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A Study of the Antiresorptive Activity of Salmon Calcitonin Microspheres Using Cultured Osteoclastic Cells

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ABSTRACT The purpose of this study was to evaluate salmon calcitonin (sCT) microspheres in vitro for their antiresorptive activity using cultured osteoclastic cells. The antiresorptive activity of sCT-loaded microspheres, prepared from a low molecular weight hydrophilic poly (lactide-co-glycolide) polymer (PLGA), was studied using bone marrow culture cells harvested from juvenile rats and cultured on slices of devitalized bone for up to 4 weeks. The resorptive activity of osteoclastic cells was quantified in terms of number and type of resorption pits and total area of resorption. Microspheres containing 5.1% sCT released 70% peptide in 2 weeks and 88% in 4 weeks. All sCT treatments inhibited total resorptive activity. A dose-dependent decrease in resorption was observed with sCT microspheres at 2 weeks. The high dose (10 mg of microspheres) produced a 99.5% decrease in resorption at 3 weeks, while the low dose (1 mg) produced an 80% reduction. Exposure of cultures to soluble sCT and sCT-loaded microspheres caused a decrease in the number of large pits, which were the predominant type formed in control cultures. Thus, this system could serve as an in vitro method to evaluate the antiresorptive effect of PLGA-sCT microspheres.

KEYWORDS: Salmon calcitonin (sCT), poly (lactide-co-glycolide) polymer (PLGA) microspheres, cell culture, osteoclasts, bone resorption.

INTRODUCTION Salmon calcitonin (sCT) is an antiresorptive agent approved by the Food and Drug Administration (FDA) for the treatment of established osteoporosis. sCT inhibits bone resorption by reducing the activity, motility, and rate of formation of osteoclasts [1-5]. It is currently formulated as a sterile solution for intramuscular or subcutaneous injection or as a nasal spray. Being a polypeptide, sCT is subjected to digestive degradation and has a very short half-life in the body; approximately 15 to 20 minutes following parenteral administration [6]. These properties represent drawbacks for the long-term treatment of osteoporosis, especially when a daily injection regimen of sCT is required that may severely limit patient compliance. Hence, there is a therapeutic advantage in developing sustained-release delivery systems that can effectively deliver sCT. The use of biodegradable polymers, such as poly (D,L-lactic-co-glycolic acid) (PLGA), has been very popular among researchers in developing sustained-release delivery systems for sCT. Although there have been some studies evaluating PLGA delivery systems containing sCT, with respect to sustained hypocalcemic effect and sustained serum sCT levels [7,8], surprisingly, the pharmacological (antiresorptive) effect of such systems has not been investigated in vivo, presumably because in vivo evaluation is very tedious and expensive. Hence, simple, inexpensive, and reliable in vitro assay methods would be helpful in evaluating the antiresorptive effect of PLGA delivery systems containing sCT.

A bone marrow culture system was used previously to investigate the effects of metal ions on the formation of osteoclastic cells from hematopoietic precursors in vitro [9]. The purpose of the present study was to utilize this system to evaluate the antiresorptive effect of biodegradable PLGA microspheres (ms) containing sCT.

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MATERIALS AND METHODS

Materials

Salmon calcitonin (sCT, MW. 3431.9, >99% peptide purity) was purchased from Bachem, Inc (Torrance, CA). 50:50 Poly(d,l-lactide-co-glycolide) (PLGA) RG502H polymer (Lot # 34035, MW. 7800) was obtained from

Boehringer Ingelheim, Inc (Ingelheim, Germany) and was used for microsphere preparation. Polyvinyl alcohol (PVA) (average molecular weight 30 000-70 000 daltons) was obtained from Sigma Chemical Co (St Louis, MO). The solvents and other excipients were analytical grade and were purchased from commercial sources. Female Sprague-Dawley rats, 90 days old and weighing approximately 250 gm, were purchased from Harlan Labs (Indianapolis, IN), and cells were harvested at the University of Kentucky Animal Research Facility in accordance with Institutional guidelines.

