone density) associated with estrogen deficiency and senescence and of osteopetrosis (a condition in which osteoclastic bone matrix resorption is deficient). Animal studies suggest that loss of ovarian function is associated with an increase in osteoclastogenesis mediated by an increase in the production of the osteoclastogenic cytokine interleukin-6 (IL-6) produced by osteoblasts (22, 52). In contrast, senescent bone matrix loss appears to be due to a decrease in bone marrow osteoblast formation and recruitment to remodelling sites (42). Studies of experimental osteopetrosis have recently shed light on the control of osteoclast differentiation and activation. The *op*/*op* mouse strain, which lacks a functional gene for macrophage colony-stimulating factor, lacks osteoclasts and is osteopetrotic (18). Knockout of the transcription factor c-Fos, which forms part of the Fos-Jun transcriptional complex binding the AP-1 transcriptional control site on a variety of genes including, e.g., that of IL-1, also renders animals incapable of producing osteoclasts (24). In contrast, knockout of the gene for Src ($pp60^{c\text{-}src}$), a member of a large family of nonreceptor protein tyrosine kinases, fails to inhibit osteoclastogenesis, but mice are osteopetrotic. Osteoclasts lacking Src fail to develop the ruffled border membrane characteristic of resorbing oste-

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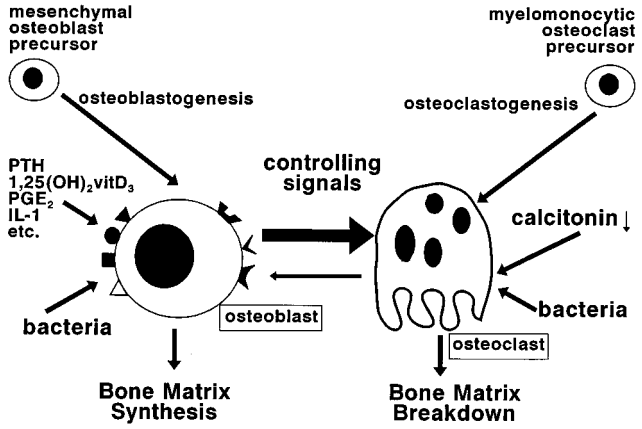

FIG. 1. Interactions between the two major bone cell populations (the osteoblast and the osteoclast) and mediators of bone remodelling including bacterial components. The osteoblast synthesizes the extracellular matrix of the bone and also plays a pivotal role in controlling the activity of the osteoclast. Bone-modulating factors (hormones such as parathyroid hormone [PTH] and 1,25-dihydroxy vitamin D_3) or local factors such as prostaglandin (PGE_2) or IL-1 can act on the mature osteoblast to induce it to produce signals which activate the osteoclast or accelerate the formation of osteoclasts. Such factors can also promote the formation of osteoblasts. It is conceivable that bacterial factors can mimic the actions of these various circulating and local host factors inducing (or inhibiting) osteoblast formation and osteoblast activation. Bacterial factors may also act directly on the osteoclast, inducing its activation and/or inducing osteoclastogenesis.

oclasts, suggesting that loss of this tyrosine kinase inhibits key cellular processes involved in bone resorption (6).

BACTERIAL INFECTIONS AND BONE PATHOLOGY

The range of bacteria involved in bone pathology is provided in Table 2. That bone infections can shape world events is seen from the recent biography of the Nazi propaganda minister Joseph Goebbels in which it is claimed that his attitude (which contributed so much to the Holocaust) was due to his need to overcome his crippled state—the result of childhood osteomyelitis (107).

The key question to be addressed in these diseases is how the bacteria stimulate pathology. A supplementary question is how bacteria get into bone in the first place. In infections of the appendicular and axial skeleton, the answer may lie in bacteria expressing receptors for bone matrix components. For example, *Staphylococcus aureus* contains receptors for fibronectin (102), laminin (87), collagen (98), and bone sialoglycoprotein (111) which presumably serve to trap blood-borne organisms in bone. This trapping is obviously not important in the periodontal diseases which are due to the buildup of bacterial biofilms subgingivally. As bacteria do not invade the periodontal tissues, the accepted paradigm is that local pathology is due to the release of soluble bacterial virulence factors (132, 137) and that this could be a general mechanism in all bone infections.

However, it is clear that at the time of writing (early 1996) we do not understand how bacteria cause pathological bone loss. Three possibilities exist: (i) bacteria directly destroy the noncellular components of bone by liberating acid and proteases, (ii) bacteria promote cellular processes that stimulate the degradation of bone, or (iii) bacteria inhibit the synthesis of bone matrix (Fig. 3). Mechanisms ii and iii may be either a direct effect of components released by bacteria or a consequence of the induction of host factors, for example, cytokines or prostaglandins which then act on bone cells. Mechanism i describes the pathology of dental caries but is likely to be only a minor mechanism in skeletal bone pathology.

