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Proliferation/differentiation of osteoblastic human alveolar bone cell cultures in the presence of stainless steel corrosion products

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Human osteoblastic alveolar bone cells were cultured for 28 days in control conditions and in the presence of three non-lethal concentrations of AISI 316L stainless steel (SS) corrosion products. Cells were exposed to SS corrosion products in two experimental situations: (i) in selected stages of the incubation time (during the first, second, third and fourth week of culture); and (ii) during the 28 days incubation period. Cultures were characterized for cell proliferation, total protein content, alkaline phosphatase activity (ALP) and ability to form mineralized deposits; culture media was analyzed for ionized calcium (Ca) and phosphorus (P) concentrations throughout the incubation period. The presence of SS corrosion products during the different stages of the incubation period did not significantly affect the cell proliferation; however, a significant dose-dependent deleterious effect was observed on the levels and pattern of ALP activity, concentration of ionized Ca and P in the culture medium and, also, ability to form mineralized deposits, especially in cultures exposed during the first and second week of culture (respectively, lag phase and exponential cell growth phase). Similar effects were observed in cultures exposed to the SS corrosion products during the 28 days incubation period. However, the presence of such products during the third week (when the mineralization process occurs) and, also, during the fourth week, resulted in little or no significant effects on the behavior of alveolar bone cells. Results suggested that SS corrosion products above certain non-lethal concentrations may disturb the proliferation/differentiation relationship of osteoblastic human alveolar bone cell cultures.

1. Introduction

The cellular events taking place at the bone/material interface can be determinant for the success of the implant's long-term performance [1–4]. There is some concern over the use of metallic biomaterials, as corrosion and fretting have been reported to occur at the biological tissues/biomaterials interface. The high surface area associated with metallic wear debris increases the potential for ion release into the physiological environment, both in tissues adjacent to implanted materials and also their distribution throughout the body via systemic circulation [5–8]. Several studies have suggested a potential role for ions and particles released from metal implants in various pathological bone and tissue conditions [9–16].

The type 316L stainless steel, iron-based alloy contains major alloying elements of iron, chromium and nickel, with deliberate addition of limited quantities of manganese, carbon, molybdenum and silicon. Both *in vivo* and *in vitro* studies have shown deleterious effects of AISI 316L stainless steel (SS) corrosion products in several organs and tissues [8, 12, 17]. *In vitro* studies suggested that such corrosion products impair the functional activity of human immune cells [18] and skin fibroblasts [19]. In addition, work performed in rodent and human osteogenic cell cultures suggested acute and long term effects of metal ions, found in commonly used metallic materials on the proliferation and function of osteoblastic lineage cells [20–27].

Previous work has shown that human alveolar bone cell cultures obtained in the presence of β-glycerophosphate, ascorbic acid and dexamethasone presented osteoblastic features, namely, high alkaline phosphatase (ALP) activity and ability to form mineralized nodules [28, 29] and, as observed with a number of bone cell systems, represent a potential *in vitro* model to study the interactions of biomaterials and their degradation products with bone cells.

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The aim of this work was to study the dose-effect of AISI 316L SS corrosion products, obtained by electrochemical means, on the proliferation and osteoblastic differentiation of human alveolar bone cell cultures. The concentrations of the major metal ions present in the SS corrosion products were in the range of iron, chromium and nickel levels found in tissues adjacent to stainless steel implants [30]. Cultures grown in control conditions and on the presence of SS corrosion products were evaluated concerning cell viability/proliferation, total protein content, ALP activity and ability to form calcium phosphate deposits; concentrations of ionized calcium (Ca) and phosphorus (P) in the culture medium collected from cell cultures throughout the incubation period were also determined.

2. Materials and methods

2.1. Stainless steel corrosion products

Type AISI 316L stainless steel (Fe 63.9%, Cr 18.0%, Ni 12.5%, Mo 2.8%, Si 1.2%, Mn 1.6%, P 0.45% and C 0.25%, weight for weight) was anodically dissolved in Hank's balanced salt solution. The resulting concentrations of the major metal ions in the AISI 316L slurry were determined by atomic absorption spectrometry: 47 μg/ml Fe $(8.77 \times 10^{-4}\,\mathrm{mol\,l^{-1}}) + 22.4\,\mu\mathrm{g\,ml^{-1}}$ Cr $(4.31 \times 10^{-4}\,\mathrm{mol\,l^{-1}}) + 15\,\mu\mathrm{g\,ml^{-1}}$ Ni $(2.55 \times 10^{-4}\,\mathrm{mol\,l^{-1}}) + 2.6\,\mu\mathrm{g\,ml^{-1}}$ Mo $(0.27 \times 10^{-4}\,\mathrm{mol\,l^{-1}})$. After pH adjustment to 7.4 and sterilization in an autoclave further solutions were obtained by successive dilutions.

2.2. Cell culture and characterization

Human alveolar bone fragments, obtained from oral surgery procedures, were washed extensively with α-minimal essential medium (α-MEM), minced into small pieces and cultured with α-MEM containing 10% fetal bovine serum, $50\,\mu\mathrm{g\,ml^{-1}}$ gentamicin and $2.5\,\mu\mathrm{g\,ml^{-1}}$ fungizone. Cell growth from the bone fragments was observed after approximately 15–20 days; primary cultures were maintained until near confluence and, at this stage, adherent cells were enzymatically released (0.04% trypsin and 0.025% collagenase). First passage cells were cultured (10^4 cells/cm^2) for 28 days in the presence of ascorbic acid ($50\,\mu\mathrm{g\,ml^{-1}}$), β-glycerophosphate (βGP, 10 mM) and dexamethasone (10 nM), in control conditions and exposed to SS corrosion products. Treated cultures were grown in the presence of three concentrations of the prepared AISI 316L slurry, namely, 0.1%

$(0.49\,\mu\mathrm{g\,ml^{-1}}\,\mathrm{Fe} + 0.22\,\mu\mathrm{g\,ml^{-1}}\,\mathrm{Cr} + 0.15\,\mu\mathrm{g\,ml^{-1}}\,\mathrm{Ni})$

0.5%

$(2.45\,\mu\mathrm{g\,ml^{-1}}\,\mathrm{Fe} + 1.12\,\mu\mathrm{g\,ml^{-1}}\,\mathrm{Cr} + 0.75\,\mu\mathrm{g\,ml^{-1}}\,\mathrm{Ni})$

and 1%

$(4.90\,\mu\mathrm{g\,ml^{-1}}\,\mathrm{Fe} + 2.24\,\mu\mathrm{g\,ml^{-1}}\,\mathrm{Cr} + 1.50\,\mu\mathrm{g\,ml^{-1}}\,\mathrm{Ni})$;

cells were exposed to the metal species in two experimental situations: (i) in selected stages of the incubation period, i.e. during the first, second, third and fourth week of culture; and (ii) during the 28 days culture time. Incubation was carried out in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C and culture medium was changed every 3 days.

