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Direct Effects of Metabolic Products and Sonicated Extracts of *Porphyromonas gingivalis* 2561 on Osteogenesis In Vitro

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It is well documented that oral microorganisms play a significant role in the initiation and progression of periodontal disease. By using various in vitro models, it has been shown that some bacteria considered periodontal pathogens or their products can stimulate bone resorption and some other parameters of osteoblast-like cell activity. However, the effects of these organisms and their products on osteogenesis itself are not known. This study was undertaken to determine the direct effects of metabolic products and sonicated extracts of *Porphyromonas gingivalis* on bone formation in the chick periosteal osteogenesis model. Cultures of *P. gingivalis* 2561 were grown under standard anaerobic culture conditions. The spent medium was collected, and following centrifugation, sonicated bacterial extracts were prepared from the bacterial pellet. These were added in various proportions to the chick periosteal osteogenesis cultures. Sonicated extracts were further fractionated into five molecular-size ranges and similarly tested. Parameters of osteogenesis, including alkaline phosphatase activity, calcium and P_i accumulation, and collagen synthesis, were measured on 6-day-old cultures. Compared with controls devoid of bacterial products, osteogenesis was inhibited significantly in cultures treated with either conditioned medium or extracts obtained from *P. gingivalis*. Various amounts of inhibitory activity were observed in the different ultrafiltration molecular-size fractions, with very profound inhibitory effects observed in the <5-kDa range. Histological observations indicated the presence of cells, some bone, and/or new fibrous connective tissue at all concentrations, indicating that toxicity was not a factor. These results suggest that periodontal pathogens such as *P. gingivalis* might contribute to the bone loss in periodontal diseases not only by stimulating resorption but, possibly, by inhibiting bone formation directly.

The association between the community of bacterial species that colonize gingival crevices and the onset and progression of periodontal diseases has been well established (40, 54). Bacteria likely stimulate tissue destruction in periodontal diseases both by acting directly on host connective tissue stromal cells and by inducing complex inflammatory responses leading to alterations in connective tissue cell metabolism (33). Although various microbial virulence factors have been identified, the complex mechanisms by which pathogenic bacteria cause connective tissue destruction have not been elucidated fully.

One of the major consequences of periodontal diseases is the loss of alveolar bone. In health, bone remodels; there is continual bone resorption and bone formation. To maintain homeostasis, the pathways for bone formation and resorption are in balance with one another and are said to be coupled (41). In periodontal diseases, net alveolar bone loss likely results from abrogation of this balance (16). Thus, understanding how bone formation may be regulated under the influence of microbial products or associated inflammatory reactions is just as significant as understanding resorption mechanisms.

While the effects of microbial products and extracts on bone resorption have been studied rather extensively, their effects on bone formation (osteogenesis) have not been examined in as much detail. This is likely due in part to the lack of reliable in vitro model systems in which mineralized bone is produced. Most model systems involving the use of whole calvarial explants may not be useful to study factors regulating formation, as little active bone formation actually occurs (23). There have also been studies assessing the effects of various bacterial factors on bone-derived cells in culture (3, 24, 25, 31, 36, 42). However, these investigations measured relatively nonspecific parameters such as cellular proliferation in isolation rather than a spectrum of parameters which might reflect bone metabolism more holistically, like alkaline phosphatase activity, calcium uptake, and α-1 collagen production.

The present investigation was undertaken to document the direct effects of metabolic products and extracts of *Porphyromonas gingivalis*, a microbial pathogen associated with periodontitis (26), on osteogenesis in an in vitro bone formation model, the chick periosteal osteogenesis (CPO) model (44, 46, 47, 50), in which the combination of the parameters of osteogenesis listed above can be quantified.

MATERIALS AND METHODS

Bacterial culture conditions. *P. gingivalis* 2561 (ATCC 33277) was originally supplied by J. Slots, State University of New York, Buffalo (currently at University of Southern California), and added to the frozen culture collection at the University of Toronto. Working stocks were grown on blood agar plates at 37°C in an anaerobic chamber in an atmosphere containing 80% N_2, 10% H_2, and 10% CO_2. After 5 to 7 days of growth, bacterial inocula were added to Trypticase-yeast extract broth which contained (per liter) 17 g of Trypticase peptone (Becton Dickinson Microbiological Systems, Cockeysville, Md.), 3 g of yeast extract (Difco, Detroit, Mich.), 5 g of NaCl, 2.5 g of K_2HPO_4, 0.084 g of $NaHCO_3$, and 2.5 g of glucose and supplemented with 5 g of hemin and 0.5 mg of menadione. The bacteria were grown in Trypticase-yeast ex-

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tract broth for a further 3 days. The purity of cultures was verified by phase-contrast microscopy, Gram stain, and subculture on blood agar plates. Bacteria were harvested by centrifugation at 10,000 × *g* for 15 min at 4°C. The supernatant medium was sterilized by filtration through a 0.45-μm-pore-size filter (Millipore) and stored at −20°C. The bacterial cell pellet was washed in phosphate-buffered saline, pH 7, and resuspended in BGJ_B tissue culture medium (GIBCO, Grand Island, N.Y.) to an optical density of 1.0 at 690 nm (model 350 spectrophotometer; G. K. Turner Associates, Palo Alto, Calif.). The bacterial suspensions were then sonicated at maximum power output by using a Biosonik IV sonicator (Bronwill Co., Rochester, N.Y.). The insoluble debris was removed by centrifugation at 10,000 × *g* for 30 min at 4°C, and the medium supernatant was filter sterilized.