TABLE 1. Mediators of bone remodelling

Mediator
Circulating factors
Parathyroid hormone
1,25-dihydroxy vitamin D_3
Calcitonin
IL-6 and other circulating cytokines
Estradiol and other steroid hormones
Local factors
Prostaglandins
Leukotrienes
Proinflammatory cytokines (IL-1, IL-6, TNF)
Growth factors
Bacterial products
LPSs
Teichoic acids
Lipid A-associated proteins
Porins
cpn60 of *A. actinomycetemcomitans* and *E. coli*
Gapstatin of *A. actinomycetemcomitans*
PMT
B. bronchiseptica DNT
Cell wall components of many bacteria
Surface-associated proteins of *A. actinomycetemcomitans*, *P. gingivalis*, *E. corrodens*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*
Capsular polysaccharide of *A. actinomycetemcomitans*
32- and 60-kDa surface-associated proteins of *Staphylococcus aureus*
43-kDa *P. gingivalis* fimbrial protein

CELLULAR AND MOLECULAR MECHANISMS BY WHICH BACTERIA CAUSE ABERRANT BONE REMODELLING

Table 1 provides a list of the various bacterial components which have been shown to interact with bone cells and cause

TABLE 2. Bacteria involved in pathological bone remodelling

Disease (reference[s])	Organism
Periodontitis (113)	*Actinobacillus actinomycetemcomitans* *Porphyromonas gingivalis* *Eikenella corrodens* *Fusobacterium nucleatum* *Prevotella intermedia* *Campylobacter rectus*, etc.
Osteomyelitis (51, 114)	*Staphylococcus aureus* *Staphylococcus epidermidis* *Salmonella* spp. *Escherichia coli*, etc.
Bacterial arthritis (41, 65)	*Staphylococcus aureus* *Neisseria gonorrhoea*[a] *Neisseria meningitidis* *Mycobacterial tuberculosis*[b] *Haemophilus influenzae* *Pasteurella multocida*, etc.
Infected metal implants (109)	*Staphylococcus aureus* *Staphylococcus epidermidis*

[a] Not associated with bone destruction.
[b] Also associated with destruction of vertebrae in Pott's disease.

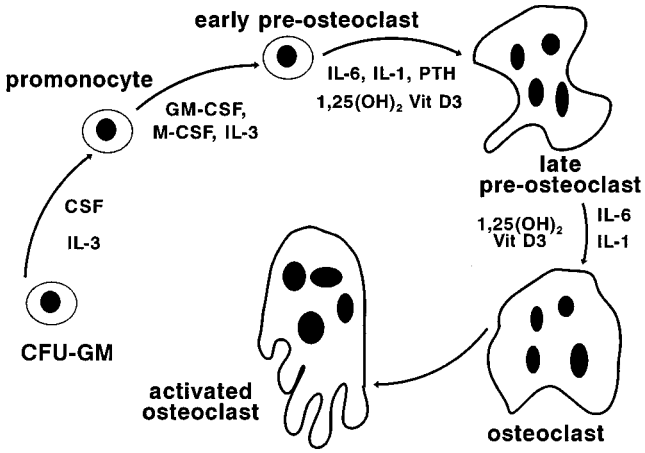

FIG. 2. Cellular pathways and factors involved in the differentiation and proliferation of the osteoclast from the CFU–granulocyte-macrophage (CFU-GM) precursor in the bone marrow. CSF, colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; PTH, parathyroid hormone.

changes in bone remodelling. A range of cell- and tissue-based assays have been used to determine the effects of these components, and the nature of these assays is briefly described in Table 3. For clarity of discussion, the bacterial factors have been divided into those that appear to promote bone resorption and those which appear to inhibit new bone formation, and these will be discussed separately.