Control and SS treated cultures were tested at days 1, 4, 7, 14, 21 and 28 concerning biochemical (viability/proliferation, total protein content and ALP activity) and histochemical (identification of the presence of ALP and calcium phosphate deposits) parameters. Also, at each medium change, culture media from control and metal-treated cultures were collected and analyzed for the quantification of ionized Ca and P; control and metal-treated culture medium were incubated in the absence of bone cells under the same experimental conditions as the cell cultures and also analyzed for ionized Ca and P concentrations.

2.2.1. Biochemical assays

MTT assay (reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple Formazan product) was used to estimate cell viability/proliferation. Cells were incubated with $0.5\,\mathrm{mg\,ml^{-1}}$ of MTT in the last 4 h of the culture time tested; the medium was then decanted, Formazan salts were dissolved with dimethylsulfoxide and the absorbance was determined at 600 nm.

ALP activity was determined in cell lysates (obtained by treatment of the cultures with 0.1% triton) and assayed by the hydrolysis of *p*-nitrophenyl phosphate in alkaline buffer solution, pH 10.3, and colorimetric determination of the product (*p*-nitrophenol) at $\lambda = 405$ nm (hydrolysis was carried out for 30 min at 37 °C); the procedure was described in detail previously [29]. Results are expressed in nanomoles of *p*-nitrophenol produced per min per cm^2 (nmol min^{-1} cm^{-2}) and also in nanomoles of *p*-nitrophenol produced per min per μg of protein (nmol min^{-1}/μg protein).

Protein content was determined in 0.1 M NaOH cell lysates according to the method of Lowry using bovine serum albumin as a standard. Results are expressed as $\mu\mathrm{g\,cm^{-2}}$.

2.2.2. Histochemical assays

For histochemical staining, cultures were fixed with 1.5% glutaraldehyde in $0.14\,\mathrm{mol\,l^{-1}}$ sodium cacodylate buffer and rinsed with distilled water.

2.2.2.1. ALP staining. Fixed cultures were incubated during 1 h in the dark with a mixture, prepared in Tris buffer pH = 10, containing $2\,\mathrm{mg\,ml^{-1}}$ of Na-α-naphtyl phosphate and $2\,\mathrm{mg\,ml^{-1}}$ of fast blue RR salt; the incubation was stopped by rinsing the samples with water. The presence of ALP was identified by a brown to black staining.

2.2.2.2. Phosphate staining. Phosphate deposits were assessed by the von Kossa technique [31] i.e. the fixed cultures were covered with a 1.0% silver nitrate solution and kept for 1 h under UV light. After rinsing, a 5.0% sodium thiosulfate solution was added for 2 min and cultures were washed again. Phosphate deposits stained black.

2.2.2.3. Calcium staining. The fixed cultures were covered with a 1.0% S alizarin sodium solution (0.028% in NH_4OH), pH = 6.4, for 2 min and then rinsed with water and acid ethanol (ethanol, 0.01% HCL) [31]. Calcium deposits stained red.

2.3. Calcium and phosphorus measurements

Ionized calcium and phosphorus were measured using, respectively, a Calcium Kit (Sigma no. 587M) and the Inorganic Phosphorus Kit (Sigma no. 670-C).

2.4. Statistical analysis

For biochemical data (MTT reduction, total protein content and ALP activity) each point represents the mean $\pm$ standard deviation of 6–8 replicates; for ionized Ca and P each point correspond to the mean $\pm$ standard deviation of three independent analyses being each individual value obtained by performing three replicates. Statistical analysis was done by one-way analysis of variance (ANOVA). The statistical difference between the different groups were determined by the Bonferroni method. P values < 0.05 were considered significant.

It is known from previous work [29] that osteoblastic alveolar bone cell cultures obtained in the experimental conditions described enter a senescence phase after the mineralization process (approximately three to four weeks of incubation) and, as a consequence, the displacement of the cell layer from the tissue culture is frequently observed; this may contribute to the high standard deviations found in some of the results concerning the measured parameters in 21 and 28 days cultures.

3. Results

Human alveolar bone cells were grown under experimental conditions known to favor the formation of osteoblast rich cultures [23, 32–34]. The first subculture was used to study the dose-effect of SS corrosion products on the proliferation and differentiation of osteoblastic cells cultured for 28 days.

3.1. Cell viability/proliferation and total protein content

Results observed for cell viability/proliferation on control and SS exposed cultures are presented in Fig. 1.

3.1.1. Control cultures

After a lag phase of 3–4 days, cells grown in control conditions proliferated gradually with incubation time and maximal values for MTT reduction were observed at day 21; cell growth showed a slow down during the third week and a stationary phase was observed on the fourth week of culture.

3.1.2. Cultures exposed to SS in different stages of the incubation period

3.1.2.1. First week. Cells exposed to 0.1% SS during the first week presented a pattern on the MTT reduction similar to that observed on cultures grown in control conditions, but increased values for cell proliferation were found from day 7 onwards (approximately, 30% at day 14). In cultures exposed to 0.5% SS, cell growth was slightly lower during the second week than that observed in control cultures but, after that, increased until day 21, reaching a stationary phase during the fourth week; at days 21 and 28, MTT reduction was similar in control and 0.5% SS treated cultures. Cells exposed to 1% SS proliferated gradually during the 28 days incubation period without reaching a stationary phase; during the second and third week, values obtained for the MTT reduction were slightly lower (ca. 20%) than those found in control conditions; however, at days 21 and 28, values were not statistically different.

3.1.2.2. Second week. As compared to control conditions, cultures exposed to SS during the second week presented a tendency for an increase on the cell proliferation during the period of SS exposure (phase of exponential cell growth) but this effect was statistically significant only in 0.1% SS treated cultures (at day 14). The highest values for MTT reduction were observed by the end of the second week decreasing after that and a stationary phase on cell growth was observed during the fourth week, in all the situations studied.

3.1.2.3. Third and fourth week. Cultures exposed to SS corrosion products during the third week of incubation showed a tendency for an increase on cell proliferation during the period of exposure, especially, 0.5% and 1% SS treated cultures; MTT reduction was maximal around day 21 and remained approximately constant until the end of the incubation period. Cells exposed to SS during the fourth week also showed a tendency for an increase on cell proliferation during this period. However, in both experimental situations, no statistical differences were observed, as compared to control cultures.