Methods

Microsphere Preparation

Salmon calcitonin microspheres were prepared by a dispersion method followed by solvent extraction and evaporation [10]. Briefly, a solution of peptide in methanol (CH_3OH) was combined with a solution of PLGA in methylene chloride (CH_2 Cl_2) and stirred until clear, then slowly injected into a 1-L reactor with baffles (Ace Glass Inc, Vineland, NJ) containing the continuous phase (CP, 0.35% [wt/vol] solution of PVA, pH 7.2) and stirred at 3500 rpm with a Silverson L4R homogenizer (Silverson Machines Ltd, Buckinghamshire, England). The temperature of the reactor was maintained initially at 25ºC for 30 minutes and then at 35ºC for 3 hours. Once the ms were formed and hardened, the contents of the reactor were transferred to a filtration apparatus equipped with a 0.8-µm membrane filter (Gelman Sciences, Ann Arbor, MI), and the filtered product was rinsed with water and then dried under reduced pressure for 48 hours at room temperature. Blank ms, without sCT, were prepared similarly.

Microsphere Characterization

sCT content within the ms was determined after dissolving the ms in methylene chloride and extracting the peptide with 0.1M acetate buffer, pH 4.0. The efficiency of the extraction method was determined by extracting known amounts of intact sCT from PLGA and was determined to be >95%. The peptide was analyzed by reversed-phase (RP)-High Performance Liquid Chromatography (HPLC) using 2 LC-6A pumps, an SIL-6B autoinjector, an SPD-6AV detector, an SCL-6B system controller (all from Shimadzu Scientific Instruments, Inc, Columbia, MA), and a Bondclone 10, C-18 reversed-phase column (150 X 3.90 mm) with a Bondclone 10, C-18 (30 X 3.90 mm) guard column (Phenomenex, Torrance, CA). Gradient elution was necessary to identify and quantify intact sCT recovered from the ms. The elution was accomplished using 0.1% trifluoroacetic acid in water (A) and acetonitrile, 0.1% trifluoroacetic acid (B), and by increasing the amount of phase B from 30% to 50% over 10 minutes at a flow rate of 1.5 mL/min. Standard curves of intact sCT ranging from 6.25 to 200 µg/mL yielded linear responses over that concentration range with detection at 220 nm. Particle size distribution was determined by laser diffractometry (Malvern 2600c Particle Sizer, Malvern, UK). Surface morphology was analyzed by scanning electron microscopy (SEM) with a Hitachi S 800 instrument (Tokyo, Japan) after palladium/gold coating on aluminum stubs. Total product yield was assessed gravimetrically on the basis of polymer/drug recovery.

Cell Culture

A bone marrow culture system was used to investigate the effects of sCT microspheres on bone resorption. Bone marrow cells were harvested from juvenile (3 months) female Sprague-Dawley rats and were cultured as described previously [9]. Briefly, the femora were removed and cleaned of soft tissues, cut through their epiphyses, and flushed using a 22G needle. Bone marrow cells were cultured in minimum essential medium, a modification (a MEM; Gibco BRL, Grand Island, NY) containing 15% fetal bovine serum (Gibco), 10^{-10} M $1,25(OH)_2$ vitamin D_3 (Calbiochem, San Diego, CA), 50 U/mL penicillin G, 50 µg/mL streptomycin, and 1 µg/mL Fungizone.

Cells were inoculated, at a density of 500 000 cells/cm^2, directly onto 8 X 8 mm wafers of devitalized bovine cortical bone contained in 24-well tissue culture plates (Costar Corp, Cambridge, MA). The bone wafers were prepared as described previously [11]. The static cultures were maintained in a 37ºC, humidified, 5% CO_2 balance air incubator. Twice per week for 4 weeks, one half of the medium was removed and replaced with fresh medium. The total volume of medium in each well was 2 mL.