Bacterial osteolytic factors. (i) Endotoxin and lipopolysaccharide (LPS). Endotoxin, a complex of LPS and proteins, was the first bacterial component shown to be capable of inducing bone resorption in vitro (23, 31–34, 43, 48, 49, 68, 85, 100, 101, 120, 121, 127). Both the LPS and the proteins of endotoxin have a myriad of actions on eukaryotic cells and are proinflammatory (38, 132). It is frequently difficult to determine if workers have used endotoxin or have purified the LPS from this mixture. However, workers such as Nishihara and colleagues (49, 95, 96) and our own group (104) have shown that highly purified LPSs have the capacity to induce calvarial bone resorption. Although LPS is identified in most people's minds as the major bacterial bone-resorbing factor, surprisingly little is known of its mechanism of action. The possibility that it has a direct effect on the osteoclast is not supported by the work of Sismey-Durrant and Hopps (121) using purified *Porphyromonas gingivalis* LPS, and we have recently failed to find a direct activation of osteoclasts with the international LPS standard, a potent and highly purified LPS from *Escherichia coli* (103b). Ueda and coworkers reported that the polysaccharide portion of *Actinobacillus actinomycetemcomitans* LPS failed to stimulate osteoclast formation in mouse bone marrow (128). LPS is a well-known stimulator of cytokine and eicosanoid synthesis by a range of cell populations (70) and in recent years has been shown to stimulate osteoblasts to secrete the osteolytic factors: IL-1 (26, 57), IL-6 (50, 64), granulocyte-macrophage colony-stimulating factor (47), prostaglandin E_2 (49), and nitric oxide (7, 12, 86, 108). This latter mediator has attracted much attention in recent years. However, its role in bone remodelling is unclear as it has a biphasic response, inhibiting bone resorption at high concentrations (7, 103). Sismey-Durrant and colleagues (120) have also demonstrated that *P. gingivalis* LPS stimulated isolated mouse osteoblasts to synthesize collagenase but not tissue inhibitor of metalloproteinases.

Thus the simplest hypothesis to explain how LPS produces bone resorption is that it activates osteoblasts to release factors which recruit and/or activate osteoclasts. This is clearly demonstrated by the inhibition of LPS-induced bone resorption by

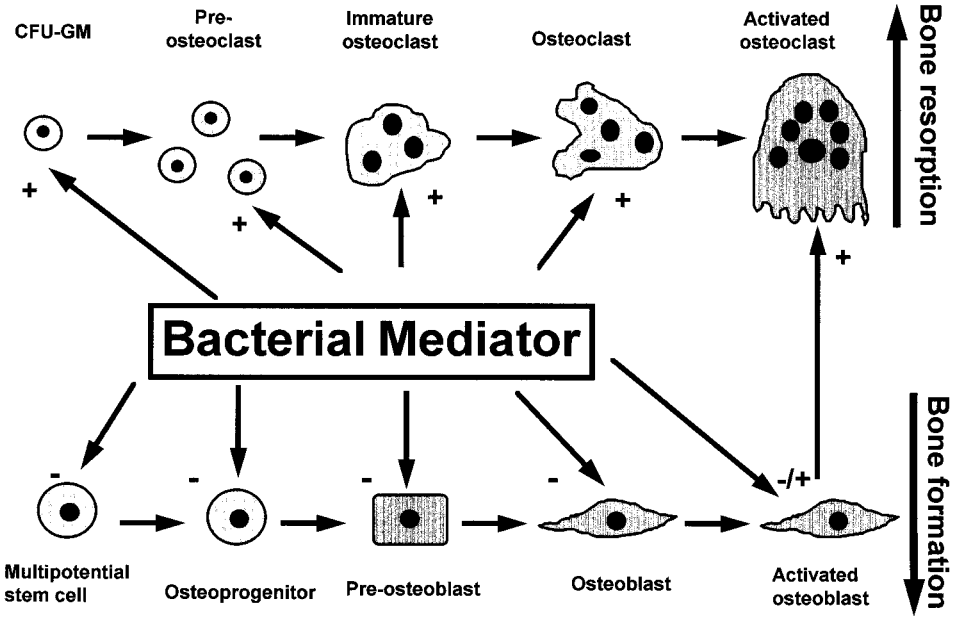

FIG. 3. The possible points of interaction between osteolytically active components of bacteria implicated in bone pathology and the two major bone cell populations. Bacterial factors can interact with either the osteoblast or the osteoclast lineages (or with both) to stimulate bone breakdown and/or inhibit the processes of bone matrix synthesis. Simplistically, bacterial components could directly increase the rate of production and activation of osteoclasts and/or osteoclast precursor cells and thus stimulate bone resorption, or they could slow down the rate of production of bone-forming osteoblasts and/or precursor cells and thus inhibit bone matrix synthesis. However, other mechanisms can come into play. For example, PMT appears to stimulate osteoblast lineage cell proliferation, and in this example, the enhanced bone resorption may be related to interference with the process of osteoblast differentiation. CFU-GM, CFU–granulocyte-macrophage.

