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Characterization of Inhibitory Effects of Suspected Periodontopathogens on Osteogenesis In Vitro

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By using an in vitro bone-forming culture system, the chick periosteal osteogenesis (CPO) model, the direct effects on osteogenesis of sonicated extracts derived from oral bacteria were examined. Both extracts from bacterial species having strong associations with periodontal diseases (*Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, and *Prevotella intermedia*, hereinafter referred to as suspected periodontopathogens) and extracts from species not correlated with periodontal disease (*Streptococcus sanguis*, *Veillonella atypica*, and *Prevotella denticola*, hereinafter referred to as nonpathogenic bacteria) were tested. All bacterial cultures were grown under standard anaerobic culture conditions. Sonicated bacterial extracts were prepared from the bacterial pellet. These were added in various proportions to the CPO cultures. Parameters of osteogenesis, including alkaline phosphatase activity, calcium and P_i accumulation, and collagen synthesis, were measured in 6-day-old cultures. Compared with controls grown in the absence of bacterial products, osteogenesis was inhibited significantly in cultures treated with extracts derived from the suspected periodontopathogens. No osteogenic inhibition was observed in cultures treated with extracts from the nonpathogenic bacteria. These results suggest that the ability to inhibit osteogenesis in vitro may be a pathogenic property shared by a limited group of species. Further characterization of the *P. gingivalis* extracts revealed that both proteinaceous and nonproteinaceous products, including lipopolysaccharide, were able to inhibit osteogenesis. *P. gingivalis* extract-mediated inhibition of osteogenesis in CPO cultures was blocked by indomethacin, implicating prostaglandins in the regulation of the bacterial effects. The bacterial extracts had either reversible or irreversible inhibitory effects on osteogenesis when added after differentiation or before/during differentiation of bone cells, respectively.

Porphyromonas gingivalis has been implicated as an important etiological agent in the initiation and progression of periodontal diseases (20, 25, 45). This bacterium, and other microorganisms associated with periodontal disease, may mediate periodontal tissue destruction either directly through their multiple virulence factors or indirectly by host-mediated inflammatory responses to, for example, lipopolysaccharides (LPSs) and fimbriae (37, 38). Regarding the direct effects, *P. gingivalis* is known to produce a number of proteinases, some vesicle associated, which can degrade various substrates, including type I collagen, which is a major constituent of periodontal connective tissues (4, 18, 30). Products of *P. gingivalis* have also been shown to inhibit type I collagen synthesis (22).

Although alveolar bone is the major mineralized tooth-supporting tissue of the periodontium, the effects of bacterial products on bone formation (i.e., osteogenesis) have not been studied in detail. In health, bone volume is maintained by a homeostatic relationship between bone resorption by osteoclasts and bone deposition by osteoblasts. These cells respond to cytokines, hormones, and matrix components by various mechanisms (29, 36). When the homeostatic relationship between bone cells is abrogated in favor of bone resorption, net bone loss will occur, as is characteristic of periodontitis (14).

To address these issues, we have demonstrated recently that metabolic products and sonicated extracts from *P. gingivalis* 2561 directly inhibited bone formation in an in vitro osteogenesis system, the chick periosteal osteogenesis (CPO) model (19). The purpose of the current study was (i) to further characterize the nature of the inhibitory effects and (ii) to determine whether inhibition of osteogenesis is restricted to putative periodontal pathogens (e.g., *Actinobacillus actinomycetemcomitans* and *Prevotella intermedia* as well as *P. gingivalis*) or whether any oral bacterium, even those not generally associated with periodontitis (e.g., *Streptococcus sanguis*, *Prevotella denticola*, and *Veillonella atypica*) might mediate such effects. Furthermore, we wished to demonstrate whether bacterial products mediated their effects strictly by influencing phenotypic expression of osteoblasts or whether these products also had effects on osteoblast cell differentiation. Finally, additional characterization of the nature of the bacterial products and their effects were studied, including determination of whether the observed inhibition of osteogenesis was mediated by prostaglandins.

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 containing an atmosphere of 80% N_2, 10% H_2, and 10% CO_2. Following 5 to 7 days of growth, bacterial inocula were added to trypticase yeast extract broth (TYB) 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 TYB for another 3 days. The purity of cultures was verified by phase-contrast microscopy, Gram stain, and subcul-

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ture on blood agar plates. Bacteria were harvested by centrifugation at 10,000 × *g* for 15 min at 4°C. The bacterial cell pellet was washed in phosphate-buffered saline (pH 7.0) and resuspended in BGJ_B medium (GIBCO, Grand Island, N.Y.), a commonly used bone tissue culture medium (3), to an optical density at 690 nm of 1.0 (model 350 spectrophotometer; G. K. Turner Associates, Palo Alto, Calif.). The bacterial suspensions were then sonicated at maximum power output in 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. *P. intermedia* 25611 and *P. denticola* 33184 stocks were grown in the same type of medium as *P. gingivalis*, and sonicated extracts were prepared in a like fashion.

A. actinomycetemcomitans 652 was supplied by J. DiRienzo, University of Pennsylvania. Working stocks were grown on blood agar plates. After 5 to 7 days, bacterial inocula were added to tryptic soy broth (Difco, Detroit, Mich.), which was supplemented with 0.6% yeast extract (Difco) and 0.04% sodium bicarbonate. The bacteria were grown for a further 3 days prior to harvest, and sonicated extracts were prepared in the same manner as for *P. gingivalis*.

S. sanguis 49295 stocks were grown in tryptic soy broth (Difco) containing 5% defibrinated sheep blood. *V. atypica* 17744 stocks were grown on *Veillonella* agar (Difco) plates. After 5 to 7 days of growth, these strains were harvested, and sonicated extracts were prepared in the same way as for *P. gingivalis*.

In previous investigations, extracts of *P. gingivalis* were subjected to ultrafiltration in order to establish molecular weight ranges for the various factors which might influence osteogenesis (19). To extend these findings, ultrafiltration (Amicon) of the *P. gingivalis* extracts was carried out to produce five fractions spanning <5, 5 to 10, 10 to 50, 50 to 100, and >100 kDa. All fractions were stored at −20°C.

CPO culture system. The CPO model system has been described in detail previously (21, 41). Ectocranial periosteal tissues were removed from calvariae derived from 17-day-old embryonic chicks after excision of most of the fibrous tissue. Removal of the fibrous tissues by microdissection significantly reduces the nonosteogenic cell content of these cultures, enhancing osteogenic purity for biochemical assessments of cultures (39). The periostea were then folded, with the side originally facing bone (i.e., the osteogenic layer of cells) in apposition. The explants, supported on a Millipore filter (HA, 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.