Free sCT was added to the cell cultures twice per week for 4 weeks. Microspheres were added using Transwell inserts (3 µm pore size; Costar Corp, Cambridge, MA). The inserts allowed the released sCT to interact with the cells, but the membrane prevented direct contact between the cells and the ms. All treatments were initiated after 1 week of culture. Cells were exposed to the following treatments (n = 4):

No sCT, no ms (negative control)
10 nM (0.03 µg/mL) free sCT (positive control 1)
250 nM (0.86 µg/mL) free sCT (positive control 2)
1 mg of blank ms
1 mg of blank ms plus 10 nM free sCT (0.03 µg/mL)
1 mg of blank ms plus 250 nM free sCT (0.86 µg/mL)
1 mg of sCT ms (51 µg sCT, low dose)
10 mg of sCT ms (510 µg sCT, high dose)
1 mg of sCT ms (51 µg sCT) given twice (double dose)

Samples were collected weekly for analysis of sCT release, cell growth, and resorption.

In vitro release from sCT ms

The release of sCT from sCT ms was studied by recovering the ms at the end of each treatment week and analyzing for the amount of intact sCT present in the ms. The amount of intact sCT present in ms was determined by RP-HPLC after dissolving the ms in methylene chloride and extracting the peptide with 0.1M acetate buffer, pH 4.0. The efficiency of the extraction

method was determined by extracting known amounts of intact sCT from PLGA and was determined to be >95%. In vitro release was calculated by subtracting the amount of intact sCT recovered at each time point from the initial amount of intact sCT present in the microspheres before culture. Previous studies had demonstrated that sCT that was encapsulated into PLGA ms remained intact, retaining its molecular structure [10]. sCT concentration in the culture medium (supernatant) under the conditions of our study was not measured since such a measurement was very difficult (and probably far less accurate) because of the presence of bovine serum proteins (the medium contained 15% fetal bovine serum). The HPLC method used in this study was very powerful in detecting intact sCT and separating it from degradation peaks. This method has been used previously to evaluate the stability of intact sCT at different pH [10].

Cell Growth

Cell growth during the entire treatment period was studied by monitoring the total intracellular protein content. Cell lysate was obtained by adding deionized water to cell cultures at the end of each treatment week followed by 3 cycles of freezing and thawing. Total protein of cell lysates was determined using the micro BCA (bicinchoninic acid) protein assay (Pierce Chemical Co, Rockford, IL). This assay is a modified Biuret assay involving incubation of an aliquot of unknown sample with working reagent in a 37ºC incubator for 90 minutes. Protein concentrations were quantified spectrophotometrically by reading absorbances at 570 nm and comparing these values with standard curves prepared with albumin. Previous work in our laboratories has shown that use of total intracellular protein as an indicator of cell growth corresponded well to other assays, such as tetrazolium and methylene blue assays [12].

Resorption Pits

Formation of resorption pits by osteoclastic cells during the 4-week culture period was evaluated using methods reported previously [9]. Briefly, cell layers were removed from bone wafers by ultrasonication in 0.25M NH_4OH for 3 minutes. Bone samples were then stained with 1% toluidine blue in 1% sodium borate for 15 minutes, and resorption features detected in light microscopic images were quantified using semi-automated image analysis. Images were obtained directly from an Olympus BH-2 microscope equipped with a Sony video camera, transferred to a Digital AXP 3000-300x UNIX workstation, and analyzed using QuantIm image processing software (Zedec Technologies, Burlington, NC). The entire surface of the bone wafers was analyzed, and the pits were classified according to their size (planar surface area). Type A pits were based on the equivalent diameter of mononuclear cells of approximately 15 µm; type B pits were selected to approximate a multinucleated cell with a diameter of less than 30 µm; and type C pits represented composite pits formed by clusters of type B pits in variable configurations. Calibration of dimensions was accomplished using a stage micrometer.

Statistical Analysis

Results for total protein and resorptive activity were first analyzed for statistical significance using 1-way ANOVA. When significant differences were obtained ($P<0.05$), data at each time point was subjected to 1-way ANOVA followed by post hoc comparison using Scheffe's test.

RESULTS

Microsphere Characterization

Blank and sCT ms were prepared with a particle size distribution in the range of 17 to 89 µm and mean particle size of 48 and 53 µm, respectively. sCT content of the microspheres was 5.1% wt/wt. Examination of the surface morphology by SEM revealed that both blank and sCT ms were essentially free of discernible surface pores.