A portion of the sonicated bacterial extract was then subjected to ultrafiltration (Amicon) to produce five fractions spanning the following molecular-size (in kilodaltons) ranges: (i) <5, (ii) 5 to 10, (iii) 10 to 50, (iv) 50 to 100, and (v) >100. All fractions were stored at −20°C.

CPO culture system. This osteogenic model system has been described in detail previously (23, 46). Ectocranial periosteal tissues were removed from calvaria derived from 17-day-old embryonic chickens after excision of most of the fibrous tissue. Removal of the fibrous tissues by microdissection significantly reduces nonosteogenic cell content of these cultures, thereby enhancing osteogenic "signal" for biochemical assessments of cultures (23, 43, 46). The periostea were then folded with the side originally facing bone tissue (i.e., the osteogenic layer of cells) in apposition. The explants, supported on a Millipore filter (0.45 μm), were held at the gas-liquid interface of the culture medium on a stainless steel grid over the center well of an organ culture dish (Falcon Plastics, Lincoln Park, N.J.) and incubated for up to 6 days at 37°C in a humidified atmosphere containing 5% CO_2 in air.

(i) Control medium. The control culture medium consisted of BGJ_B medium supplemented with 10% fetal calf serum (GIBCO), 10^{-7} M dexamethasone (Sigma, St. Louis, Mo.), (22, 44), 10 mM β-glycerophosphate (Sigma) (46), and 300 μg of L-ascorbate (GIBCO) (49) per ml.

(ii) Effects of bacterial metabolic products. To test the effects of bacterial metabolic products on osteogenic cultures, the control medium (described above) was supplemented with various proportions (volume/volume) of either uninoculated Trypticase-yeast extract broth growth medium (25%) or sterile conditioned (spent) Trypticase-yeast extract broth growth medium (12.5 and 25%).

(iii) Effects of bacterial sonicated extracts. To test the effects of sonicated bacterial extracts on osteogenic cultures, the extracts were added in various proportions (10, 20, and 40% [vol/vol]) to the control medium. The extract fractions derived by ultrafiltration were added directly to control culture medium in concentrations of 20, 40, and 60% (vol/vol). Media in all cultures were changed every 48 h.

Biochemical measurements. Single CPO explants were homogenized in 1 ml of a bicarbonate buffer (3 mM $NaHCO_3$ in 15.0 mM NaCl, pH 7.4) by using a Polytron homogenizer (Kinematica Gmbh, Littau, Switzerland). The homogenate was transferred to glass test tubes and centrifuged at 3,180 × *g* for 10 min at 6°C. The supernatant fraction was assayed for soluble protein content to estimate culture size (4), as well as alkaline and acid phosphatase activities to assess osteoblastic differentiation and activity (43, 44). Mineralization was measured by estimating the amount of acid-extractable calcium and phosphate from the pellet after overnight hydrolysis in 0.5 N HCl (46, 48). All colorimetric assays (used for determination of acid and alkaline phosphatase activity, P_i, and protein content) were carried out in Titertek 96-well plates, and optical density was measured with a Titertek Multiskan MC spectrophotometer (Flow Laboratories, Mississauga, Canada). The calcium extracted from the pellets was measured by atomic absorption spectrophotometry (Perkin-Elmer, Norwalk, Conn.).

Measurement of α-1 collagen content and synthesis. Newly synthesized collagen was labelled for a 48-h period, at 4 to 6 days, by the addition of ^{14}C-glycine (Amersham, Arlington Heights, Ill.) (10 μCi/ml, 59 mCi/mmol) to the culture medium. Following incubation, the cultures were harvested and frozen at −20°C.

Whole radiolabelled explants were demineralized by being washed briefly in 0.1 N HCl. Total protein content of the acid extract was determined by the method described by Smith et al. (39) using bicinchoninic acid in a buffer containing 0.2 N NaOH and 4% copper sulfate and read colorimetrically at 562 nm on a Titertek spectrophotometer.

To measure collagen, the explant was digested at 15°C for 4 h in pepsin (100 μl; 50 μg/ml in 1.67×10^{-5} M acetic acid), and the digest was pelleted in the Microfuge at 12,000 × *g* for 10 min. The pepsin extract was freeze-dried and reconstituted in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (Tris-glycine) for separation on Phast-Gels (Pharmacia-LKB, Uppsala, Sweden) and then stained with Coomassie blue. The α-1 bands were scanned on an E.C. 910 densitometer (Pharmacia) and quantified by using an integration program (GelScan; Pharmacia). Fluorographs were made from the same gels, and assessed densitometrically by using the same scanner and integration program. This approach allowed for simultaneous measurement of both total and newly synthesized α-1 type I collagen.

Histology. To confirm that osteogenesis occurred and that cultures were indeed viable, five to eight cultures per group were fixed in neutral buffered formalin and processed for routine paraffin sections (5 μm). They were subsequently stained with hematoxylin and eosin and by the von Kossa method to demonstrate mineral deposits.

Statistical analysis. Values for the various biochemical parameters were used to calculate a mean and standard error for each group (8 to 10 cultures), and the differences between means were evaluated by Student's *t* test for comparisons between specific experimental groups and the control or analysis of variance for multiple comparisons. Significance was assigned at the $P < 0.05$ level. All values in tables and figures represent means ± standard errors of the means (SEM).