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.), 10 mM β-glycerophosphate (Sigma), and 300 μg of L-ascorbate (Gibco) per ml (40, 41).

Effects of sonicated bacterial extracts. To test the effects of sonicated bacterial extracts on osteogenic cultures, extracts from *P. intermedia*, *A. actinomycetemcomitans*, *P. denticola*, *S. sanguis*, and *V. atypica* were added in various proportions (20, 40, and 60%, vol/vol) to the control medium throughout the incubation period in separate experiments. Since the bacterial extracts were prepared in BGJ_B medium, there would be no dilution of the essential nutrients contained in this medium when the dilutions were prepared. The medium in all cultures was changed every 48 h.

Effects of time of addition of *P. gingivalis* extracts. Bone formation by intramembranous ossification in the CPO model may be divided into three dominant phases (with some overlap), including (i) differentiation of osteoprogenitors into osteoblasts (days 0 to 2), (ii) osteoid matrix production (days 2 to 4), and (iii) mineralization of the osteoid matrix (days 4 to 6). It is possible to test the effects of various factors on relatively specific stages of osteogenesis by adding (or removing) them at various times of incubation. To extend previous findings obtained with *P. gingivalis* (19), whole unfractionated and <5-kDa sonicated *P. gingivalis* extracts at a concentration of 40% (vol/vol) were added to CPO culture medium on days 0 to 6, 0 to 2, 0 to 4, 2 to 4, 2 to 6, and 4 to 6 (Fig. 1) in CPO cultures grown for a total of 6 days. These cultures were subjected to various biochemical analyses (see below) at the end of the 6-day culture period. As some biochemical measurements might not reflect immediate effects of sequential incubation (for example, mineral content at day 6 essentially represents total mineral accumulation and might not reflect the immediate effects of a factor on active mineral deposition if added at an earlier time, e.g., over days 2 to 4), some cultures were stopped at the end of the extract challenge period. Thus, cultures (with appropriate controls) were also terminated after 2 or 4 days.

Further characterization of osteogenesis-inhibitory factors from *P. gingivalis*. Sonicated extracts from *P. gingivalis* 2561 fractionated by molecular size were previously shown to possess different degrees of ability to inhibit osteogenesis (19). To determine whether inhibition was mediated by protein-like factors, the five extracts were subjected to heat treatment sufficient to denature and thereby inactivate all proteinaceous components, including various enzymes (10). The extracts were heated to 90°C for 30 min. They were added to the culture medium of CPO cultures in concentrations of 20, 40, and 60% (vol/vol). Cultures were analyzed at the end of the 6-day culture period.

The effects of *P. gingivalis* LPS on osteogenesis were also tested. LPS from *P. gingivalis* 2561 was extracted by phenol-water extraction techniques as described by Westphal and Jann (46) and added to the culture medium for final concentrations of 0.1, 1, and 10 μg/ml for a period of 6 days. LPS was analyzed by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) procedure of Laemmli (17), by biochemical analysis for protein, carbohydrate, and nucleic acid content, and morphologically by transmission electron microscopy.

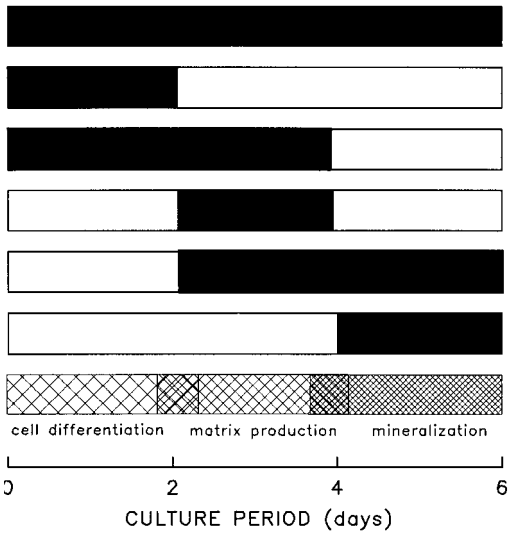

FIG. 1. Schematic representation of the timing and sequence of the three principal stages of osteogenesis (cell differentiation, matrix production, and mineralization) during the 6-day CPO culture period. Solid bars demonstrate the various times during the 6 days of the incubation that the cultures were grown in the presence of the bacterial extracts.

Effects of indomethacin. Since factors contained in the extracts might mediate their effects indirectly through the generation of prostaglandins, some cultures treated with extracts or fractions were coincubated with indomethacin, a cyclooxygenase inhibitor (15). Indomethacin (Sigma, St. Louis, Mo.) was added at a concentration of 1 μM to control culture medium as well as to medium supplemented with either whole bacterial extract obtained from *P. gingivalis* (40% [vol/vol] unfractionated) or the <5-kDa fraction (40% vol/vol), also obtained from *P. gingivalis*.

Analysis of osteogenesis. The effects of the various extracts on osteogenesis were assessed by measuring multiple parameters of bone cell activity and bone formation, including alkaline phosphatase activity, calcium and phosphate accumulation (mineralization), and collagen synthesis (osteoid matrix production). This was done to obtain a broader assessment of osteogenesis, since assessment of only one parameter could lead to inappropriate conclusions.

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]) with a Polytron homogenizer (Kinematica GMBH). 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 (6) and for alkaline and acid phosphatase activity to assess osteoblastic differentiation and activity (39, 40). Mineralization was measured by estimating the amount of acid-extractable calcium and phosphate in the pellet after overnight hydrolysis in 0.5 N HCl (41, 42). All colorimetric assays (used for determination of acid and alkaline phosphatase activity and P_i and protein content) were carried out in 96-well plates, and optical density was measured with a Titertek Multiskan MC spectrophotometer (Flow Laboratories, Mississauga, Ontario, Canada) as described earlier (2). 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 (during the final 2 days of culture) by the addition of [^{14}C]glycine (10 μCi/ml; 59 mCi/mmol; NEN Dupont, Mississauga, Ontario, Canada) to the culture medium. Following incubation, the cultures were harvested and stored at −20°C.