In vitro release from sCT ms

Figure 1 shows the in vitro release of sCT from sCT ms. Seventy percent of the encapsulated sCT was released at the end of treatment week 2 and 90% was released at the end of week 3. The release was complete at the end of week 4, when 98% of the encapsulated sCT had been released. This confirmed that most of the encapsulated sCT had been released over the duration of the study.

Cell Growth

A similar pattern of intracellular protein contents (reflecting cell number) was observed for all treatments. As shown in **Figure 2**, although all cultures exhibited decreased protein levels between weeks 1 and 2, levels then increased or remained constant through the end of

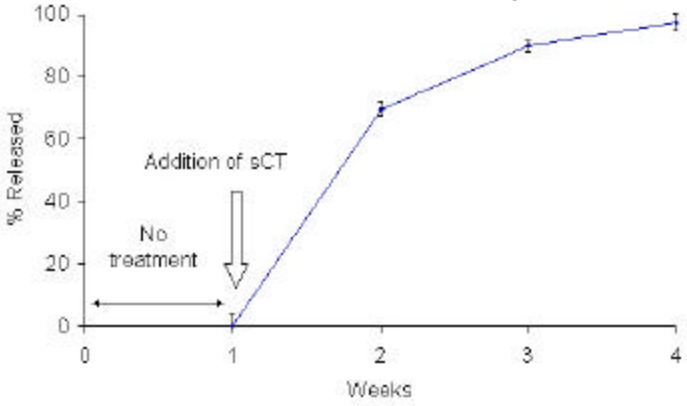

Figure 1. In vitro release from sCT ms in culture medium at 37°C (pH 7.4). Cell cultures were incubated with the culture medium for 1 week. At the end of week 1, sCT ms were added and % sCT released was measured at the end of weeks 2, 3, and 4. Data are mean ± SD (n=4).

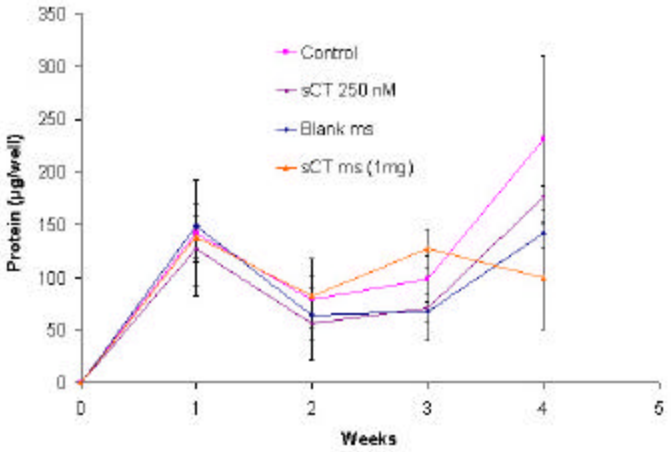

Figure 2. Representative results of total intracellular protein, reflecting cell growth, in bone marrow cell cultures. Cell cultures were incubated with the culture medium for 1 week and treatments were initiated at the end of week 1. Data are mean ± SD (n=4).

week 4. All treatments resulted in statistically similar cell growth.

Resorption

Results for resorptive activity were compared using 2 measures: (1) the total area of resorbed bone surface (expressed as percent resorption compared with control cultures) and (2) the number and type of pits formed.

As expected, the area of resorption was low at the end of week 2 (0.3 mm ^{2}). As the cells acquired resorptive ability, the area of resorption increased to 1.0 mm ^{2} at the end of 3 weeks and to 1.3 mm ^{2} at the end of week 4. Addition of free sCT at 10 and 250 nM caused a 60% to 90% decrease in the total amount of resorption (**Figure 3**). At the end of 4 weeks, the decrease was significantly different from control cultures for 10-nM concentration. A dose-dependent decrease was not observed at the 2 sCT concentrations studied.