3.1.3. Cultures exposed to SS during the 28 days incubation

As compared to control cultures, cells exposed to 0.1% SS slurry showed increased values for MTT reduction during the exponential phase of cell growth (ca. 30% at day 14), and cultures growing in the presence of 0.5% and 1% SS a decrease on cell proliferation (ca. 35% at day 14); in 0.5% SS cultures, cell growth increased throughout the 28 days incubation and at days 21 and 28 no statistical differences were found as compared to control cultures; however, in cultures exposed to 1% SS, MTT reduction remained approximately constant from day 14 onwards.

Results observed for total protein content in control and SS exposed cultures followed a similar pattern to that observed for the MTT reduction in the various experimental situations, providing comparable information (results not shown).

3.2. ALP activity

Results concerning ALP activity determined in control and SS exposed cultures are presented in Fig. 2a and b, expressed, respectively, in nmol min^{-1} cm^{-2} and in nmol min^{-1} μg protein; in this case, results are shown from day 7 onwards, since in all the situations tested, the levels of the enzyme were very low during the first week of culture.

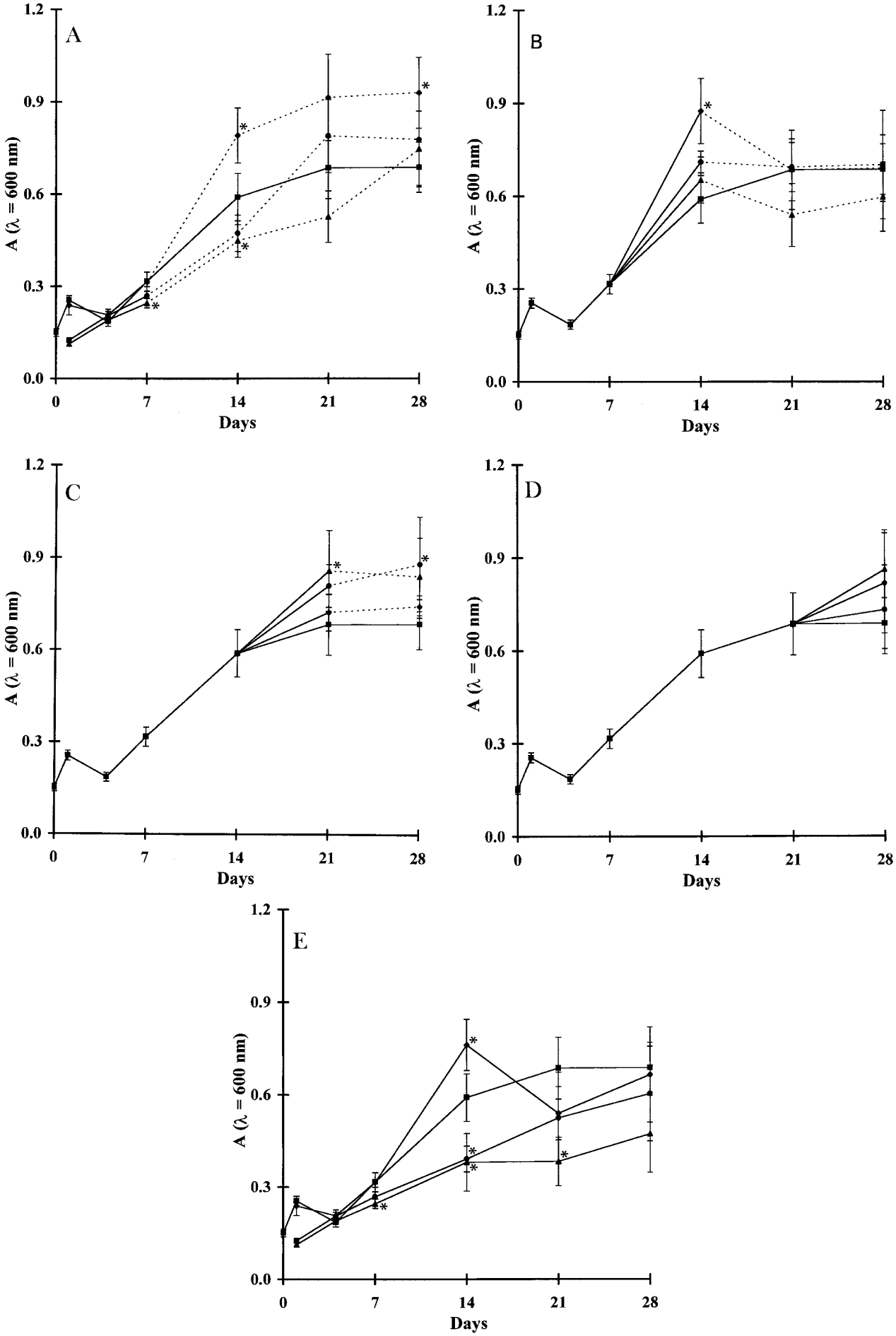

Figure 1 Cell viability/proliferation of human alveolar bone cells cultured for 28 days in control conditions and in the presence of stainless steel corrosion products. Cells exposed to SS during the first (A), second (B), third (C) and fourth (D) week of culture; period of SS exposure is marked with a continuous line. (E) cells exposed to SS during the 28 days incubation period. Control cultures (■) and cultures exposed to 0.1% SS (◆), 0.5% SS (●) and 1% SS (▲). *Significantly different from control cultures.