Whole radiolabelled explants were demineralized by incubation in 100 μl of 0.1 N HCl for 30 min (the total noncollagenous protein content of the acid extract was determined by the method of Smith et al. [34], with 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 microcentrifuge at 12,000 × *g* for 10 min. The pepsin extract was freeze-dried, reconstituted in SDS-PAGE sample buffer (Tris-glycine) for separation on Phast-Gels (Pharmacia), 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-LKB, Uppsala, Sweden). Fluorographs were made from the same gels and assessed densitometrically with the same scanner and integra-

TABLE 1. Effects of sonicated extracts of oral bacteria on parameters of osteogenesis of CPO cultures[a]

Extract	Concn (%)	AP activity[b]	Calcium[c]	P_i[d]	Total collagen[e]	Collagen synthesis[e]
P. intermedia	20	69 ± 9	50 ± 8*	63 ± 16*	69 ± 9	33 ± 9*
	40	45 ± 8*	55 ± 7*	72 ± 12	45 ± 8*	12 ± 3*
	60	40 ± 9*	20 ± 3*	31 ± 4*	40 ± 9*	17 ± 6*
A. actinomycetemcomitans	20	77 ± 8	47 ± 12*	58 ± 13	51 ± 14*	35 ± 3*
	40	47 ± 6*	42 ± 2*	52 ± 4*	23 ± 4*	28 ± 5*
	60	36 ± 4*	22 ± 4*	31 ± 5*	16 ± 3*	16 ± 3*
S. sanguis	20	78 ± 10	96 ± 15	75 ± 15	97 ± 14	110 ± 13
	40	83 ± 11*	110 ± 10	95 ± 16	80 ± 13	102 ± 24
	60	109 ± 11*	114 ± 7	121 ± 20	85 ± 9	81 ± 15
V. atypica	20	81 ± 4	73 ± 17	66 ± 19	95 ± 18	52 ± 13*
	40	88 ± 5*	90 ± 13	82 ± 3	88 ± 12	92 ± 14
	60	92 ± 4*	70 ± 19	63 ± 13	72 ± 6	95 ± 24
P. denticola	20	79 ± 5	98 ± 38	119 ± 47	78 ± 19	79 ± 12
	40	89 ± 4*	61 ± 13	66 ± 13	69 ± 7	87 ± 10
	60	106 ± 7*	105 ± 15	100 ± 16	72 ± 11	69 ± 36

[a] Mean ± SEM for 8 to 10 cultures. *, significantly different from the value for the control of the respective experiment ($P < 0.05$) values for each bacterial group.
[b] For alkaline phosphatase (AP) activity, all values are expressed as a percentage of control values.
[c] All values are expressed as a percentage of control values.
[d] All values are expressed as a percentage of control values.
[e] All values are expressed as a percentage of control values.

tion program. This approach allowed simultaneous measurement of both total and newly synthesized (i.e., radiolabelled) α-1 type 1 collagen.

Statistical analysis. Values for the various parameters were used to calculate a mean and standard error for each group (8 to 10 CPO cultures per experimental group), and the differences between means were evaluated by using Student's *t* test for comparisons between specific experimental groups and control. Significance was assigned at the $P < 0.05$ level. All values in tables and figures represent the mean ± standard error of the mean (SEM). All values have been presented as a percentage of the control value for the respective experiment (control = 100 ± SEM) to facilitate comparisons between different experiments.

RESULTS

Effects of sonicated extracts of oral microorganisms on osteogenesis. One of our goals was to determine whether sonicated extracts from oral bacteria other than *P. gingivalis* affected osteogenesis. Alkaline phosphatase activity was reduced by up to 60% in CPO cultures grown for 6 days in the presence of 20, 40, and 60% concentrations of sonicated extracts derived

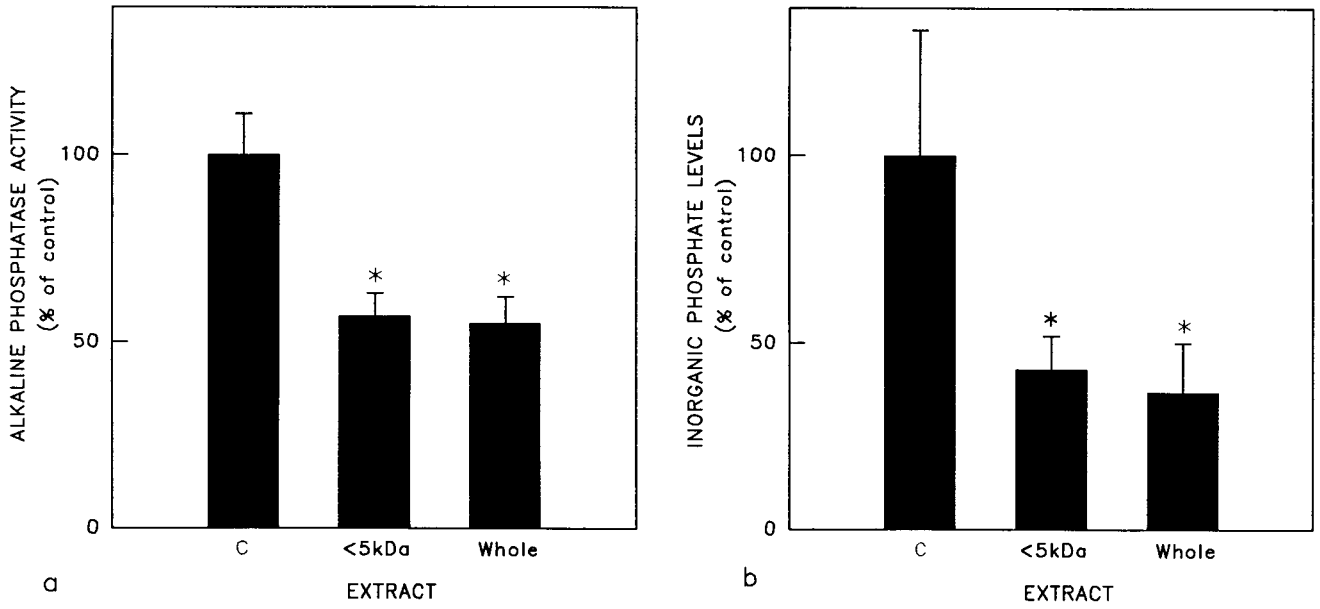

FIG. 2. Biochemical parameters of 2-day-old cultures. C, control (100% plain BGJ_B medium). A 40% concentration of sonicated extracts of *P. gingivalis* (<5-kDa fraction and whole [unfractionated] sonicates) was tested. (a) Alkaline phosphatase activity. Alkaline phosphatase activity was significantly different ($P < 0.05$, *) between treated and control cultures. (b) P_i level. Significant decreases from control levels were seen in both extract treatment groups for P_i accumulation (*). Calcium accumulation levels (data not shown) paralleled those of P_i. The data are means and SEM for 8 to 10 cultures. All numerical values have been expressed as a percentage of control values.