TABLE 3. Methods used to determine bone remodelling

Method
Bone resorption assays
Release of calcium from cultured murine calvaria measured by colorimetric assay
Release of ^{45}Ca from prelabelled long bones or calvaria of rodents
Osteoclast and osteoclast recruitment assays
Capacity of chick, quail, or rat long bone cells to produce pits in dentine slices. Active cells are tartrate-resistant acid phosphatase positive and multinucleated, and the ability to induce resorption pits can normally be blocked by calcitonin (in rodent systems).
Formation of tartrate-resistant acid phosphatase-positive multinucleated cells capable of forming pits in dentine slices from murine or human bone marrow
Bone formation assays
Measurement of connective tissue component (e.g., collagen) synthesis in explants of cultured bone or in isolated osteoblast cultures
Production of calcified nodules in cultures of osteoblasts
Production of calcified bone from cultured periosteal explants

the cyclo-oxygenase inhibitor indomethacin or with anti-IL-1 antiserum (49). We have also found that, in contrast to the bone resorption induced by the calcitropic hormones, parathyroid hormone and 1,25-di-(OH) vitamin D_3, or the osteolytic cytokine IL-1, the bone resorption induced by LPS from oral bacteria is inhibited by selective inhibitors of the arachidonate-oxidizing enzyme 5-lipoxygenase (77). This enzyme produces 5-hydroxyeicosatetraenoic acid and the leukotrienes, a family of lipids and peptidolipids which have potent inflammatory actions (37), and we were the first to demonstrate that products of 5- and 12-lipoxygenases are extremely potent inducers of bone resorption in vitro (78). Leukotrienes have been shown to activate osteoclasts (20). A recent report has shown that inhibitors of 5-lipoxygenase block osteoclast-induced bone resorption in vivo (19). This raises the possibility that bacterially induced bone resorption promoted by LPS could be treated with the selective 5-lipoxygenase inhibitors that are expected to be in clinical use in the next few years (76).

Nishihara and coworkers (96) have recently shown that *A. actinomycetemcomitans* LPS, which can induce murine calvarial bone resorption (49), can stimulate the murine macrophage cell line P388D to synthesize the natural IL-1 receptor antagonist (IL-1ra) and that this, in turn, can switch off the osteolytic activity of the LPS. This may be an important control mechanism in a disease in which there is constant exposure of host tissues to LPS, limiting the osteolytic activity of this bacterial constituent.

In terms of activating myelomonocytic cells, free LPS is relatively inactive and requires complexation with CD14 (catalyzed by LPS-binding protein) for maximal cellular effect (115). The report that fetal calf serum or human serum blocked the bone-resorbing activity of LPS from oral bacteria and from *Salmonella typhimurium* suggests that CD14 is not involved in pathways of bone resorption (48). However, more detailed investigation of the role of CD14 in LPS-induced bone remodelling is required before this conclusion can be reached.

We have also demonstrated, for the first time, that the non-LPS component of endotoxin, i.e., lipid A-associated protein, from a number of periodontopathic bacteria can stimulate bone resorption (104) and also induce synthesis of IL-6 (105), a cytokine whose involvement in bone resorption has been discussed elsewhere (22, 50, 52, 68). In these studies, the lipid A-associated proteins proved to be more active bone-resorbing agents than the corresponding protein-free LPS. As lipid A-associated proteins are present in endotoxin preparations, it necessitates a reevaluation of the hypothesis that the bone-resorbing activity of endotoxin is due exclusively to LPS.

(ii) Other cell surface components. It is a common misconception that endotoxin-LPS is the only bacterial component capable of inducing bone resorption. Many microbiologists and bone biologists and/or clinicians are unaware of the fact that there are an increasing number of bacterial structures, components, and products able to induce bone resorption (Table 1). Some of these components are extremely potent inducers of bone resorption. Many of these non-LPS components are also potent inducers of cytokine synthesis (38, 39). Examination of gram-positive bacteria has revealed that unspecified cell wall components from *Streptococcus mutans* and *Streptococcus sanguis* (62, 82) and sonicated extracts of *Staphylococcus aureus* and *Staphylococcus epidermidis* (63) stimulate bone resorption. Lipoteichoic acid (31), muramyl dipeptide (13), and an amphipathic antigen from *Actinomyces viscosus* also have been reported to induce bone resorption (32). However, in our experience, lipoteichoic acid from *Staphylococcus aureus* was a weak inducer of calvarial bone resorption (93).

P. gingivalis fimbriae (composed of a 43-kDa fimbrillin monomer) stimulate fibroblasts and monocytes to synthesize osteolytic and chemotactic cytokines (27, 29, 30). In a recent report, fimbriae have been reported to stimulate murine calvarial bone cells to produce resorption pits in bone, a process inhibited by neutralizing antisera to IL-1 or granulocyte-macrophage colony-stimulating factor (56). These cytokines are known inducers of osteoclast differentiation (56). On the basis of the finding that Src-deficient mice lack functional osteoclasts, Hanazawa et al. (28) examined the effect of the non-selective tyrosine kinase inhibitor genistein on the capacity of isolated murine calvarial bone cells to form pits on dentine slices after stimulation with *P. gingivalis* fimbriae. Significant inhibition of bone resorption was produced by this inhibitor, demonstrating that this bacterial component is activating some cellular tyrosine kinase (possibly Src) to promote osteoclastic bone resorption (28).