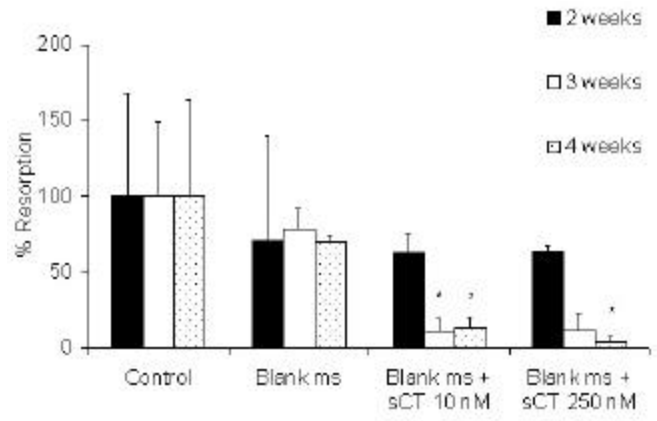

Figure 4. Percent resorption (compared with controls) in bone marrow cell cultures treated with blank ms either alone or with 10 and 250 nM of free sCT. * Significantly different from control cultures (P < .05). Cell cultures were incubated with the culture medium for 1 week and treatments were initiated at the end of week 1. Data are mean ± SD (n=4).

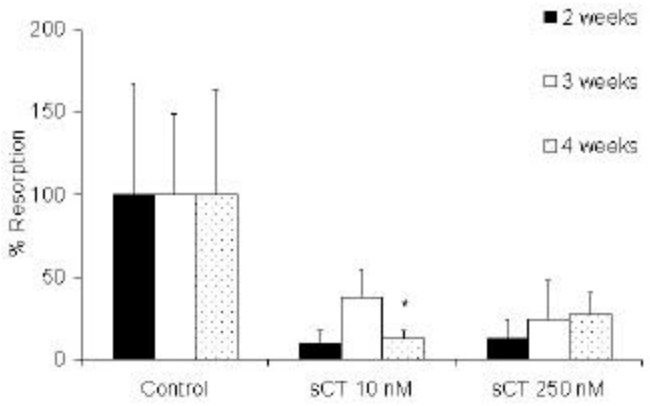

Figure 3. Percent resorption (compared with controls) in bone marrow cell cultures treated with 10 and 250 nM of free sCT. * Significantly different from control cultures (P < .05). Cell cultures were incubated with the culture medium for 1 week and treatments were initiated at the end of week 1. Data are mean ± SD (n=4).

As seen in **Figure 4**, at the end of 2 weeks, addition of free sCT (at 10 and 250 nM) along with blank ms (1 mg) did not decrease the total amount of resorption as much as with the addition of free sCT alone. The insignificant decrease in total amount of resorption was similar to that with cultures exposed to blank ms alone. At the end of weeks 3 and 4, however, the decrease in the total amount of resorption at both concentrations was either comparable with or greater than that with free sCT alone. The decrease in resorption was significantly different from control cultures for 10-nM concentration at the end of weeks 3 and 4 and for 250-nM concentration at the end of week 4. At the end of weeks 2, 3, and 4, the total amount of resorption after exposure to blank ms was not statistically different from control cultures.

Exposure to sCT ms decreased the total amount of resorption at the end of weeks 2, 3, and 4 (**Figure 5**). A dose-dependent decrease was observed at the end of week 3, meaning the decrease (99.5%) in resorption with the high dose was not only significantly different from control cultures but also lower than with the low dose (80%). At the end of week 4, both doses resulted in significantly decreased total resorption compared with control cultures (85% with the low dose and 99.5% with the high dose). The decrease in total amount of resorption through 4 weeks after treatment with the double dose was similar to treatment with the single low dose.