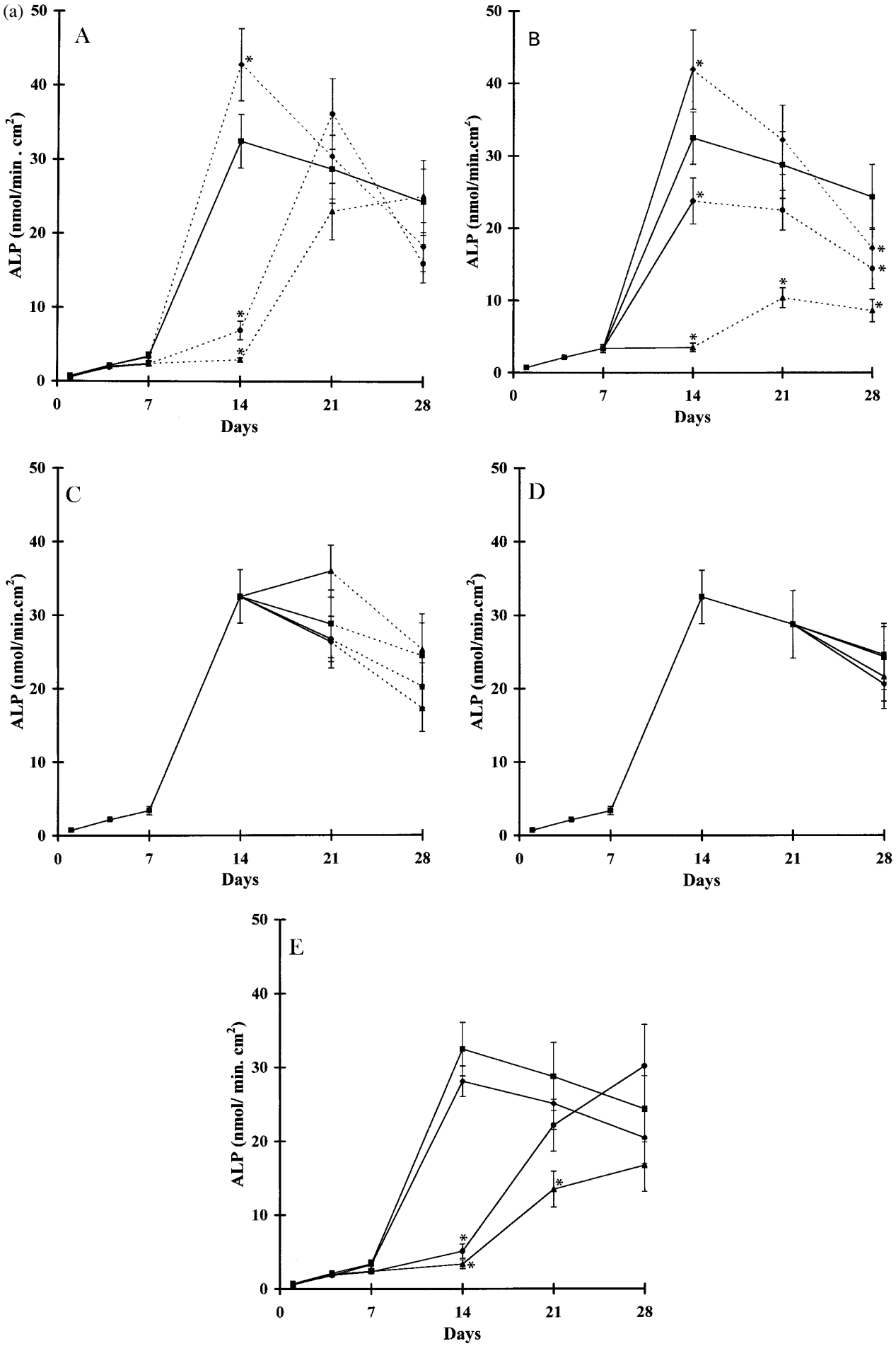

Figure 2 ALP activity of human alveolar bone cell cultures grown for 28 days in control conditions and in the presence of stainless steel corrosion products: (a) results expressed in nmol min^{-1} cm^{-2}; (b) results expressed in nmol min^{-1}/μg protein. Cells exposed to SS during the first (A), second (B), third (C) and fourth (D) week of culture; period of SS exposure is marked with a continuous line. (E) cells exposed to SS during the 28 days incubation period. Control cultures (■) and cultures exposed to 0.1% SS (◆), 0.5% SS (●) and 1% SS (▲). *Significantly different from control cultures.

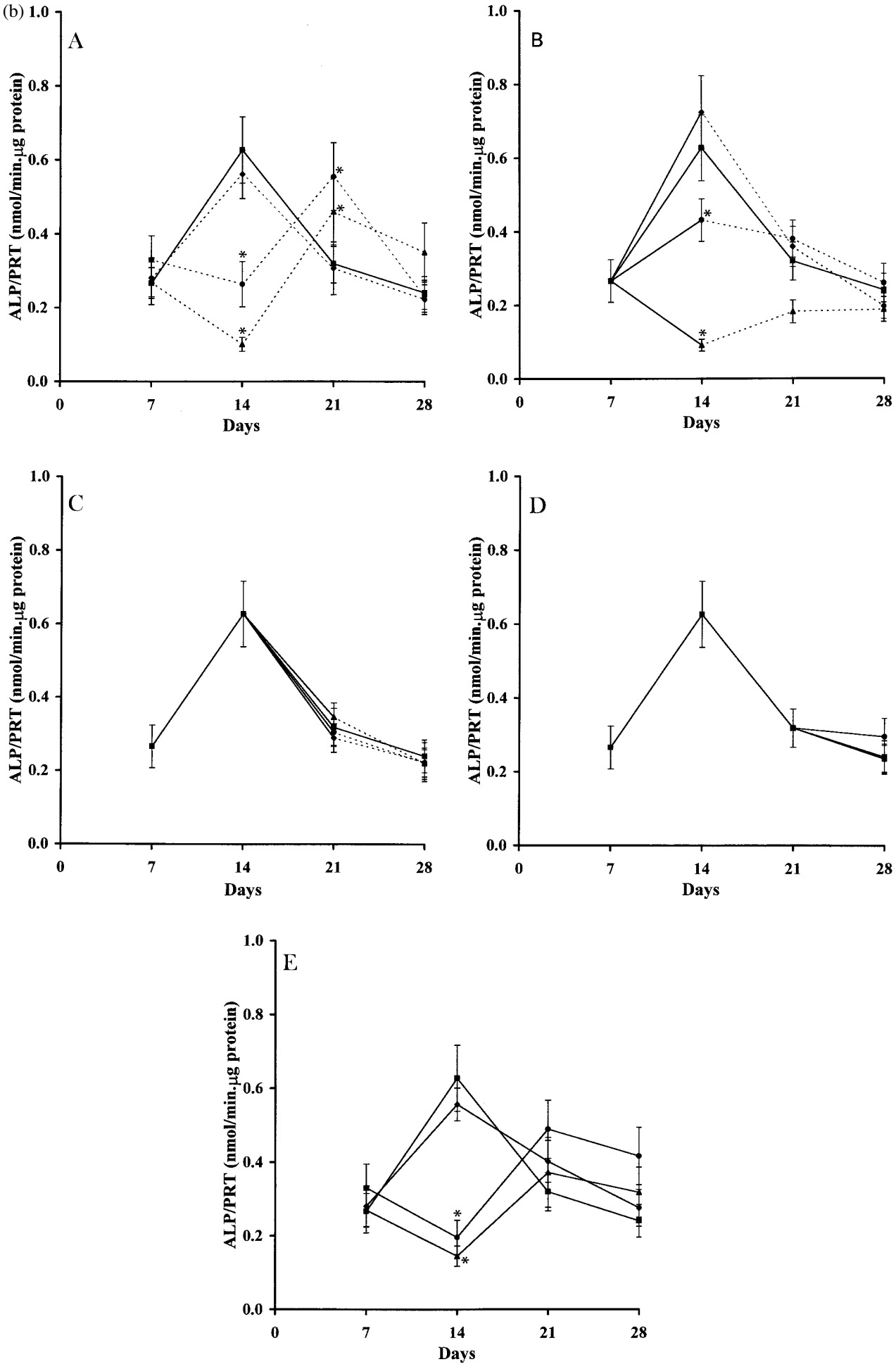

Figure 2 (Continued)

3.2.1. Control cultures

ALP activity was low during the first week, increased significantly during the second week (maximal levels were observed around day 14) and decreased after that.