RESULTS

***P. gingivalis* metabolic products.** Growing CPO cultures in BGJ_B medium containing dilutions of *P. gingivalis* growth medium yielded biochemical parameters of osteogenesis that were reduced in a dose-dependent pattern. Alkaline phosphatase activity was reduced and acid phosphatase activity increased significantly in the presence of 12.5 and 25% concentrations of *P. gingivalis* conditioned medium (Fig. 1). The nonconditioned bacterial medium also produced some reduction of osteogenesis in CPO cultures, but inhibition of alkaline phosphatase and mineral uptake (Fig. 2) by the bacterially conditioned medium was more profound.

***P. gingivalis* whole sonicated extracts.** The addition of sonicated extracts from pellets of *P. gingivalis* to CPO cultures also yielded reductions in most parameters of osteogenesis (Fig. 3 and 4). Statistically significant reductions in alkaline phosphatase activity, calcium, and phosphate were observed. In addition, there was at least a 40% reduction in total and newly

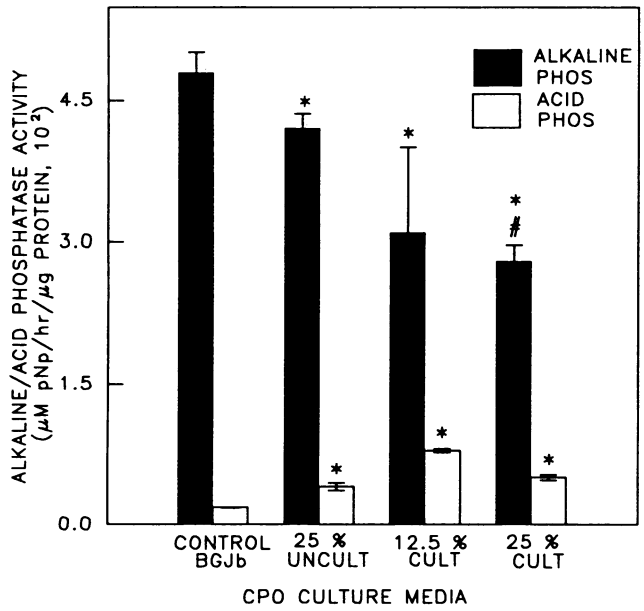

FIG. 1. Alkaline and acid phosphatase activities for control, 25% unconditioned (uncultured) bacterial medium, 12.5% conditioned (cultured) bacterial medium, and 25% conditioned medium groups. Alkaline phosphatase (AP) activity is significantly different ($P < 0.05$) (*) between control and all other treatment groups. There was a similar statistically significant difference in AP activity between the unconditioned control group and 25% conditioned medium ($P < 0.05$) (#). Acid phosphatase activity was increased in the three treatment groups over the control ($P < 0.05$) (*) but was much lower than AP activity within the same group. The data are means and SEM for 8 to 10 cultures. pNp, paranitrophenol.

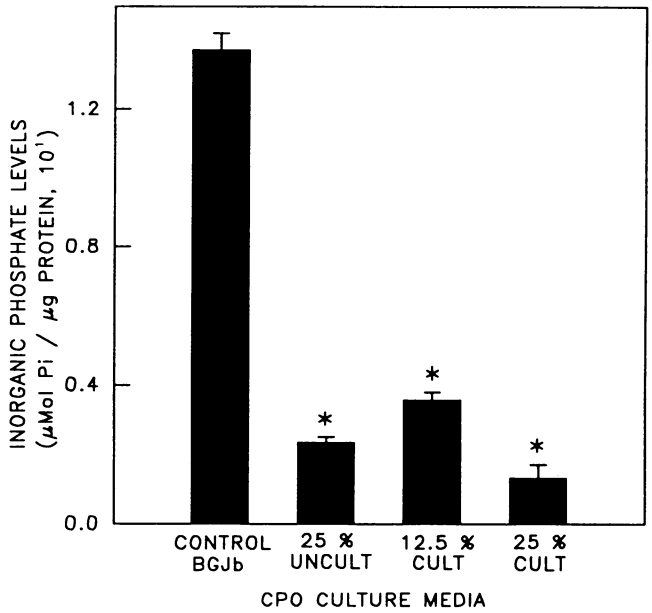

FIG. 2. P_i levels for control, unconditioned, and conditioned groups (see the legend to Fig. 1 for descriptions). A significant decrease from control levels is seen for all treatment groups ($P < 0.05$) (*). The data are means and SEM for 8 to 10 cultures.

synthesized radiolabelled α-1 collagen as assessed by densitometry of Coomassie-stained gels and fluorographs, respectively. Although there was an apparently dose-dependent relationship between concentration of extract and inhibition of parameters of osteogenesis, the relationship was not entirely linear, probably as a result of the heterogeneous nature of the extracts.

Cultures grown in the presence of the various whole extracts appeared to maintain viability, as histological observation indicated that sections of all cultures contained mineralized bone (Fig. 5).

Effects of various molecular-weight fractions on osteogenesis. Whole sonicated extracts were separated by ultrafiltration into different molecular-size fractions. The most significant decrease in alkaline phosphatase activity was observed in CPO cultures grown in the presence of the 60% concentration in all five fractions (Table 1). Yet, significant decreases were observed even at lower extract concentrations. Alkaline phosphatase activity was reduced most dramatically (more than eightfold; $P < 0.001$) and almost to the detection threshold in the presence of the <5-kDa fraction, regardless of the concentration used.