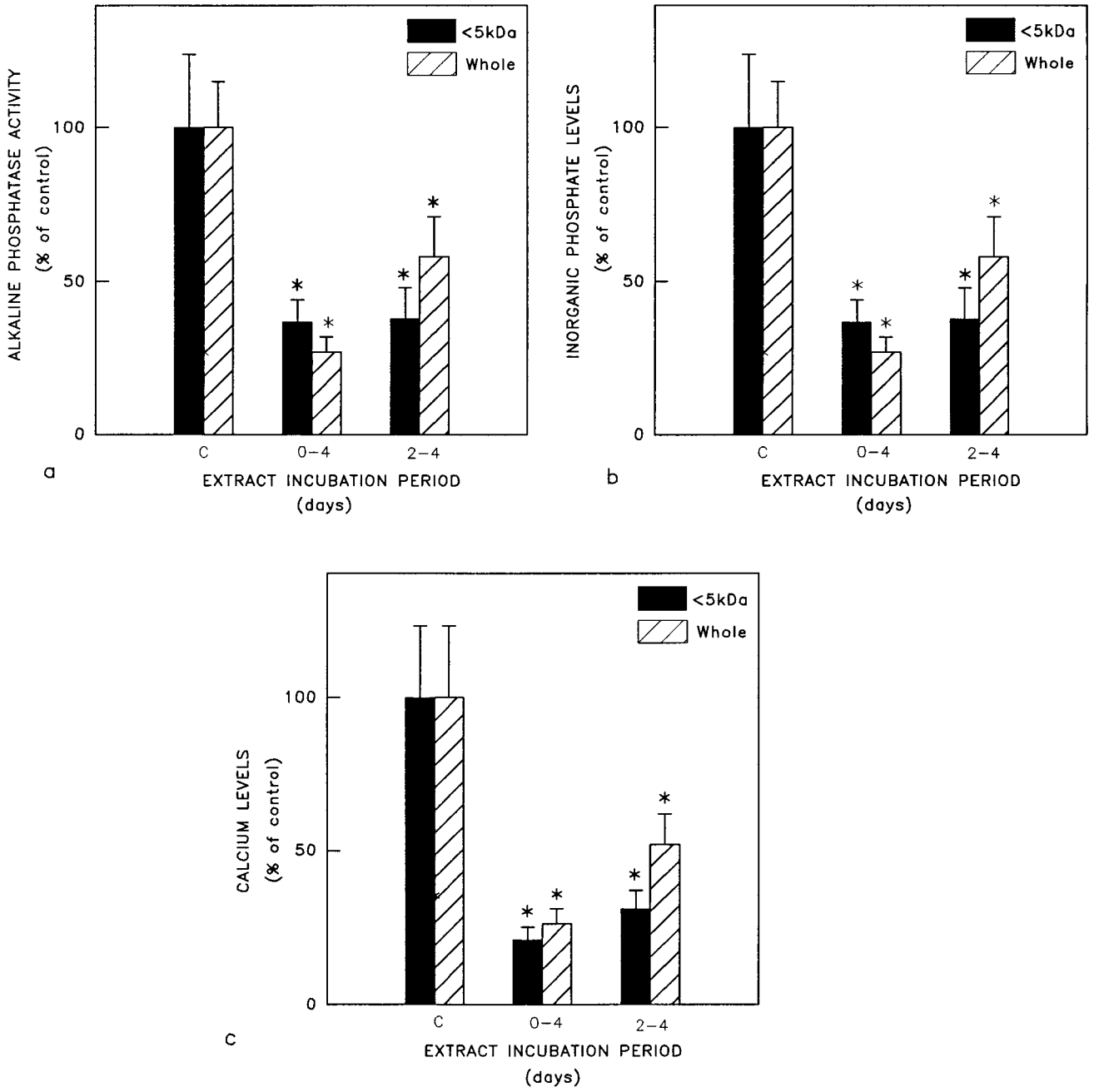

FIG. 3. Biochemical parameters of 4-day-old cultures. C, control (100% plain BGJ_B medium). A 40% concentration of sonicated extracts (<5-kDa fraction and unfractionated [whole]) of *P. gingivalis* was tested. (a) Alkaline phosphatase activity. Alkaline phosphatase activity was significantly reduced ($P < 0.05$, *) when cultures were incubated with either extract for the entire 4-day period. (b) P_i level. (c) Calcium level. Significant decreases from control levels were seen for both treatment periods for P_i and calcium accumulation. The data are means and SEM for 8 to 10 cultures. All values have been expressed as a percentage of control values.

from *P. intermedia* and *A. actinomycetemcomitans*, putative periodontal pathogens (Table 1). In contrast, there was virtually no reduction in alkaline phosphatase activity in CPO cultures grown in the presence of extracts derived from the species considered nonpathogenic, with the exception of the lowest dose of *P. denticola* (notably, there were no reductions in any of the other parameters of osteogenesis at this dose of *P. denticola*; see below) (Table 1).

Calcium accumulation was significantly reduced for cultures grown in the presence of most concentrations of extracts derived from suspected periodontopathogens, as was collagen accumulation (0 to 6 days) and newly synthesized collagen content (labelled over days 4 to 6) (Table 1). No effects were observed in cultures grown in the presence of the extracts derived from any of the nonpathogenic bacteria (Table 1).

Effect of time of addition of *P. gingivalis* extracts to CPO cultures. The effects of the bacterial extracts on the different phases of osteogenesis were examined by treating the cultures with these extracts at various times (Fig. 1). All measured parameters of osteogenesis (i.e., alkaline phosphatase activity