Good evidence for the role of fimbriae in bone resorption associated with periodontal disease is shown by experiments in which (i) immunization of gnotobiotic rats with *P. gingivalis* fimbriae protected against *P. gingivalis*-induced periodontal tissue destruction (16) and (ii) the inactivation of the *fimA* gene coding for the major fimbrial subunit blocked the *P. gingivalis*-induced alveolar bone loss in gnotobiotic rats (69). These findings suggest the possibility of vaccination against the *P. gingivalis* fimbriae as a treatment for the periodontal diseases. Unfortunately, these diseases are rarely the result of single bacteria and immunization against a single component from one organism is unlikely to be of therapeutic value to more than a small proportion of patients.

In collaboration with Maria Tufano, University of Milan, we have shown that a purified porin preparation from *Salmonella typhimurium* is osteolytically active at nanomolar concentrations (76a). The mechanism of action of these components remains undefined.

A capsular polysaccharide from *A. actinomycetemcomitans* has also been shown to stimulate bone resorption by a prostaglandin-dependent mechanism (97, 108). This polysaccharide can induce the formation of osteoclasts from mouse bone marrow by a process which is driven by the formation of IL-1α and prostaglandin E_2, but not IL-1β or IL-6. Inhibitors of cyclic AMP (cAMP)-dependent kinases inhibited this osteoclast differentiation.

At the Eastman Dental Institute, we have been studying the bone-modulating effects of proteins associated with the bacterial surface. All the bacteria examined had significant amounts of protein on their surfaces, and this could be released by gently stirring bacteria in saline. With bacteria such as *A. actinomycetemcomitans*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*, up to 80 proteins have been isolated from their surfaces (59, 93). Periodontopathic bacteria such as *A. actinomycetemcomitans* (133, 134) and *Eikenella corrodens* (79) release surface-associated proteins with potent bone-resorbing activity while that from *P. gingivalis* is 1 log order less active (135). In contrast, the surface-associated proteins from *Prevotella intermedia* and *Campylobacter rectus* were only very weakly active in the calvarial bone resorption assay (104). Surface proteins from the gram-positive bacteria *Staphylococcus aureus* (93) and *Staphylococcus epidermidis* (77) showed substantial osteolytic activity.

A direct comparison has been made of the mediators involved in the bone resorption induced by the surface-associated proteins from the gram-negative oral bacteria *A. actinomycetemcomitans*, *E. corrodens*, and *P. gingivalis*. With indomethacin to block cyclo-oxygenase, IL-1ra to antagonize IL-1, and a neutralizing monoclonal antibody to murine tumor necrosis factor (TNF) alpha, completely different patterns of inhibitory responses were seen with these three bacteria. It was not possible to block the osteolytic activity of the surface proteins from *A. actinomycetemcomitans* (which, as reviewed below, has now been established to be due to chaperonin 60 (cpn60) by any of these inhibitors. Only the anti-TNF antibody blocked the bone-resorbing activity of *E. corrodens* surface proteins. In contrast, all three inhibitors potently blocked the bone-resorbing activity of the surface-associated proteins from *P. gingivalis* (79, 135). This suggests that the surface proteins (cpn60) from *A. actinomycetemcomitans* fail to stimulate the synthesis of the three major osteolytic mediators examined. In contrast, *E. corrodens* surface proteins induce TNF synthesis, which then induces the cellular and molecular cascade leading to bone resorption. Surface proteins from *P. gingivalis* appear to induce an explosion of mediators, any one of which, if blocked, totally inhibits bone resorption. We speculate that the prostanoids, IL-1, and TNF must be acting in synergy and that removal of any one of them leads to complete collapse of the osteolytic mechanism. Thus the surface protein fraction from these various bacteria demonstrates the variety of secondary mediator-induced mechanisms which can be activated in bone by bacterial products. This variety of mechanisms, if relevant clinically, could prove a problem for the therapy of the periodontal diseases (137).

A similar picture emerges with the surface proteins eluted from gram-positive bacteria. Those from *Staphylococcus aureus* are potent stimulators of murine calvarial bone resorption and are exquisitely inhibited by nanomolar concentrations of indomethacin but require very high levels of IL-1ra or anti-TNF to block resorption. Indeed, the surface-associated protein fraction from this bacterium is a fairly weak stimulator of human fibroblast or monocyte cytokine synthesis (93). In contrast, the surface-associated protein fraction from *Staphylococcus epidermidis* is only minimally inhibited by indomethacin but is sensitive to neutralization of TNF (77). Sonicates of *Staphylococcus aureus* (which, because of the method of handling bacteria, probably contain very little of surface-associated proteins) stimulate bone resorption by a cyclo-oxygenase-independent mechanism (63).