Calcium and phosphate accumulation levels were reduced significantly in all five fractions at concentrations of 40 and 60% (Table 2). For both parameters, greater effects were observed at the higher extract concentration. Calcium and P_i were below the detection threshold for all <5-kDa fraction treatment groups.

Both total and radiolabelled α-1 collagen content extracted by pepsin digestion were reduced in the presence of all sonicated extracts (Table 3). Inhibition of collagen production was greater at higher extract concentrations for most of the fractions. There was at least a 10-fold decrease in collagen production by CPO cultures grown in the presence of the <5-kDa fraction compared with controls ($P < 0.001$). Degradation products of collagen were not observed.

Noncollagenous protein content was reduced significantly in all fractions at most extract concentrations, with the exception of the <5-kDa fraction, for which values did not differ significantly from those for the control cultures (data not shown).

Histological observation by light microscopy demonstrated apparently normal bone formation in all extract fraction groups except the <5-kDa group. The latter cultures contained extensive areas of nonmineralized fibrous tissue and thin seams of osteoid but little or no mineralized bone tissue (Fig. 5). However, the appearance of the cells and tissues was within normal limits, and there was no evidence for toxicity. The bone matrix in the other extract fraction groups appeared to be highly cellular and remarkably similar in appearance to that of chondroid bone (2).

DISCUSSION

We have found that products derived from conditioned media or extracts of *P. gingivalis* have the capacity to inhibit bone formation directly. All measured parameters of osteogenesis were reduced. These findings suggest the possibility that the bone loss observed in periodontitis might be related not only to increased resorption but also to decreased bone formation.

There have been numerous investigations of bacterially stimulated bone resorption. For example, *P. gingivalis* elaborates several extracellular and cell-associated products which have been reported to stimulate bone resorption in vitro (3, 25, 42). Lipopolysaccharide (LPS) isolated from *P. gingivalis* 381 has also been found to stimulate resorption in fetal rat bone

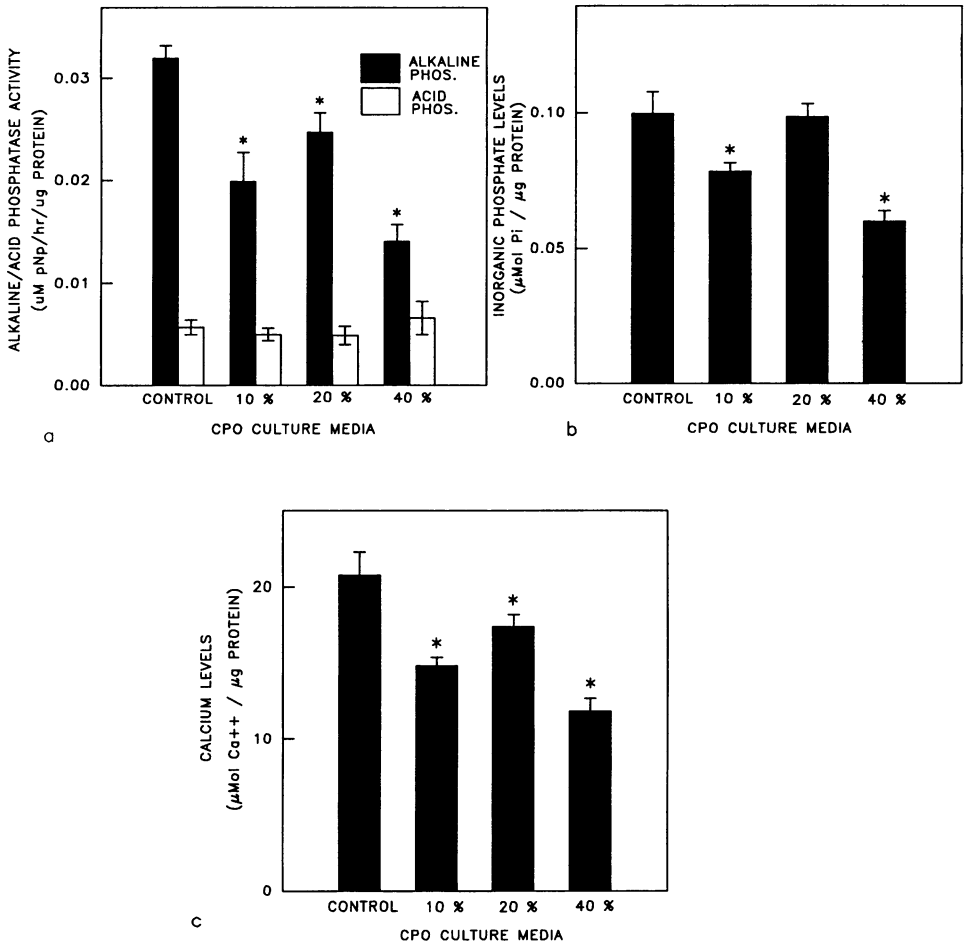

FIG. 3. Biochemical parameters for control (100% plain BGJ_B medium) and 10, 20, and 40% concentrations of BGJ_B medium sonicated extracts of *P. gingivalis*. (a) Alkaline phosphatase (AP) and acid phosphatase activities. A significant difference in AP activity is seen between the control and all treatment concentrations ($P < 0.05$) (*). Acid phosphatase activity did not differ significantly between control and test groups. pNp, paranitrophenol. (b) P_i levels. A significant decrease from control levels is seen for both the 10 and the 40% concentrations ($P < 0.05$) (*). (c) Calcium levels. A significant decrease from control levels is seen for all treatment concentrations ($P < 0.05$) (*). The data are means and SEM for 8 to 10 cultures.

cultures (28). Moreover, LPS extracted from *P. gingivalis* W83 has been shown to stimulate human fibroblast production of prostaglandin E_2 and interleukin 1β, both of which are considered important local mediators of bone resorption (7, 35, 37). Fimbriae derived from *P. gingivalis* have also been shown to trigger interleukin 1β production by monocytes/macrophages (15).