3.2.2. Cultures exposed to SS in different stages of the incubation period

3.2.2.1. First week. Fig. 2 shows that, as compared to control cultures, 0.1% SS treated cultures presented a similar pattern of behavior concerning ALP activity, although, higher levels of the enzyme were observed on day 14 (ca. 30%), event most probably related with the increase on cell proliferation observed in these cultures at this stage (Fig. 1). In 0.5% and 1% SS treated cultures, levels of ALP were very low during the first two weeks, and, at day 14, values were, respectively, 80% and 90% lower than those observed in control cultures, however, ALP activity increased significantly during the third week and maximal levels of the enzyme were observed by day 21. Fig. 2a and b show that the highest levels of the enzyme were observed at day 14 in control and 0.1% SS cultures and, at day 21 in cultures exposed to 0.5% and 1% SS.

3.2.2.2. Second week. Cells exposed to 0.1% and 0.5% SS during the second week presented a pattern of ALP activity similar to that observed in control cultures, although, 0.1% SS cultures showed an increase and 0.5% SS cultures a decrease on the levels of the enzyme during the incubation period (differences statistically significant at day 14); however, it is interesting to note that in these two situations, maximal activity of the enzyme was attained by day 14 (Fig. 2a and b), as in control cultures, contrasting with that observed in cultures exposed to 0.5% SS during the first week of incubation. Cultures exposed to 1% SS showed significantly lower ALP activity throughout the incubation period, as compared to control cultures (Fig. 2a), and the highest levels of the enzyme were observed by days 21–28.

3.2.2.3. Third and fourth week. Cultures exposed to SS corrosion products during the third and also the fourth week of culture presented a behavior similar to that observed in control cultures (Fig. 2) and differences in ALP activity in control and treated cultures were not statistically significant; however, cultures exposed to 1% SS during the third week showed a tendency for an increase in ALP levels during the period of exposure, similarly to the observed value for cell proliferation, attaining maximal levels at day 21 and decreasing after that.

3.2.3. Cultures exposed to SS during the 28 days incubation

As compared to control cultures, cultures exposed to 0.1% SS presented a similar pattern of ALP activity throughout the incubation, i.e. the maximal levels were observed around day 14 and decreased after that, but, levels of the enzyme were slightly lower (differences were not statistically significant). In cultures exposed to 0.5% and 1% SS, ALP was very low during the first two weeks but, after that, increased progressively until the end of the culture period; at days 21 and 28 levels of ALP were similar in control and 0.5% SS cultures, but lower in 1% SS cultures. Results concerning ALP/μg protein (Fig. 2b) clearly showed that 0.5% and 1% SS treated cultures presented a significant decrease on the levels of the enzyme around day 14 and that the highest ALP activity was observed by day 21 (contrasting with that observed in control and 0.1% SS exposed cultures).

3.3. Ionized calcium and phosphorus evaluation

Results concerning the levels of ionized Ca and P in the culture medium collected from control and SS treated cultures during the incubation period (and also in control and treated medium incubated in the absence of cells) are presented, respectively, in Fig. 3a and b. Levels measured were not cumulative, as culture medium was totally replaced every 3 days; values shown reflect changes occurring in intervals of 3 days throughout the culture period.

In the absence of bone cells, levels of ionized Ca and P in control and metal-treated culture medium did not change significantly during the incubation period, ranging from ca. 1.5 to 2.0 $\mathrm{mmol\,l^{-1}}$ for Ca and from ca. 1.3 to 2.0 $\mathrm{mmol\,l^{-1}}$ for P.

3.3.1. Control cultures

Culture medium collected from control cultures showed an increase in the concentrations of ionized P until approximately day 16 (from ca. 1.9 to 8.1 $\mathrm{mmol\,l^{-1}}$); increase in the levels of Pi in the medium results from the hydrolysis of β-glycerophosphate added to the culture medium (10 mM), most probably by ALP present in high levels in the cell cultures at this stage. During this period, levels of ionized Ca did not change significantly. However, from day 16 onwards, a significant decrease on the levels of ionized Ca and P was observed, an event that reflects the mineralization process, i.e. the formation of calcium phosphate deposits in the cultures.

3.3.2. Cultures exposed to SS in different stages of the incubation period

3.3.2.1. First week. In cultures exposed to 0.1% SS, concentrations of ionized Ca and P in medium followed a similar pattern to that observed in control cultures, although, the increase on the levels of P_i was slightly lower and maximal values attained later. Cultures exposed to 0.5% SS presented increased levels of P_i in the medium from approximately 2 weeks onwards (period during which ALP activity increased, Fig. 2), attaining maximal levels around days 22–25 and decreasing slightly after that; however, levels of P_i were significantly lower than those observed in control cultures. Levels of Ca_i were approximately constant until around day 25 and decreased afterwards. In 1% SS exposed cultures, levels of ionized Ca and P remained approximately constant during the 28 days incubation and were similar to those found in the absence of bone cells.

3.3.2.2. Second week. Cultures exposed to 0.1% and 0.5% SS during this period followed a similar pattern to that observed in control cultures, concerning ionized Ca