(iii) Other components or products. Although many of the bone-resorbing factors identified in bacteria appear to be part of, or associated with, the bacterial surface, there are reports of other components or products with osteolytic properties. For example, a 24-kDa protein which is secreted by *P. gingivalis*, promotes fibroblast proliferation, and in consequence has been termed fibroblast-activating factor (83) has been shown to promote in vitro bone resorption and also stimulate the formation of tartrate-resistant acid phosphatase-positive multinucleated cells. However, the presence of classic osteoclast resorption pits was not reported in this study and so osteoclast formation has not been conclusively demonstrated (84). It is not clear how fibroblast-activating factor stimulates osteoclast-like cell formation, but the most likely mechanism is an indirect one via stimulation of bone marrow stromal cells, which then produce factors which promote the formation of the myelomonocytic osteoclast precursor cells.

One of the most potent bacterial bone-resorbing factors described to date is *Pasteurella multocida* toxin (PMT) produced by the said organism. This bacterium is responsible for swine atrophic rhinitis, a disease in which there is severe loss of the bone of the nasal conchae (2, 10, 110), and has been reported to produce septic arthritis in humans (3). PMT has been purified, cloned, and expressed and is a protease-insensitive (123) 146-kDa protein which is an extremely potent mitogen (124) capable, at concentrations as low as 1 pM, of stimulating anchorage-independent cell growth (40). Indeed, this bacterial protein is significantly more potent a mitogen than mammalian mitogens such as epidermal growth factor and platelet-derived growth factor (40). Mitogenic activity appears to be dependent on the entry of the toxin into the vacuolar apparatus of the cell and the stimulation of inositol trisphosphate and protein kinase C, with an associated elevation of intracellular calcium levels (124). While a number of bacterial toxins, including PMT, contain a His-Glu-Trp motif, which exhibits ADP-ribosylation activity, it has been shown, by site-directed mutagenesis, that this activity is not required for the mitogenic action of PMT (130). It is assumed that the mitogenic activity of PMT is related to its potent ability to promote bone resorption. In experimental studies in which PMT has been administered to experimental animals, bone resorption was associated with an increased number of osteoclasts in affected bones (1, 14, 21, 73, 74). A surprising finding was that intraperitoneal injection of PMT resulted in increased osteoclast density and bone loss both in the maxillofacial skeleton and in the appendicular skeleton of rats (73). The activity of PMT has been tested in the two major in vitro bone resorption assays—the murine calvarial assay and the murine long bone assay (17). These bones have a different embryonic derivation and often respond differently to the same osteolytic mediator (e.g., TNF or epidermal growth factor, etc.). However, in both assays, PMT produced maximal bone resorption at the extremely low concentration of 5 ng/ml (approximately 30 pM [17]). We have shown that recombinant PMT stimulates significant murine calvarial bone resorption at concentrations as low as 0.1 ng/ml (<1 pM [76b]). Thus this protein is as active a mediator of bone resorption as the most potent osteolytic cytokine, IL-1 (116, 125). PMT-induced bone resorption was completely inhibited by calcitonin, a circulating hormone able to inhibit osteoclast function, and inhibitors of cyclo-oxygenase partially inhibited this bone resorption (17). While *Pasteurella multocida* infections in pigs are associated only with nasal turbinate atrophy, it appears from this and in vivo data that PMT can act on bones other than the nasal bones. Whether PMT acts directly on mature osteoclasts to activate them, or to induce their proliferation, or acts indirectly to promote osteoblast proliferation with consequent osteoclast recruitment, is still to be established. PMT was reported to reduce alkaline phosphatase levels in the rat osteoblast-like cell line ROS 17/2.8 (126), and in a detailed recent study, PMT was shown to

be a potent dose-dependent mitogen for primary chicken osteoblasts, inducing elevation of inositol phosphates but not cAMP. Of note was the finding that PMT down-regulated the expression of several markers of osteoblast differentiation including alkaline phosphatase and type I collagen and inhibited osteoblastic bone formation in an in vitro assay of bone formation (88). As has been discussed, bone remodelling requires the coordinated differentiation of osteoblast and osteoclast lineages. Any interference with such lineage development is likely to result in altered bone remodelling kinetics, and this may be the mechanism of action of PMT.