In this study, we have used the CPO model, a bone formation system in which osteoclasts have not been demonstrated. Thus, reductions of mineral or collagen content in the CPO cultures grown in the presence of bacterial metabolites are in all probability not related to induction of osteoclast differentiation or activity.

Apart from their apparent ability to stimulate osteoclast-mediated bone resorption, there is ample evidence indicating that various periodontal pathogens produce proteases which may degrade extracellular matrices in bone and soft connective tissues (10, 12–14, 52). Thus, apart from mediating effects on osteoblastic differentiation or function, it is conceivable that bacterial proteases might also degrade extracellular products of osteoblasts, leading to the decreases in net bone matrix formation in vivo or, as in this study, in vitro. However, in this investigation, the presence of serum in the culture medium makes this less likely, because of its collagenase-inhibitory factors. Moreover, diffusion of large proteolytic molecules through the multilayer of osteoblasts surrounding the bone (5, 30) would not be very efficient, diminishing the effects that these could have on measurements of collagen synthesis in bone tissues. In addition, we have shown previously that the majority of collagen synthesis in the modified microdissected CPO model is attributable to osteoblasts (23). Therefore, it is unlikely that changes in radiolabelled collagen synthesis are due to degradation of collagen produced in the fibroblastic exogenous layers which might be more accessible to the proteases.

Although, there is less known about the direct effects of bacteria or bacterial products on osteogenesis, there have been a few relevant reports. For example, interleukin 1β has also been shown to inhibit DNA synthesis and proliferation of osteoblast-like cells, an observation which might have implications with respect to inhibition of osteogenesis (15). In addition, Norton et al. (31) reported that bone formation in the rat