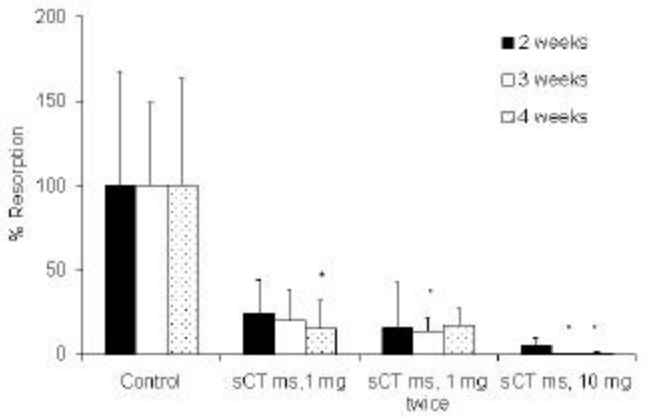

Figure 5. Percent resorption (compared with controls) in bone marrow cell cultures treated with sCT ms. * Significantly different from control cultures ($P < .05$). Cell cultures were incubated with the culture medium for 1 week and treatments were initiated at the end of week 1. Data are mean ± SD (n=4).

Bone marrow cell cultures subjected to different sCT treatments were also analyzed for the number and type of resorption pits (**Figure 6 a, b, c**). Although all 3 types of pits were observed, type C pits were the predominant type found in control cultures, with type A and B pits being observed less frequently. The number and type of pits after exposure to blank ms through 4 weeks was similar to control cultures. Addition of free sCT at 10 and 250 nM decreased the total number of pits through 4 weeks. At the end of week 2, addition of free sCT (at 10 and 250 nM) along with blank ms, resulted in pits with type and number similar to that in control cultures. At the end of weeks 3 and 4, however, the pit number decreased, similar to that found after addition of free sCT alone at the 2 concentrations. Exposure to sCT ms also decreased the total number of pits through 4 weeks. The decrease was more prominent with increasing sCT ms dose (from 1 to 10 mg). Also, at the high dose of sCT ms, there was a slight shift from larger type C pits to smaller type A and B pits.

DISCUSSION The results of this study demonstrate that salmon calcitonin microspheres decreased resorptive activity of cultured osteoclastic cells. Hematopoietic stem cells of bone marrow can advance along multiple differentiation pathways, one of which gives rise to the osteoclastic lineage [13], thereby permitting investigation of the effects of various factors on the formation and function of osteoclastic cells. The present model also allows interaction between different cell types in bone marrow, most importantly stromal cells, which are critically important for differentiation of osteoclasts. Therefore, this is a useful model for evaluating antiresorptive sustained-release delivery systems.

The total intracellular protein assay, which reflects cell growth, showed that the cells in control as well as treated cultures proliferated during the treatment period. This confirmed the viability of the bone marrow cells throughout the treatment period. The decrease observed between weeks 1 and 2 likely reflects disturbance of the cultures when the Transwell inserts were introduced. The fact that decrease was observed even in control cultures suggests that it was an artifact of introducing the inserts. This effect was transient, however, and the cells readily recovered.

All sCT treatments, except the addition of free sCT along with blank ms at the end of 2 weeks, inhibited total resorptive activity. At the end of 2 weeks, addition of free sCT at the 2 concentrations along with blank ms did not decrease the total amount of resorption as much as that with the addition of free sCT at 10 and 250 nM. Previous adsorption studies of sCT to blank ms had shown that as much as 20 µg of sCT was bound to 1 mg of blank ms at pH 7.4 (pH of the culture medium) [10]. Therefore, at concentrations of 10 nM (0.03 µg sCT/mL) and 250 nM (0.86 µg sCT/mL) almost all of the sCT should be adsorbed to 1 mg of blank ms (at the end of week 2) leaving none or very little available for binding to cells. Degradation of blank ms prepared using PLGA RG502H polymer (MW 7800), because of hydrolytic cleavage, is expected to be complete within 21 days. So, as the blank ms degraded with time (at the end of weeks 3 and 4), the bound sCT would have been released and would then be available to inhibit resorption. Hence, the decrease in total amount of resorption at the end of weeks 3 and 4 was either comparable with or greater than that with free sCT alone.