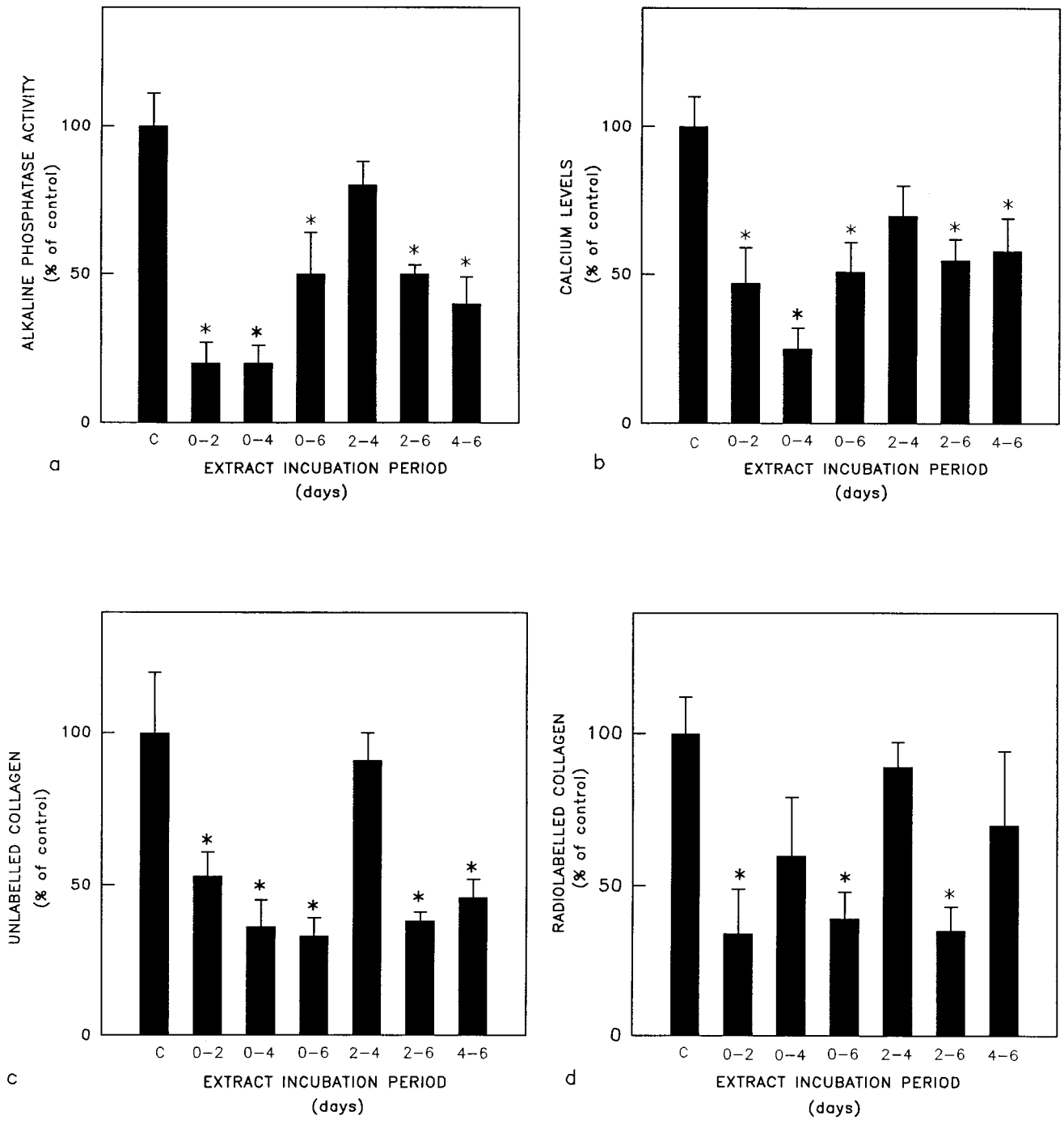

FIG. 4. Osteogenic parameters of 6-day-old cultures treated with the <5-kDa fraction. C, control (100% plain BGJ_B medium). A 40% concentration of sonicated extracts of the <5-kDa fraction of *P. gingivalis* was tested. (a) Alkaline phosphatase activity. (b) Calcium level. (c) Densitometric evaluation of Coomassie blue-stained minigels run on SDS-PAGE as a control. (d) Densitometric evaluation of fluorographs of SDS-PAGE gels. Significant ($P < 0.05$, *) decreases from control levels were seen for most treatment periods for all measured parameters. Notably, when the extract was present only on days 2 through 4, all parameters returned to control levels. The data are means and SEM for 8 to 10 cultures. All values have been expressed as a percentage of control values.

and P_i and calcium accumulation) were significantly reduced in 2-day cultures treated continuously with both the whole extract- and the <5-kDa fraction-treated groups (Fig. 2) compared with the levels in controls.

Cultures grown for 4 days were incubated with unfractionated (whole) *P. gingivalis* extract or the <5-kDa fraction over days 0 to 4 or 2 to 4. The results were different for each of the extracts tested. When CPO cultures were grown in the presence of whole extracts, significant decreases in all parameters were found only when the bacterial extract was present for the entire 4 days (Fig. 3). When the extract was first added on day 2 of culture (i.e., just after the differentiation stage), no differences in alkaline phosphatase levels were observed. However, significant decreases in initial mineral deposition were noted.