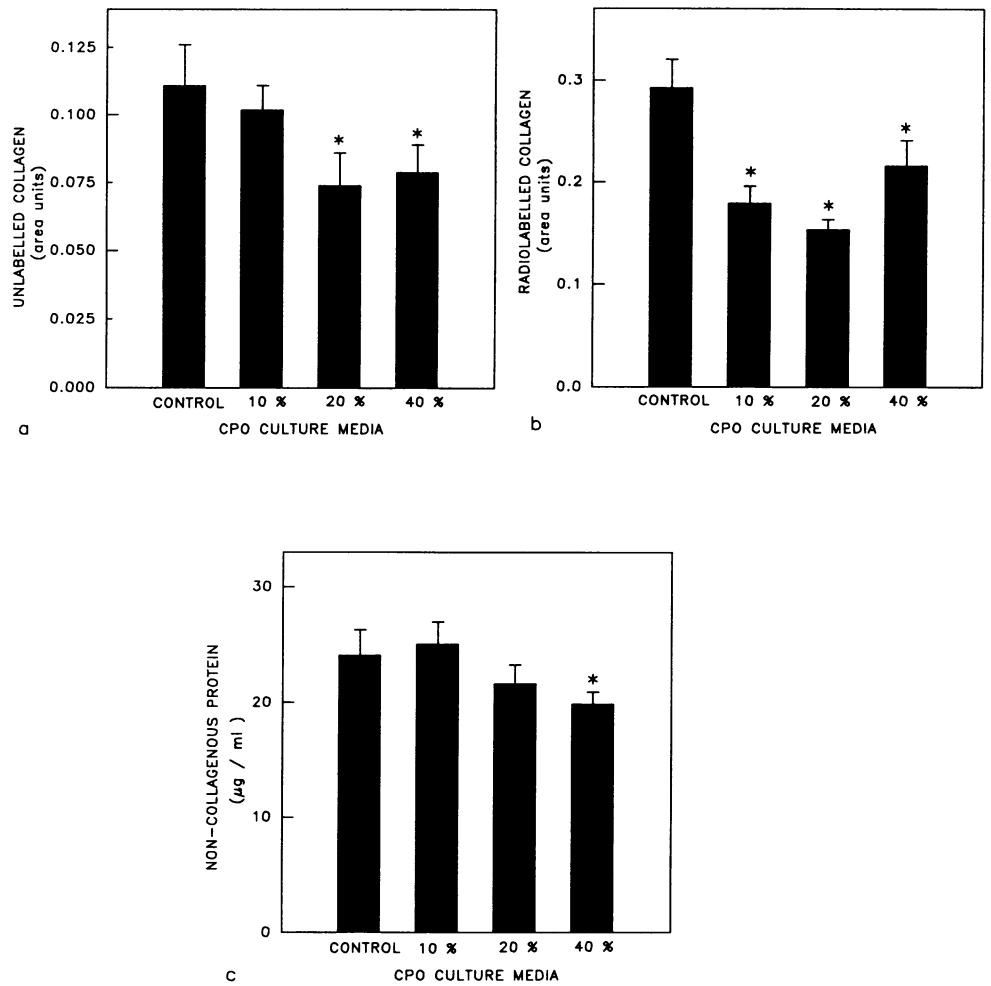

FIG. 4. Collagen and noncollagenous protein evaluations for control (100% plain BGJ_B medium) and 10, 20, and 40% concentrations of BGJ_B medium sonicated extracts of *P. gingivalis*. (a) Densitometric evaluation of Coomassie blue-stained minigels run on SDS-PAGE for control. A significant decrease from control levels is seen for both the 20% and 40% concentrations ($P < 0.05$) (*). (b) Densitometric evaluation of fluorographs of SDS-PAGE gels. A significant decrease from control levels is seen for all treatment concentrations ($P < 0.05$) (*). (c) Noncollagenous protein levels. A significant decrease from control levels is seen at the 40% concentration ($P < 0.05$). The data are means and SEM for 8 to 10 cultures.

forepaw was inhibited in vitro in the presence of LPS derived from *Escherichia coli*. However, this could have been due to stimulation of resorption, inhibition of bone formation, or possibly even cartilage formation (i.e., endochondral ossification).

Others have shown direct inhibitory effects of various bacterial products including bacterial plaque on bone-derived cells or tissues. Multanen et al. (27) reported that low concentrations of dental plaque could inhibit type I collagen synthesis in cultured fetal rat calvaria, but the cellular source of collagen, whether osteoblastic or fibroblastic, was not known. Denatured plaque extracts were shown to be equally effective, suggesting that some component other than protein was likely the causative agent. Others have shown that culture medium filtrates from *P. gingivalis* can inhibit matrix production by chicken embryo cartilage cells in vitro (51). When the filtrates were heated at 100°C, the inhibitory activity was not abolished, again suggesting that the inhibitory factors were not proteinaceous.

In another investigation (25) the effects of two separate species (based on molecular weight and carbohydrate/fatty acid ratios) of *P. gingivalis* 381 LPS on bone formation in fetal rat long bones were evaluated. LPS induced a 30-to-40% reduction in net collagen formation at a concentration of 10 µg/ml. However, resorption and low levels of bone deposition occur simultaneously in the long-bone model, making it difficult to discriminate agents affecting resorption and formation.

In an attempt to identify the bacterial products that might influence osteogenesis directly or indirectly, Bom-van Noorloos et al. (3) tested the direct and immune-cell-mediated effects of *P. gingivalis* on bone metabolism in fetal long-bone rudiments in vitro. Metabolic products from spent medium, with a molecular size under 1 kDa, not only induced bone resorption but also appeared to inhibit mineralization. Conditioned media of bacterium-activated spleen cells strongly enhanced bone resorption and increased osteoclast numbers while inhibiting mineral formation. Metabolically inactivated bacteria had no effect when added directly to the cultures (3). From this it was concluded that bacteria and their products do not have direct effects on bone metabolism but that their effects must be mediated through stimulating the immune

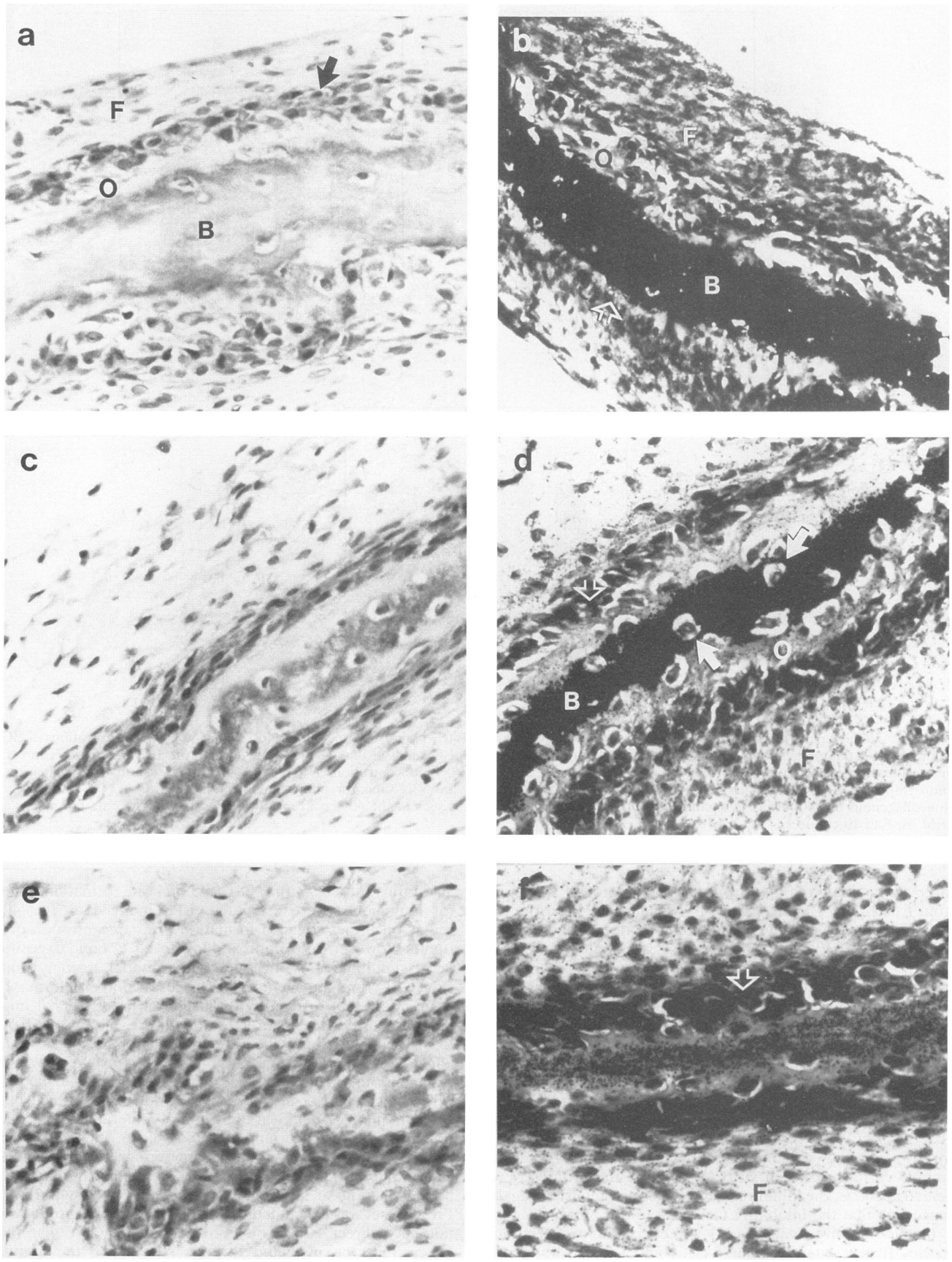
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TABLE 1. Effects of fractionated *P. gingivalis* sonicated extracts on alkaline and acid phosphatase activities in CPO cultures