Interestingly, a dose-dependent response was not observed at the 2 sCT concentrations that were added either alone or along with blank ms. In contrast, Sugawara et al reported a dose-dependent effect between 10 and 100 nM sCT [14]. The different culture models used may at least partially explain the differences. Whereas only bone marrow cells were used in the present work, Sugawara et al used cocultures of bone marrow and calvaria-derived osteoblastic cells. Furthermore, free sCT, either alone or along with blank ms, was added to the cultures only twice a week for 4 weeks. Previous studies at pH 7.4, 37°C had shown that almost 37% of sCT degraded within 24 hours and 100% by 3 days [10]. Consequently, the frequency of addition of free sCT was less than optimal and could have resulted in the lack of dose response. However, it should be emphasized that even the addition of 10 nM of free sCT alone, only twice a week for 4 weeks, was enough to significantly decrease (inhibit) the total amount of resorption at the end of 4 weeks as compared with control cultures.

A dose-dependent decrease in resorption was observed with sCT ms (1 and 10 mg) through 4 weeks, confirming that the sCT ms were efficacious in inhibiting resorption throughout the culture period. In vitro release studies

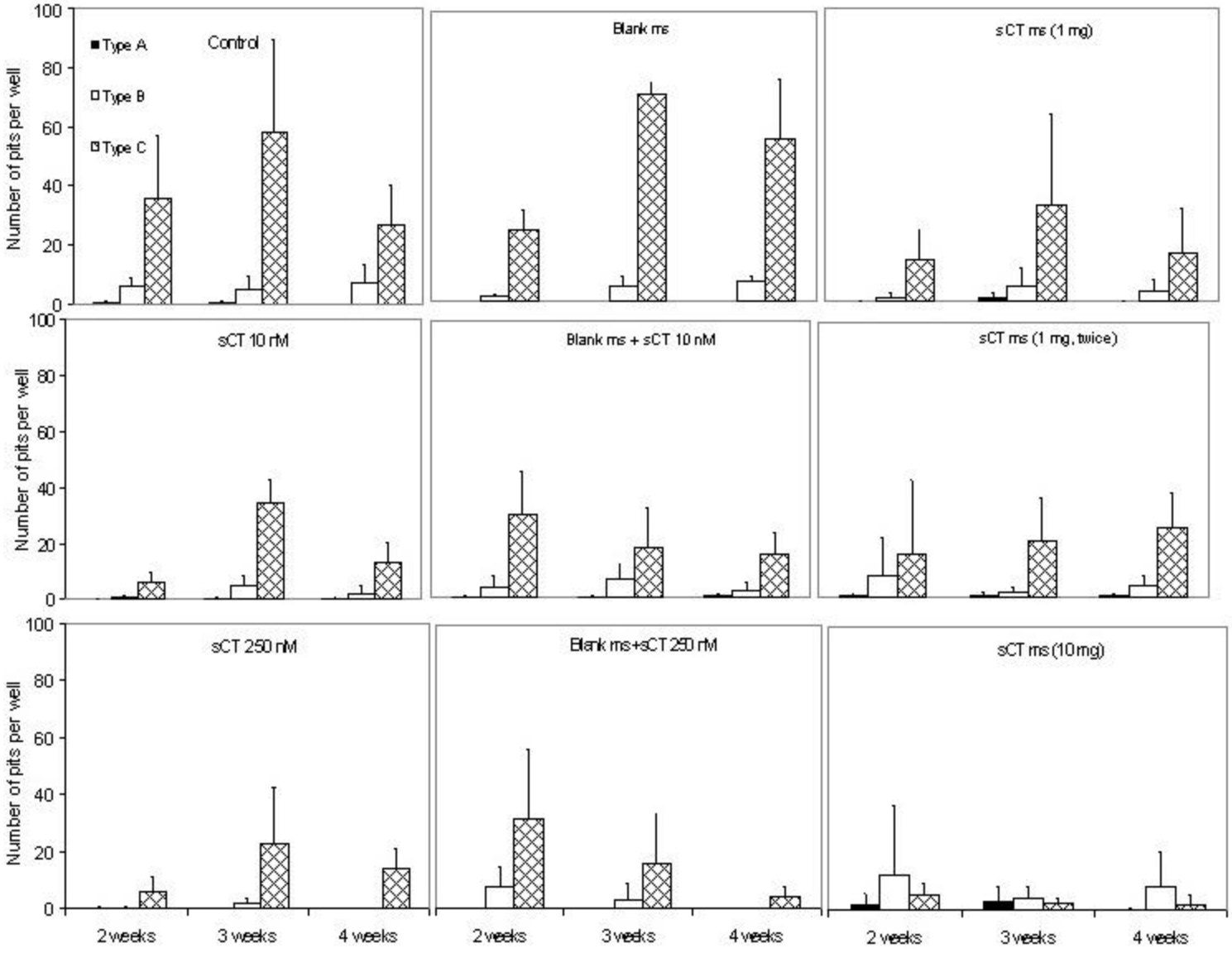

Figure 6. Number of pits per well in control and treated bone marrow cell cultures. Cell cultures were incubated with the culture medium for 1 week and treatments were initiated at the end of week 1. Data are mean ± SD (n=4).

confirmed that most of the encapsulated sCT had been released over the duration of the study, which caused suppression of resorption at all time points examined. The 1-mg sCT ms dose (at 5.1% loading) contained 51 µg of sCT that was released over 21 days (3 weeks), approximately at an average daily rate of 2.4 µg/day. This translated to an sCT concentration of approximately 1.2 µg/mL in each well (total volume of medium within each well was 2 mL) and was roughly comparable with an sCT concentration of 0.86 µg/mL in the 250 nM control. A 10-fold higher sCT dose (10 mg) in the sCT ms group was included to study the dose-dependency of inhibition of resorption due to sCT ms treatment. An exact dose-matching would be almost impossible to achieve with a continuous-release system.