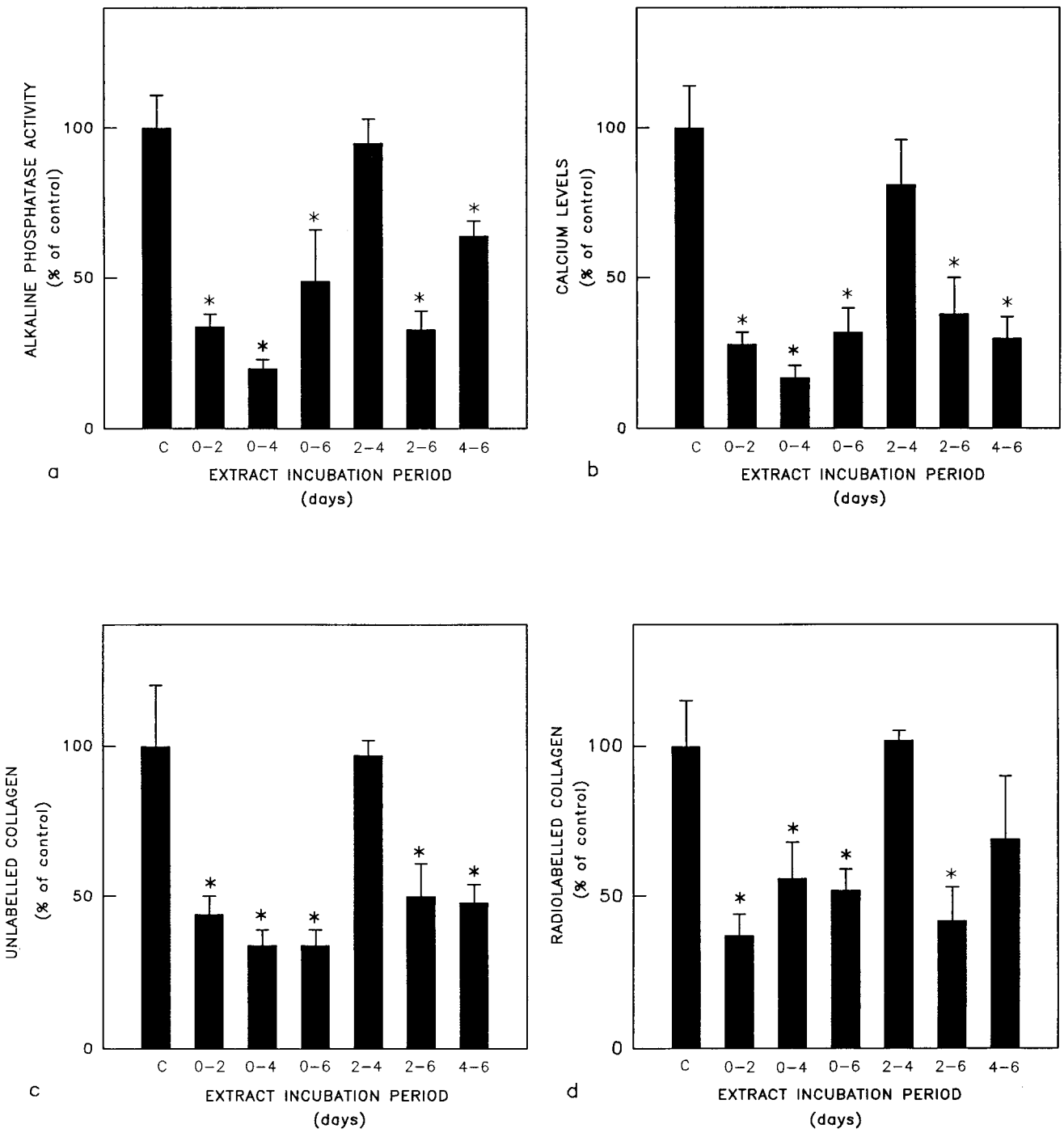

FIG. 5. Osteogenic parameters of 6-day-old cultures treated with unfractionated *P. gingivalis*. C, control (100% plain BGJ_B medium). A 40% concentration of sonicated extracts of unfractionated *P. gingivalis* was tested. (a) Alkaline phosphatase activity. (b) Calcium level. (c) Densitometric evaluation of Coomassie blue-stained minigels run on SDS-PAGE as a control. (d) Densitometric evaluation of fluorographs of SDS-PAGE gels. A significant ($P < 0.05$, *) decrease from control levels was seen for most treatment periods for all measured parameters. The presence of the extract on days 2 through 4 did not cause any significant reductions in any measured biochemical parameter. The data are means and SEM for 8 to 10 cultures. All values have been expressed as a percentage of control values.

In contrast, when the <5-kDa fraction was tested, addition of the extract either at the start of culture or at day 2 caused significant reductions in the parameters of osteogenesis (Fig. 3).

A significant decrease in the measured parameters of osteogenesis was demonstrated in cultures grown for 6 days in the presence of the <5-kDa *P. gingivalis* extract (Fig. 4). When the extract was present only on days 2 to 4, the cultures recovered, and by day 6 there were no significant differences from the controls. In contrast, complete recovery was not found when cultures were incubated with this extract during days 0 to 2 or 0 to 4. Furthermore, measures of bone formation were reduced

TABLE 2. Effects of fractionated *P. gingivalis* heat-treated sonicated extracts on parameters of osteogenesis of CPO cultures[a]

Fraction (kDa)	Concn (%)	Alkaline phosphatase activity[b]	Total collagen[b]	Collagen synthesis[b]
Whole (control)		100 ± 11	100 ± 6	100 ± 18
<5	20	99 ± 13	33 ± 4*	27 ± 6*
	40	56 ± 17*	31 ± 4*	49 ± 5*
	60	13 ± 10*	29 ± 4*	16 ± 4*
5 to 10	20	83 ± 11	51 ± 10*	52 ± 18
	40	57 ± 7*	49 ± 6*	48 ± 11*
	60	42 ± 12*	29 ± 4*	18 ± 3*
10 to 50	20	44 ± 17	57 ± 6	35 ± 4*
	40	36 ± 8*	51 ± 14	25 ± 4*
	60	36 ± 9*	25 ± 5	14 ± 2*
50 to 100	20	117 ± 10	93 ± 10	63 ± 19
	40	70 ± 32	104 ± 6	60 ± 13*
	60	112 ± 17	110 ± 8	86 ± 6*
>100	20	110 ± 11	102 ± 8	46 ± 4*
	40	100 ± 5	71 ± 8*	18 ± 3*
	60	105 ± 11	59 ± 8*	15 ± 3*

[a] Mean ± SEM for 8 to 10 cultures. *, significantly different from the value for the control ($P < 0.05$).
[b] All values are expressed as a percentage of control values.

when the cultures were exposed to this bacterial extract during days 2 to 6 or 4 to 6. Similar findings were obtained with cultures grown in the presence of the whole bacterial extract (Fig. 5).

Further characterization of *P. gingivalis* extracts. (i) Effect of heat treatment of sonicated extracts. Heat denaturation either eliminated (50 to 100 and >100 kDa), reduced (<5 and 5 to 10 kDa), or had no effect (10 to 50 kDa) on the osteogenic inhibitory effects of the various fractions. For example, alkaline phosphatase activity remained significantly reduced when cultures were grown with higher concentrations of both heated <5- or 5- to 10-kDa extract fractions and at all concentrations of the heated 10- to 50-kDa extract fraction (Table 2). No reductions in alkaline phosphatase activity were found at any concentration tested of the heated 50- to 100- and >100-kDa extract fractions, suggesting complete abrogation of the inhibitory factor effects in the latter fractions by heating. Unlike alkaline phosphatase, mineral accumulation, as measured by calcium and phosphate accumulation, remained significantly reduced with all five heated fractions (data not shown).

Both total and newly synthesized radiolabelled α-1 collagen contents were reduced in the presence of all heated extracts except the 50- to 100-kDa fraction (Table 2). However, in most cases, higher concentrations of most heated extract fractions were required to inhibit collagen synthesis and accumulation. Degradation products of collagen were not detected.

(ii) Effect of *P. gingivalis* LPS. Significant reductions in all measured parameters of osteogenesis were observed in CPO cultures incubated continuously with 1 and 10 μg of LPS per ml (Table 3). There were no detectable effects at 0.1 μg/ml. There were no significant differences in acid-extracted noncollagenous protein levels between any of the culture groups.

(iii) Effects of indomethacin. Indomethacin alone had no effect on osteogenesis (Fig. 6). However, the inhibitory effects of the bacterial extracts on alkaline phosphatase activity, calcium and P_i accumulation, and collagen synthesis (both unlabelled and labelled) were blocked by indomethacin (Fig. 6).