Extract (kDa)	Concn (%)	Activity (nmol of pNp/μg of protein/h)[a]	
		Alkaline phosphatase	Acid phosphatase
Control		4.2 ± 0.6	0.6 ± 0.1
<5	20	0.5 ± 0.3*	0.1 ± 0.1
	40	0.5 ± 0.9*	0.1 ± 0.1
	60	0.6 ± 0.4*	0.2 ± 0.1
5–10	20	4.1 ± 1.0	0.5 ± 0.1
	40	3.6 ± 0.9	0.1 ± 0.1
	60	2.9 ± 0.6*	0.6 ± 0.1
10–50	20	3.8 ± 0.7	0.2 ± 0.1
	40	3.4 ± 0.8	0.6 ± 0.1
	60	2.3 ± 1.1*	0.2 ± 0.1
50–100	20	2.1 ± 0.7*	0.2 ± 0.1
	40	2.6 ± 0.6*	0.9 ± 0.1
	60	1.7 ± 0.6*	0.2 ± 0.1
>100	20	4.0 ± 0.9	0.4 ± 0.1
	40	3.0 ± 0.6	0.6 ± 0.1
	60	2.1 ± 0.7*	0.3 ± 0.1

[a] Mean ± SEM for 8 to 10 cultures. pNp, paranitrophenol. *, significantly lower than the value for the control ($P < 0.05$).

TABLE 2. Effects of fractionated *P. gingivalis* sonicated extracts on calcium and P_i in CPO cultures[a]

Extract (kDa)	Concn (%)	Calcium (μmol of Ca^{2+}/μg protein)	P_i (nmol/μg of protein)
Control		98.56 ± 10.49	134.0 ± 5.0
<5	20	BDT	BDT
	40	BDT	BDT
	60	BDT	BDT
5–10	20	85.98 ± 6.20	134.9 ± 5.0
	40	70.23 ± 6.40*	98.1 ± 4.7*
	60	70.63 ± 7.82*	14.8 ± 4.9*
10–50	20	83.00 ± 5.10	78.9 ± 17.4*
	40	71.60 ± 8.61*	45.9 ± 6.1*
	60	60.71 ± 8.55*	26.4 ± 9.3*
50–100	20	71.81 ± 8.66*	52.0 ± 11.0*
	40	66.83 ± 5.15*	38.9 ± 7.8*
	60	51.74 ± 8.50*	35.6 ± 7.2*
>100	20	94.76 ± 11.10	125.0 ± 15.0
	40	77.12 ± 7.45*	110.0 ± 22.0
	60	65.66 ± 4.53*	38.0 ± 18.0*

[a] The data are means ± SEM for 8 to 10 cultures. *, significantly lower than the value for the control ($P < 0.05$). BDT, below detection threshold.

system. Clearly, the results reported in this investigation do not support this contention.

Previous investigations have led to a clearer understanding of the more direct effects of microbial products and extracts on bone cell metabolism. However, the effects of these factors on osteodifferentiation and osteogenesis cannot be ascertained with certainty because of some inherent deficiencies of the model systems used. While all model systems have drawbacks, the CPO model has been demonstrated clearly to be reliable for osteogenic cell differentiation and mineralized bone formation (6, 43, 44). In fact, the bone formed in this model is virtually indistinguishable from the bone synthesized in vivo on the basis of ultrastructural criteria (49). Although it is an avian system, there is substantial evidence that issues addressed with this model are relevant to mammalian systems. For example, glucocorticoid effects in this model have been replicated in various mammalian bone cell models (24, 45). In addition, the necessity for the presence of an organic phosphate source for the induction of morphotypic mineralization in vitro was first demonstrated with the CPO model, and this has now been replicated in cell lines and osteogenic models derived from mammalian species (1, 8, 17, 32, 53) including humans (9, 11, 29). Thus, it would seem appropriate that findings obtained with this model with respect to bacterial regulation of osteogenesis are relevant to what might be expected for mammalian systems under the same conditions. However, it must be recognized that the levels of bacterial products used in this investigation may substantially exceed the levels expected to occur in an area adjacent to alveolar bone in vivo. This is a common problem encountered with the use of in vitro models. Thus, direct extrapolation of findings obtained by the use of any in vitro model must be interpreted with some caution.