We have recently identified the proteins on the surface of *Staphylococcus aureus* and *A. actinomycetemcomitans* responsible for stimulating bone resorption. In the case of the former bacterium, a small number of proteins have osteolytic activity, with the most potent being a 32- to 36-kDa heterodimeric protein (93). The osteolytic protein on the surface of *A. actinomycetemcomitans* has now been conclusively identified as cpn60, a homolog of *E. coli* GroEL (59). This is a surprising finding as cpn60 is an intracellular oligomeric protein, composed of 14 60-kDa subunits forming a molecular aggregate of around 850 kDa. cpn60 is a molecular chaperone, i.e., "a protein that assists the non-covalent assembly of other protein-containing structures but which is not a component of these structures when they are carrying out their normal biological functioning" (11, 36). It is not clear why substantial amounts of this molecular chaperone are on the outside of this bacterium. This external location has also been determined by immunogold labelling of *A. actinomycetemcomitans* using a monoclonal antibody to cpn60 (4). The possibility exists that the extracellular cpn60 is required to fold the large number of proteins found in or on the cell wall of *A. actinomycetemcomitans*. The most interesting finding was that this chaperonin possessed potent osteolytic activity. None of the other bacteria which we have shown to possess osteolytic surface proteins contain cpn60 in this surface fraction. When we examined the cpn60 from other bacteria, we were surprised to find that the *E. coli* cpn60-GroEL was also an extremely potent inducer of calvarial bone resorption active at concentrations as low as 1 ng/ml (i.e., in the low picomolar range). The possible role of LPS in the bone-resorbing activity of cpn60, either as a contaminant or as a synergistic complex, has been ruled out by appropriate control experiments (59).

It has been reported that certain cpn60s can stimulate cytokine synthesis (39, 106). The bone-resorbing activity of the surface-associated protein fraction of *A. actinomycetemcomitans*, which is attributable to cpn60, is not inhibited by indomethacin, IL-1ra, or neutralizing anti-TNF antibodies, while the osteolytic activity of GroEL is inhibited by indomethacin and IL-1ra (reference 79 and unpublished data). In contrast to these cpn60s, the cpn60 (hsp65) from *Mycobacterium tuberculosis* and *Mycobacterium leprae* failed to show any bone-resorbing activity. This may relate to differences in the oligomerization of the gram-negative and mycobacterial cpn60s (59). However, the mycobacterial cpn60 cochaperone, cpn10, is a potent inducer of murine calvarial bone resorption (76b).

The finding that the ubiquitous gram-negative cpn60s can stimulate bone resorption at picomolar concentrations opens up a new vista in our understanding of the mechanisms by which bone remodelling can be controlled by bacteria. It also raises the question of whether mammalian cpn60s can also control bone remodelling and, if so, whether they are involved in idiopathic diseases of bone such as osteoporosis. Our current research strongly suggests that the action of cpn60 on bone may be due to the direct activation of osteoclasts and osteoclast recruitment (103a).

THE CAPACITY OF BACTERIA AND THEIR PRODUCTS TO INHIBIT BONE FORMATION

In addition to stimulating in vitro bone resorption, endotoxin-LPS has also been reported to inhibit bone collagen and noncollagenous protein synthesis (85, 136). A number of reports have suggested that extracts of dental plaque or of cultured periodontopathic bacteria can inhibit bone matrix synthesis (44, 89). Surface-associated proteins from oral bacteria are also able to inhibit bone matrix synthesis (81).

In a recent report, sonicated extracts from bacteria implicated in the pathology of periodontitis (*P. gingivalis*, *A. actinomycetemcomitans*, and *Prevotella intermedia*) were found to inhibit osteogenesis in the chick bone-forming culture system (Table 3). In contrast, organisms not implicated in these diseases (*Streptococcus sanguis*, *Veillonella atypica*, and *Prevotella denticola*) had minimal effect (66, 67).

Certain periodontopathic bacteria produce factors which have general antiproliferative activity (35, 53, 54, 61, 80, 112, 118). These could possibly play a role in inhibiting osteoblast proliferation and thus impair bone remodelling. These various biological activities have not been purified or characterized. However, we have recently isolated an antiproliferative protein from *A. actinomycetemcomitans* which is most active against human osteoblast-like cell lines, suggesting some specificity for bone. This 8-kDa protein (131) termed gapstatin (130a) has a novel mechanism of action. It does not inhibit DNA synthesis directly but inhibits cell cycle progression by blocking cells in the G_2 phase of the cell cycle. Moreover, kinetic studies of synchronized cell populations revealed that gapstatin acts only on cells in S phase. This is an unusual, probably unique, mechanism of cell cycle inhibition, and we speculate that this molecule may act by inhibiting the synthesis of cyclin B1, a protein required to ensure that cells make the transition from G_2 to mitosis. As bone remodelling and matrix synthesis require the continued production of osteoblasts and osteoclasts, the action of gapstatin could inhibit new bone matrix formation. Such an effect would be particularly damaging if it were to occur in concert with molecules stimulating bone breakdown, such as cpn60. It is possible that gapstatin could inhibit the formation of osteoclasts. However, in studies of the effect of *A. actinomycetemcomitans* surface proteins on osteoclastogenesis, using the standard murine bone marrow proliferation assay, this surface fraction stimulated, not inhibited, osteoclast formation, suggesting that gapstatin does not block the processes involved in the formation of bone marrow polykaryons (79). Of interest, in this context of influencing cell cycle control, is the recent report that *A. actinomycetemcomitans* can induce apoptosis in cultured murine macrophages (55).