TABLE 3. Effects of *P. gingivalis* LPS on osteogenic parameters in CPO cultures[a]

Parameter	Control value	Value in CPO culture treated with *P. gingivalis* LPS at (μg/ml):		
		0.1	1.0	10.0
Alkaline phosphatase	100 ± 3	85 ± 11	55 ± 11*	50 ± 7*
Acid phosphatase	100 ± 13	113 ± 10	107 ± 15	118 ± 22
Calcium	100 ± 7	101 ± 24	56 ± 12*	45 ± 8*
P_i	100 ± 6	104 ± 6	49 ± 10*	59 ± 10*
Total collagen	100 ± 20	107 ± 18	61 ± 7	45 ± 6*
Collagen synthesis (from days 4 to 6)	100 ± 15	110 ± 13	102 ± 24	81 ± 15
Noncollagenous proteins	100 ± 9	129 ± 12	128 ± 11	99 ± 16

[a] Mean ± SEM for 8 to 10 cultures. All values are expressed as a percentage of control values. *, significantly different from the value for the control ($P < 0.05$).

DISCUSSION

We have shown previously that *P. gingivalis* has the capacity to inhibit bone formation directly (19). As there was some question of whether inhibition of bone formation could be mediated by any oral species, the effects of extracts from putative periodontal pathogens (*A. actinomycetemcomitans* and *P. intermedia* as well as *P. gingivalis*) and organisms not associated with periodontal diseases (*S. sanguis*, *V. atypica*, and *P. denticola*) were tested. The data suggest that among the strains tested, the ability to inhibit bone formation was restricted to the suspected periodontal pathogens tested here. Thus, it appears that these bacteria have the capacity to contribute to the bone loss observed in periodontitis by inhibiting bone formation in addition to stimulating bone resorption (5, 14, 38). These findings are consistent with results derived from clinical and animal investigations that have suggested that similar bacteria play a major role in the initiation and progression of periodontal diseases (25, 35, 48).

Effects on differentiation. Bone formation in the CPO model system can be divided into three relatively discrete phases. By testing the various extracts at different times in culture, it was possible to determine their effects on different stages of bone formation, such as differentiation (days 0 to 2), matrix production (days 2 to 4), and mineralization (days 4 to 6). Because of the large number of possible permutations and combinations (i.e., with various extracts and time points or periods), this aspect of our investigation was restricted to studying only the unfractionated extract and the <5-kDa fraction derived from *P. gingivalis*, as these have been shown to have the most profound inhibitory effects of the periodontal extracts studied (19). The results revealed that osteogenesis was inhibited irreversibly when either extract or fraction was added during the differentiation stage. In contrast, the inhibitory effects of the extracts appeared to be reversible when cultures were exposed during the matrix production stage (i.e., after differentiation). Finally, the extracts directly inhibited mineralization, but because of the length of the culture period, it was not determined whether such inhibition was reversible. Together, the findings suggest that the factors contained in the *P. gingivalis* extract as well as the <5-kDa fraction may inhibit osseodifferentiation and therefore the development of functional osteoblasts. When this occurs, the inhibitory effects on osteogenesis are largely irreversible. Alternatively, the extracts also seem to inhibit phenotypic expression by differentiated osteoblasts (e.g., matrix formation, alkaline phosphatase activity, and min-