The results obtained in these experiments demonstrate clear and direct inhibition of osteogenesis. Although a linear concentration-dependent relationship between extracts or products and inhibition of osteogenesis was not observed, inhibition was greatest at the higher concentrations for every parameter of osteogenesis tested. The nonlinear findings are most likely due to the rather crude nature of the extracts.

To begin identification of inhibitory factors, sonicated extracts were fractionated into five molecular-size ranges. While inhibition of osteogenesis appeared to follow similar trends for the fractions greater than 5 kDa, there were some noteworthy differences. For example, pronounced inhibitory effects for all parameters were observed at lower concentrations for the 10- to 50-kDa fraction than for the other three fractions. This might be due to the multitude of proteolytic enzymes and fimbrial and other outer membrane and extracellular vesicle proteins contained within this fraction (12, 13, 18, 34, 38).

The results obtained with the <5-kDa fraction were unique in that virtually complete inhibition of osteogenesis was ob-

FIG. 5. Light-microscopic photomicrographs of CPO cultures grown in control medium (a and b), in medium containing 40% 50- to 100-kDa sonicated ultrafiltered extract (c and d), or in medium containing 40% <5-kDa sonicated ultrafiltered extract (e and f). Sections from each test group were stained with hematoxylin and eosin (a, c, and e) and by the von Kossa method (b, d, and f). The control culture appears to contain fully mineralized bone (B), as shown by the black von Kossa staining, surrounded by a thin seam of osteoid (O) and osteoblast-like cells (arrow). Fibrous tissue (F), seen in all cultures, is found peripherally to the osteoblast layers. The culture grown in the 50- to 100-kDa extract (d) has a bone matrix that appears to be extremely cellular, with high numbers of large-size osteocytes (arrows) and a larger than average osteoid seam, indicating that a greater proportion of the osteoid had yet to mineralize. The culture grown in the <5-kDa extract appeared to be minimally mineralized, with only small spotty areas of von Kossa-positive material (f). Magnification, ×570.

TABLE 3. Effects of fractionated *P. gingivalis* sonicated extracts on total collagen and collagen synthesis from day 4 to 6 in CPO cultures[a]

Extract (kDa)	Concn (%)	Total collagen (Absorbance U × mm)	Collagen synthesis (Absorbance U × mm)
Control		0.0780 ± 0.0094	0.211 ± 0.032
<5	20	0.0014 ± 0.0003*	0.037 ± 0.007*
	40	0.0012 ± 0.0002*	0.027 ± 0.006*
	60	0.0010 ± 0.0002*	0.032 ± 0.006*
5–10	20	0.0490 ± 0.0044	0.233 ± 0.011
	40	0.0294 ± 0.0031	0.092 ± 0.019*
	60	0.0208 ± 0.0040*	0.086 ± 0.014*
10–50	20	0.0525 ± 0.0038	0.142 ± 0.032*
	40	0.0311 ± 0.0027	0.089 ± 0.010*
	60	0.0204 ± 0.0030*	0.069 ± 0.013*
50–100	20	0.0560 ± 0.0010*	0.099 ± 0.008*
	40	0.0297 ± 0.0023*	0.063 ± 0.007*
	60	0.0210 ± 0.0028*	0.031 ± 0.003*
>100	20	0.0551 ± 0.0073	0.185 ± 0.027
	40	0.0362 ± 0.0041	0.124 ± 0.007*
	60	0.0277 ± 0.0034*	0.084 ± 0.010*

[a] The data are means ± SEM for 8 to 10 cultures. *, significantly lower than the value for the control ($P < 0.05$).

served at all tested concentrations. It did not appear that the cultures were killed by the extract, as collagen production still continued, as did alkaline phosphatase production, albeit at a lower level than that observed for the other fractions. In addition, histological observations suggested the presence of healthy cells without the presence of cellular debris, and noncollagenous protein synthesis was not inhibited in CPO cultures treated with the <5-kDa fraction. These findings indicate that, although bone formation was inhibited profoundly, the cultures were able to maintain their viability. Nonetheless, these results do suggest inhibition of osteogenic cell differentiation. The active compounds contained in this fraction were probably neither protein nor intact LPS, as their molecular weights would likely be too great. However, this fraction may contain high levels of organic acids. Production of nonvolatile organic acids, for example, phenylacetic acid and others, by *P. gingivalis* had been found to be exceedingly high (19–21) and likely has the potential to affect osteoprogenitor cell differentiation profoundly.

In conclusion, the data indicate that metabolic products and sonicated extracts of *P. gingivalis* directly inhibit osteogenesis in vitro. This might have implications for the progression of periodontitis-associated bone loss if these factors were to possess similar activities in vivo. Such bone loss may be related not only to increased levels of resorption but also to direct inhibition of bone formation. Future studies in this area are in progress to further define and test the constituents of the extract fractions.

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