Bordetella bronchiseptica produces a 145-kDa dermonecrotic toxin (DNT) which has actions similar to those of PMT and is also responsible for turbinate atrophy in swine atrophic rhinitis (2, 15). Histologically, the lesions induced by *B. bronchiseptica* suggest impaired osteoblastic function (119), which contrasts with the histological picture of increased osteoclastic activity in animals exposed to PMT (discussed above). There is only one report of the effect of DNT on cultured bone, and its effects were not particularly striking (58). However, when added to the murine osteoblastic cell line MC3T3-E1 it caused changes in cellular architecture and potently inhibited (50% inhibitory concentration, 100 pg/ml) the osteoblasts' capacity to produce both alkaline phosphatase and collagen (45), an action that could seriously affect bone remodelling if replicated in vivo. Surprisingly, it has recently been reported that DNT is a potent stimulator of tritiated thymidine incorporation into MC3T3-E1 cells with a 50% effective dose of approximately 1 ng/ml. In

spite of this incorporation of label, the numbers of MC3T3-E1 cells in culture did not increase. The major consequence of exposure to DNT was the appearance of multinucleated osteoblasts. Tritiated thymidine incorporation could be inhibited by drugs known to block DNA synthesis. It therefore appears that DNT inhibits the process by which cells divide into two daughter cells (cytokinesis), but it is not clear how this is achieved or how it relates to the inhibition of osteoblast function, such as collagen synthesis (46). Another cell cycle-inhibitory protein has recently been isolated from the periodontopathic bacterium *Fusobacterium nucleatum*. This protein blocks human T lymphocytes in the mid-G_1 phase of the cell cycle (117). While it has not been shown to be able to stimulate bone resorption, we would predict that it would have this mode of action.

It is now becoming clear that bacteria produce a range of proteins which are able to interfere with the mammalian cell cycle, and we suggest that the activity of these proteins represents a new bacterial virulence mechanism. The importance of the proliferation and maturation of bone cell lineages in bone remodelling is presumably the reason that the bacterial cell cycle-modulatory proteins discovered to date induce bone pathology or come predominantly from bacteria implicated in diseases involving bone matrix loss.

SUMMARY

Normal bone remodelling requires the coordinated regulation of the genesis and activity of osteoblast and osteoclast lineages. Any interference with these integrated cellular systems can result in dysregulation of remodelling with the consequent loss of bone matrix. Bacteria are important causes of bone pathology in common conditions such as periodontitis, dental cysts, bacterial arthritis, and osteomyelitis. It is now established that many of the bacteria implicated in bone diseases contain or produce molecules with potent effects on bone cells. Some of these molecules, such as components of the gram-positive cell walls (lipoteichoic acids), are weak stimulators of bone resorption in vitro, while others (PMT, cpn60) are as active as the most active mammalian osteolytic factors such as cytokines like IL-1 and TNF. The complexity of the integration of bone cell lineage development means that there are still question marks over the mechanism of action of many well-known bone-modulatory molecules such as parathyroid hormone. The key questions which must be asked of the now-recognized bacterial bone-modulatory molecules are as follows: (i) what cell population do they bind to, (ii) what is the nature of the receptor and postreceptor events, and (iii) is their action direct or dependent on the induction of secondary extracellular bone-modulating factors such as cytokines, eicosanoids, etc. In the case of LPS, this ubiquitous gram-negative polymer probably binds to osteoblasts or other cells in bone through the CD14 receptor and stimulates them to release cytokines and eicosanoids which then induce the recruitment and activation of osteoclasts. This explains the inhibitor effects of nonsteroidal and anticytokine agents on LPS-induced bone resorption. However, other bacterial factors such as the potent toxin PMT may act by blocking the normal maturation pathway of the osteoblast lineage, thus inducing dysregulation in the tightly regulated process of resorption and replacement of bone matrix. At the present time, it is not possible to define a general mechanism by which bacteria promote loss of bone matrix. Many bacteria are capable of stimulating bone matrix loss, and the information available would suggest that each organism possesses different factors which interact with bone in different ways. With the rapid increase in antibiotic resistance, particularly with *Staphylococcus aureus* and *M. tuberculosis*, organisms responsible for much bone pathology in developed countries only two generations ago, we would urge that much greater attention should be focused on the problem of bacterially induced bone remodelling in order to define pathogenetic mechanisms which could be therapeutic targets for the development of new treatment modalities.

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