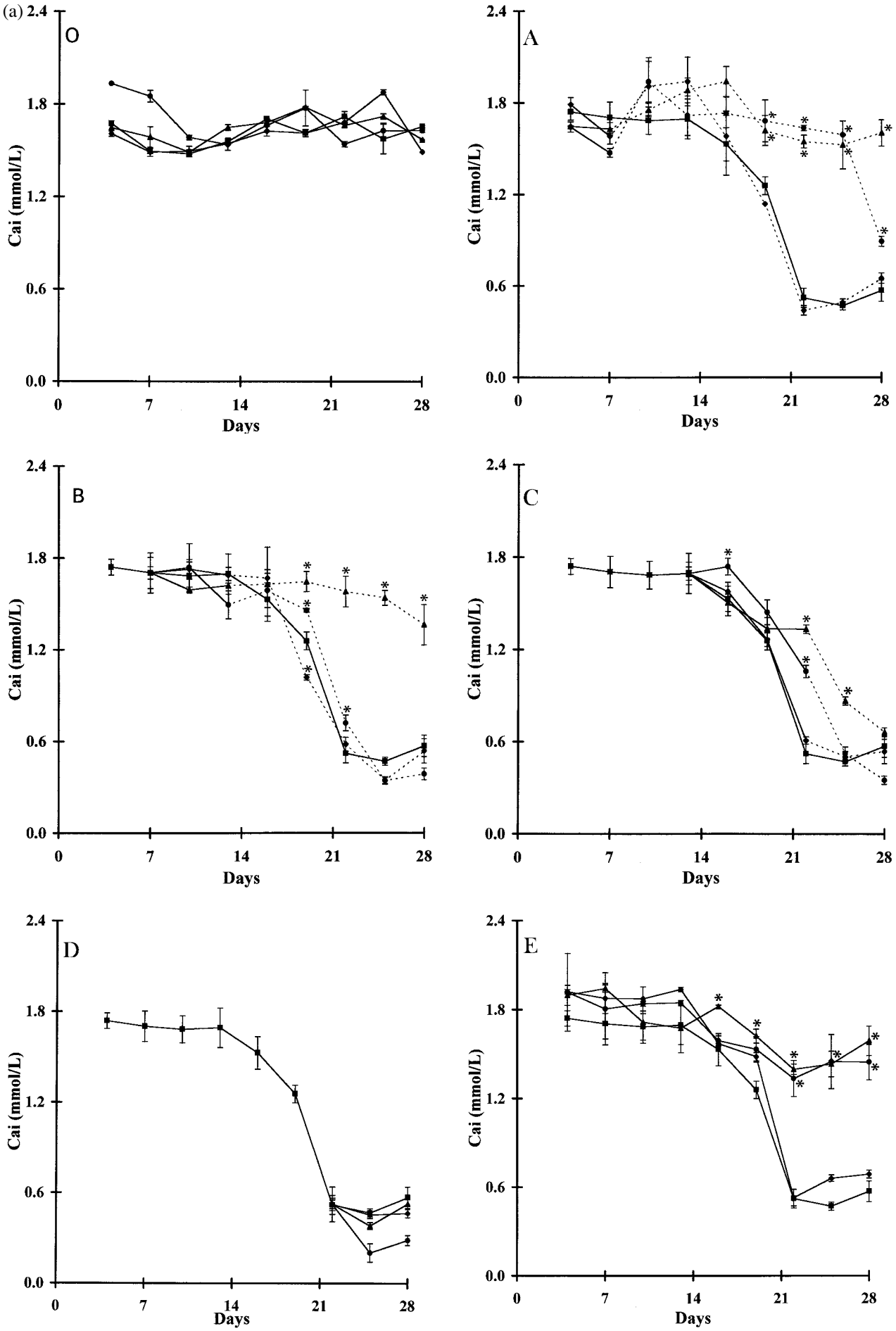

Figure 3 Levels of ionized calcium (a) and phosphorus (b) in the culture medium collected from human alveolar bone cell cultures grown for 28 days in control conditions and in the presence of stainless steel corrosion products. Absence of bone cells (O). Cells exposed to SS during the first (A), second (B), third (C) and fourth (D) week of culture; period of SS exposure is marked with a continuous line. (E) cells exposed to SS during the 28 days incubation period. Control cultures (■) and cultures exposed to 0.1% SS (♦), 0.5% SS (●) and 1% SS (▲). *Significantly different from control cultures.

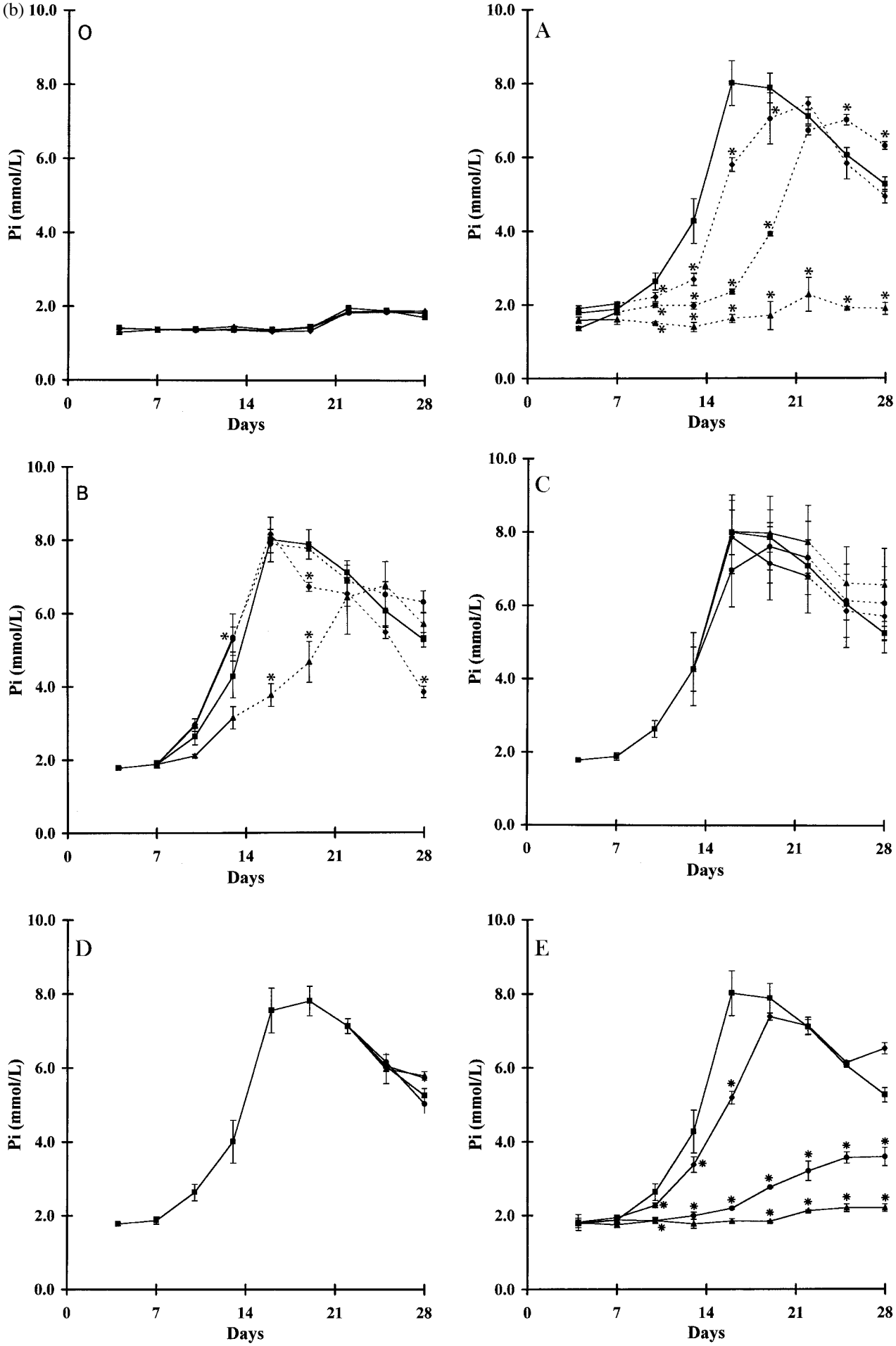

Figure 3 (Continued)

and P in the medium. Cultures exposed to 1% SS presented an increase in P_i levels almost until the end of the incubation period, with a little decrease in the last 3 days, but, during the first 3 weeks, values were significantly lower than those found in the previous situations; Ca_i levels remained approximately constant during the incubation, decreasing slightly in the last days.