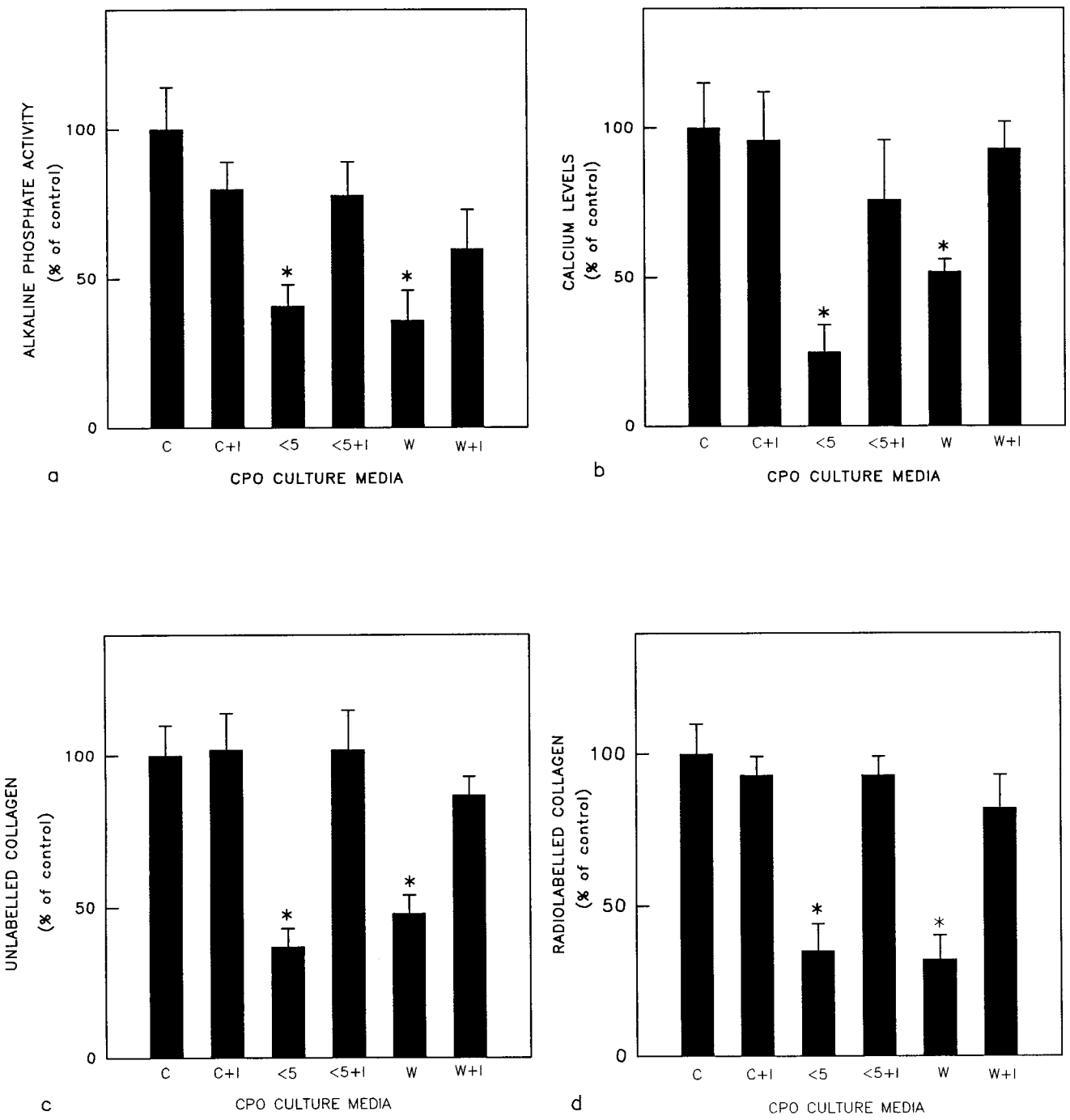

FIG. 6. Osteogenic parameters for control (C, 100% plain BGJ_B medium) and 40% concentrations of sonicated extracts of *P. gingivalis* (<5-kDa fraction [<5] and unfractionated [W] sonicates) incubated in the presence or absence of 1 μM indomethacin (I). (a) Alkaline phosphatase activity. (b) Calcium level. (c) Densitometric evaluation of Coomassie blue-stained minigels run on SDS-PAGE as a control. (d) Densitometric evaluation of fluorographs of SDS-PAGE gels. Significant ($P < 0.05$, *) decreases from control levels were seen for both tested extracts for all measured parameters of osteogenesis activity. The addition of indomethacin blocked the inhibitory activity of the bacterial extracts. Indomethacin alone had no effect on osteogenesis. The data are means and SEM for 8 to 10 cultures. All values have been expressed as a percentage of control values.

eralization), but this effect is essentially reversible once the inhibitory agent is removed.

Characterization of extracts. Heat treatment caused virtually complete loss of the inhibitory properties in the 50- to 100-kDa fraction, implying that heat-labile compounds might be responsible for some of the observed reductions in bone formation by CPO cultures treated with its unheated counterpart. *P. gingivalis* produces various proteolytic enzymes in this molecular size range (9). It has also been shown by others (23) that heat treatment of a 24-kDa protein isolated from the outer membrane of *P. gingivalis* W50 caused loss of the ability to induce bone resorption in vitro. In other fractions, notably the

>100-kDa fraction, only incomplete abrogation of the inhibitory effects was observed, suggesting that proteinaceous factors might be only partially responsible for inhibition of osteogenesis by this fraction. In contrast, heat treatment did not inactivate the inhibitory potential in the <5-kDa *P. gingivalis* extract fractions. Thus, the factor(s) in this fraction was not likely proteinaceous and yet had rather profound effects on differentiation and phenotypic expression of bone cells. These findings seem to parallel other reported results indicating that low concentrations of dental plaque can inhibit type I collagen synthesis in cultured fetal rat calvariae and that heat-denatured plaque extracts are equally effective (26). This suggests that some component other than protein is likely the causative agent. Similarly, the inhibition of matrix production by chick embryo cartilage cells in vitro was not abolished when culture medium filtrates from *P. gingivalis* were heated to 100°C (44). Of further interest was the finding that various parameters of osteogenesis were affected differently and at different doses (e.g., heat-treated extract effects on alkaline phosphatase activity versus collagen synthesis). This underscores the need to assess more than one parameter of bone cell function when assessing the effects of a particular factor on osteogenesis.

Inhibitory activity was not completely eliminated by heating, and therefore other bacterial factors, such as LPS, must also be considered. The results indicated that all parameters of osteogenesis were reduced significantly at LPS concentrations of 1 and 10 μg/ml. No decreases were evident when a very low concentration was used (0.1 μg/ml). Extensive research has been performed on the potential inhibitory effects of LPS on periodontal tissues, including bone. LPS isolated from *P. gingivalis* 381 and W83 has been found to stimulate resorption in fetal rat bone cultures (16, 27). Millar et al. (24) found that two separate species (based on molecular weight and carbohydrate-fatty acid ratios) of *P. gingivalis* 381 LPS induced a 30 to 40% reduction in net collagen formation at a concentration of 10 μg/ml in a long-bone model. However, this long-bone model cannot be used to differentiate between the effects on bone deposition and resorption, as both occur simultaneously.

Although LPS may have direct effects on osteoblastic phenotypic expression (and possibly differentiation), there is evidence which suggests a more indirect effect. LPSs extracted from *P. gingivalis* W83 and other pathogenic bacteria have been shown to stimulate human fibroblast and monocyte/macrophage production of prostaglandin E_2 and interleukin-1β, both of which are considered important local mediators of bone resorption (7, 13, 33, 47). In addition, LPSs from both invasive and noninvasive strains of *P. gingivalis* were able to activate the alternative complement pathway (31). It is conceivable that LPS exerts its inhibitory effects on bone formation through its ability to stimulate cytokine production by target cells other than osteoblasts. This could be mediated through fibroblast products, such as prostaglandin E_2, since the outer cell layers of the folded periosteal cultures contain large numbers of fibroblasts (even after microdissection) (43).

Although the inhibition of osteogenesis by the bacterial extracts was probably not due to direct toxicity, this is still a possibility. Our findings showing that bone-forming cultures could recover from the inhibitory bacterial effects when the extracts were added after differentiation tend to support the notion that toxicity is not involved. This finding, in addition to the lack of effect on noncollagenous protein levels and our previous histological analysis (19), implies that the inhibitory effects of the *P. gingivalis* extracts on CPO osteogenesis were likely cytopathic rather than cytotoxic.

In conclusion, the data indicate that sonicated extracts derived from bacteria implicated in the pathogenesis of periodontal diseases were capable of inhibiting bone formation. Conversely, bacteria which are not associated with periodontal diseases did not have this property, which lends support to the concept that infection with specific bacteria is required to initiate or at least aggravate the bone loss associated with periodontal disease. These bacteria may not only contribute to the progression of periodontal diseases by stimulating bone resorption, as shown by others (14, 24, 27), but also inhibit bone formation itself, thus upsetting the balance in favor of bone loss. In addition, there are also data implicating the same species in infective failures of osseointegrated dental implants, a therapy that relies on osteogenesis at the interface with biomaterials for success (1, 8, 28). Furthermore, there is reason to believe that these organisms might have a negative impact on treatment outcome for the so-called guided tissue regeneration procedures in periodontal therapy (11, 12, 32). Inasmuch as these bacteria seem to have, in essence, a direct inhibitory effect on bone formation, the latter findings should not be surprising, as both osseointegration and guided tissue regeneration procedures rely on bone formation either in whole (osseointegration) or in part (guided tissue regeneration). If regeneration of resorbed bone is to be a realistic goal of therapy, it would seem prudent that this be attempted in the absence of bacteria which have profound inhibitory effects on osteogenesis.

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