Also, the data presented in this manuscript clearly demonstrates that the sCT, which is released from the PLGA ms, is capable of inhibiting bone resorption in a dose-dependent manner. This is evidence that the sCT, which is released from the ms, is intact and bioactive. Given the limited stability of unencapsulated sCT in the culture medium, once it is released from the ms, it is expected to degrade over time. However, the data clearly show that there is enough time for intact sCT exposure to cultured osteoclastic cells to effect inhibition of bone resorption. This is confirmed further by the recovery of intact sCT, which was encapsulated within the ms, during the in vitro release studies at the end of weeks 2, 3, and 4.

For control cultures, resorption pits were observed starting at week 2. As expected, the pits grew in number from week 2 to 3, but decreased at the end of week 4. In light of the greater amount of resorbed area, this decrease reflects a lower number of larger pits resulting from the merging of smaller pits. Differentiation of osteoclast progenitors into osteoclasts capable of resorption in vitro is thought to take approximately 17 to 18 days: active proliferation of active osteoclast progenitors through day 15; acquisition of tartrate-resistant acid phosphatase activity by mononucleated pre-osteoclasts by day 17; and formation of mutinucleated osteoclasts at day 18 [15]. Thus, the appearance of resorption features in this model roughly corresponded to the differentiation time course of osteoclasts. As described for a study of avian osteoclastic cells [16], type A pits are probably formed as mononuclear osteoclast progenitors begin to develop resorption capability. Type B pits are formed from larger cells further along the differentiation pathway resulting from mutinucleated cells formed by the fusion of 2 or 3 mononuclear cells. Type C pits are formed by motile bone-resorbing osteoclastic cells, which move along the bone surface forming large trails or areas of resorption.

In general, exposure of bone marrow cultures to sCT treatments caused a decrease in the number of resorption pits formed (except at the end of 2 weeks after addition of free sCT at 10 and 250 nM along with blank ms). The measured decrease in the number of pits, especially large type C pits, explains the reduction in the total amount of resorption with cultures exposed to sCT treatments. This observation reflects the known mechanisms of calcitonin action, which are to decrease the activity and mobility of osteoclasts [1-5].

CONCLUSION sCT ms were capable of inhibiting bone resorption in a dose-dependent manner in cultured osteoclastic cells. Blank ms did not significantly affect bone resorption. Cultured osteoclastic cells could serve as a simple and inexpensive in vitro method to evaluate the antiresorptive effect of PLGA delivery systems containing antiresorptive agents, such as salmon calcitonin.

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