3.3.2.3. Third and fourth week. Levels of ionized Ca and P in the medium collected from cultures exposed to SS during the third week followed a pattern similar to that observed in control cultures; however, some differences were evident. In 0.5% and 1% SS cultures, the rate of decrease on the levels of ionized Ca and P was lower than that observed in control and 0.1% SS cultures, effect that appears to be dose-dependent and was especially evident for ionized Ca.

The presence of SS corrosion products during the fourth week did not affect significantly the pattern of variation of the levels of ionized Ca and P, as compared to control cultures.

3.3.3. Cultures exposed to SS during the 28 days incubation

Levels of ionized P and Ca in the medium collected from 0.1% SS cultures followed a pattern of variation similar to that observed in control cultures, although, with a lower increase in the P_i levels during the first 19 days and maximal levels attained later. In cultures exposed to 0.5% SS, levels of P_i did not change significantly during the first 16 days but, after that, increased gradually until the end of the incubation period, although, values were significantly lower than those observed in control cultures. In 1% SS cultures, levels of P_i in the medium were similar to those observed in the absence of bone cells. Concerning the levels of ionized Ca in 0.5% and 1% SS exposed cultures, only a very little tendency for a decrease was observed in the last days of the culture period.

3.4. Histochemical assays

Control and SS-exposed cultures were stained histochemically for the presence of ALP and calcium and phosphate deposition. Cultures stained for ALP in the tested days showed results that were in agreement with those observed in the biochemical determination of the enzyme, giving the same kind of information and are not shown. Results concerning the histochemical staining of calcium phosphate deposition (von Kossa assay) in 21 and 28 days cultures are shown in Fig. 4. Staining of the cultures by the Alizarin red assay showed similar results (results not shown).

The presence of 0.1% SS, in all the situations tested, did not affect the formation of mineralized calcium phosphate deposits (Fig. 4). In cultures exposed to 0.5% and 1% SS during the different stages of the incubation period, it was observed that exposure during the first and, also, the second week of culture, resulted in deleterious effects on the mineralization process; this effect was dose-dependent and appeared to be more pronounced in cultures exposed during the first week. In contrast, exposure to SS during the third and also the fourth week

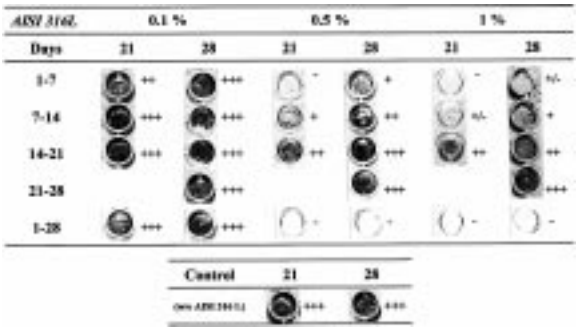

Figure 4 Histochemical staining of calcium phosphate deposits (von Kossa assay) in 21 and 28 days cultures grown in control conditions and in the presence of stainless steel corrosion products. Intensity of staining was graded as follows: −, negative staining; +/−, weak to undetectable staining; +, definite staining but of low intensity; ++, moderate staining; +++, intense staining.

resulted in little or no detectable effect on the formation of mineralized deposits. However, cultures growing in the presence of 0.5% and 1% SS during the 28 days incubation failed to form calcium phosphate deposits.

4. Discussion

The results reported in the previous section showed that human alveolar bone cells grown in control conditions, i.e. in the presence of ascorbic acid, β-glycerophosphate and dexamethasone presented osteoblastic features, showing a similar behavior concerning cell proliferation/differentiation to that reported previously for this culture system [29] and other similar bone nodules forming cultures [23, 32–34]. After a lag phase of 3–4 days, bone cells enter a period of active proliferation that is functionally related to the synthesis of a bone-specific extracellular matrix and by the third and fourth week, a tendency for a stationary phase on cell growth is observed, most probably, as a result of the accumulation and maturation of the extracellular matrix [35–37]. ALP expression followed a similar pattern of variation and results concerning ALP/μg protein (that provides information about the mean ALP content per cell) showed that maximal activity of the enzyme was observed by day 14, decreasing after that. The significant increase on the levels of this enzyme during the second week of incubation (Fig. 2) suggested that cells were shifting to a more differentiated state [36–38]. ALP appears to play a crucial role in the initiation of matrix mineralization providing localized enrichment of Pi and, after that, expression of the enzyme is down-regulated [38, 39]. These cultures presented a high ability to ionize the β-glycerophosphate present in the culture medium, as shown by the high levels of P_i observed in the medium during the first 16–19 days of incubation, most probably by ALP which is known to have a high efficacy in hydrolyzing this substrate [38]. This ester phosphate provides a potential source of phosphate ions required for the mineralization process in bone nodules forming cultures (deposition of calcium phosphate) [38–41]. In this culture system, the mineralization process, an osteoblast-specific function, occurred during the third week, as demonstrated by the strong positive staining reactions for calcium phosphate deposition at day 21

(Fig. 4) and, also, the significant consumption of ionized Ca and P from the culture medium during this phase of the culture (Fig. 3). Evaluation of the concentration of ionized Ca and P in the incubation medium throughout the culture period may be regarded as a measure of the mineralization process ocurring in these cell cultures, as calcium phosphate deposition in the extracellular space requires consumption of Ca and phosphate ions from the culture medium.

The behavior of alveolar bone cell cultures was not significantly affected by the presence of 0.1% SS, corresponding to $0.49\,\mu g\,ml^{-1}$ Fe + $0.22\,\mu g\,ml^{-1}$ Cr + $0.15\,\mu g\,ml^{-1}$ Ni; however, as compared to control cultures, exposure to the SS slurry during the first and, also, the second week, resulted in increased cell proliferation during the exponential cell growth phase that was accompanied by a similar increase in the ALP levels. Also, cultures exposed during the 28 days incubation period showed a similar increase on cell proliferation. In 0.1% SS cultures, formation of mineralized deposits also followed maximal ALP activity (around day 14) and 21 days cultures showed a positive staining for calcium phosphate deposits.

In cultures exposed to 0.5% and 1% SS during the different stages of the incubation, cell proliferation was similar to that observed in control cultures, although, exposure during the first week resulted in a slight decrease on cell growth during the second week of culture. In contrast, a significant effect was observed on the levels and pattern of ALP activity, concentration of ionized Ca and P in the medium and, also, ability to form mineral deposits, especially in cultures exposed to the SS corrosion products during the first and second week of incubation.

In cultures exposed to 0.5% SS during the first week (Figs 2–4), ALP activity was very low during the first two weeks, increasing after that and the highest levels were observed around day 21, decreasing afterwards; P_i levels increased during the third week and levels of ionized Ca and P began to decrease from about day 25 onwards; these results are in agreement with that observed in the histochemical assays, showing that some mineralization occurred during the fourth week (staining was negative at day 21 and slightly positive at day 28). Exposure to SS during the second week caused a less pronounced effect on the parameters studied and the behavior of the treated cultures approximates that observed in control cultures (Figs 2–4), i.e. maximal ALP activity tends to be observed around day 14 and the pattern of variation on the levels of ionized Ca and P in the medium was similar to that observed in control conditions; however, some impairment on the osteoblastic differentiation is apparent as histochemical staining for calcium phosphate deposition was only slightly positive at day 21.

In cultures exposed to 1% SS during the first week (Figs 2–4), ALP increased from 2 weeks onwards and the highest levels were obtained around day 21, although they were lower than those observed in 0.5% SS cultures. Ionized Ca and P remained approximately constant during the culture period (with a little increase on P_i levels in the third week) and, accordingly, negative staining for the presence of mineralized deposits was observed both in 21 and 28 days cultures. Exposure during the second week (Figs 2–4) resulted in lower levels of ALP than those observed in 0.5% SS cultures, but maximal activity was also attained by day 21; P_i levels increased slowly during approximately three weeks and the decrease on the levels of ionized Ca and P was more evident in the last days of the culture; histochemical staining was weak to undetectable at day 21 and slightly positive at day 28.

The behavior of alveolar bone cells exposed to 0.5% and 1% SS during the third week was similar to that observed in cultures grown in control conditions, except for a lower rate of decrease on the levels of ionized Ca and P, effect, apparently, dose-dependent. Exposure to SS during the fourth week did not affect significantly the behavior of alveolar bone cell cultures.

The results described above suggest that the presence of SS corrosion products at the concentrations studied did not interfere significantly with the proliferation of alveolar bone cells in the experimental conditions tested, except a tendency for an increase on the cell growth in the presence of 0.1% SS. However, above certain concentrations (0.5% and 1% of the SS slurry prepared), the presence of such products, during the first and also the second week of incubation, significantly disturbed the osteoblastic differentiation of bone cells. The most significant effect was an evident reduction of ALP activity around day 14 (maximal ALP activity in control cultures) and, in treated cultures, expression of the enzyme tended to occur during the third week. As cell proliferation was not significantly affected, and considering that ALP has been regarded as a marker of osteoblastic differentiation *in vitro* [33, 36, 37, 42], these results suggested that the cell population exposed to the SS corrosion products during the initial stage of the culture (first week) and, also, during the exponential cell growth (second week), differentiated later than that growing in control conditions. This is also supported by the results observed for the pattern of variation on the levels of ionized Ca and P in the medium and by the histochemical staining of the cultures, showing that the formation of mineralized deposits tends to occur later in SS exposed cultures. This was a dose-dependent effect and for a given concentration of SS (0.5% or 1% SS) appeared to be more pronounced when the corrosion products were present during the first week, in the phase of cell adaptation to the culture conditions.

As reported in the previous section, in control cultures, the mineralization process occurred during the third week of culture and, in this context, it could be anticipated that non-lethal levels of SS corrosion products would not significantly influence this culture system, when present after osteoblastic differentiation had already occurred.

Results concerning cell cultures exposed to 0.5% SS during the 28 days incubation showed that cells proliferated slowly but gradually without showing any tendency for a stationary phase, and ALP increased continuously from two weeks onwards (and also P_i levels in the medium). This suggests that the cell population growing in these conditions appears to be in a less differentiate stage in the osteoblastic lineage and cultures would probably mineralize later [35–37]; alternatively, as cell proliferation increases so slowly, production of an

extracellular matrix in quantity and quality for mineralization to occur is probably not observed and, as a consequence, differentiation is never accomplished [35–37]. Cultures grown in the presence of 1% SS during the 28 days incubation showed a similar pattern of behavior, although deleterious effects on cell proliferation and ALP activity were more pronounced.

Previous work has examined the dose-dependent effect of SS corrosion products on the behavior of osteoblastic cells derived from rat bone marrow, in similar experimental conditions [26, 27]. It was observed that the addition of the SS corrosion products to the control medium resulted in a small increase in the Fe content of the medium (about 45%) when compared to the increases observed in the Cr and Ni contents (threefold for Cr and fivefold for Ni). Determination of the concentrations of Fe, Cr and Ni ions on the incubation medium throughout the culture period and also observation of the cultures by transmission electron microscopy suggested a biologically induced deposition of Cr and Ni, but not Fe, a process that may probably be associated with the marked increase on the levels of Cr and Ni ions, leading to the formation of more proportionally, Cr and Ni organometallic complexes as compared to those formed with Fe [26, 27]. In addition, as reported by other authors [7, 43–45], uptake of Cr and Ni species from its complexes by cells via a transport system may occur with consequent interferences in the cellular metabolism. Merrit *et al*. [46] showed that fretting of AISI 316L in serum resulted in corrosion products containing Cr^{6+} which is able to penetrate the cell membrane. These hypotheses may help to explain the cytotoxicity of SS, in combination with previous results obtained by Lucas *et al*. [47] that indicate *in vitro* induction of cellular ultrastructural damage by Ni, and also the low levels of Cr found in culture media exposed to SS and osteogenic cells.

It should be considered that a key factor in the toxicity of AISI 316L would be the relative proportions of the various metal species formed during the degradation of this metallic alloy in the biological fluids and, also, that some of the species can exist in a variety of valence states with different toxicity potential [45, 47, 48]. In addition, metal species react in biological systems and as different species are formed, significant changes in toxicity may occur. Understanding of these processes will be incomplete until the relative proportions and different chemical species (of the same element) formed during implant degradation will be determined/characterized.

5. Conclusions

Results reported suggested that AISI 316L stainless steel corrosion products above certain non-lethal concentrations may disturb the proliferation/differentiation relationship of osteoblastic human alveolar bone cell cultures, in a dose-dependent manner.

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