Sustained and Localized In Vitro Release of BMP-2/7, RANKL, and Tetracycline from FlexBone, an Elastomeric Osteoconductive Bone Substitute

Jianwen Xu,[1,2] Xinning Li,[1] Jane B. Lian,[1,2] David C. Ayers,[1] Jie Song[1,2]

[1]Department of Orthopedics, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, Massachusetts 01655, [2]Department of Cell Biology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, Massachusetts 01655

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ABSTRACT: We tested the hypothesis that synthetic composites containing a high percentage of osteoconductive biominerals well-integrated with a hydrophilic polymer matrix can be engineered to provide both the structural and biochemical framework of a viable synthetic bone substitute. FlexBone, an elastic hydrogel-mineral composite exhibiting excellent structural integration was prepared by crosslinking poly(2-hydroxyethyl methacrylate) hydrogel in the presence of 25 wt% nanocrystalline hydroxyapatite and 25 wt% tricalcium phosphate. Biologically active factors tetracycline, BMP-2/7, and RANKL that stimulate bone formation and remodeling were encapsulated into FlexBone during polymerization or via postpolymerization adsorption. SEM and dynamic mechanical analyses showed that the encapsulation of tetracycline (5.0 wt%) did not compromise the structural integrity and compressive behavior of FlexBone, which could withstand repetitive megapascal-compressive loadings and be securely press-fitted into critical femoral defects. Dose-dependent, sustained in vitro release of tetracycline was characterized by spectroscopy and bacterial inhibition. A single dose of 40 ng BMP-2/7 or 10 ng RANKL pre-encapsulated with 50 mg FlexBone, released over 1 week, was able to induce local osteogenic differentiation of myoblast C2C12 cells and osteoclastogenesis of macrophage RAW264.7 cells, respectively. With a bonelike structural composition, useful surgical handling characteristics, and tunable biochemical microenvironment, FlexBone provides an exciting opportunity for the treatment of hard-to-heal skeletal defects with minimal systemic side effects. © 2009 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 27:1306–1311, 2009

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Synthetic bone substitutes are attractive alternatives to autografts and allografts in treating volumetric bone loss, as they have the potential to reduce patient morbidity and overcome the inadequate supply and high failure rates associated with traditional bone grafts.[1] Bone is a structural composite of collagen matrix and apatite minerals. The content of biomineral and its integration with the organic matrix are essential in both determining the mechanical properties and defining the biochemical microenvironment of the calcified tissue.[2] Existing synthetic bone substitutes used in clinical practice are mostly weak gel foams, brittle ceramics, or a coarse combination of these components. With little resemblance to the structural or biochemical sophistication of natural bone, these materials often lead to long-term graft failure due to both unstable graft fixation and inadequate positive tissue-graft interactions.[3]

Inspired by natural bone, we recently developed a class of hydrogel-mineral composites with a high content of hydroxyapatite (HA), termed FlexBone.[4] Prepared by crosslinking hydrophilic poly(2-hydroxyethyl methacrylate) (pHEMA) hydrogel in the presence of nanocrystalline HA, the composite exhibits excellent resistance to repetitive moderate compressive stress under physiological conditions (37°C in water) without exhibiting brittle fractures. The elasticity enables convenient press-fitting of FlexBone into sites of segmental bony defect. When subcutaneously implanted in rats, the osteoconductive composite supports osteoblastic differentiation of preseeded bone marrow stromal cells.[4]

In this study, we tested the hypothesis that the excellent structural integration of the hydrogel and mineral components of FlexBone, coupled with the intrinsic affinity of apatite minerals for a range of biomolecules,[5,6] would enable straightforward encapsulation and sustained release of therapeutic agents. The strategy of locally delivering exogenous signaling molecules to elicit proper host cell responses and expedite skeletal repair has been explored with other synthetic bone substitutes.[7] The poor integration of soluble factors with most biomaterial carriers, however, has limited the success of retaining and releasing these molecules in a confined environment over a biologically relevant time period.[8] Consequently, therapeutic agents such as bone morphogenetic proteins (BMPs) need to be incorporated with carriers such as collagen meshes and biodegradable polylactides in doses far exceeding their physiological concentrations.[9,10] Systemic side effects of the quickly diffused BMPs could outweigh the benefit of the local treatment in many cases.

In this study, we examined the in vitro release of BMP-2/7 heterodimer, receptor activator of nuclear factor κB ligand (RANKL), and tetracycline pre-encapsulated with FlexBone. BMP-2 promotes osteogenesis and initiates fracture repair, and has been clinically used as an adjuvant for spinal fusion and fracture union. We chose BMP-2/7 heterodimer, a more potent osteogenic recombinant protein,[11] to augment the osteointegration potential of FlexBone. In parallel, we used RANKL for potential modulation of in vivo remodeling of FlexBone. In combination with vascular

Additional Supporting Information may be found in the online version of this article.

Correspondence to: Jie Song (T: 508-334-7168; F: 508-334-2770; E-mail: jie.song@umassmed.edu)

endothelial growth factor (VEGF), RANKL has recently been suggested to cause revitalization of processed cortical bone.[12] Tetracycline was chosen as a model therapeutic for not only its broad spectrum antibiotic activity and high affinity for calcified matrices, but also its capacity to reduce pathological bone resorption via matrix metaloproteinase (MMP) inhibition[13] and enhanced bone formation.[14,15]

MATERIALS AND METHODS

Materials

HEMA and ethylene glycol dimethacrylate (EGDMA) were purified via distillation and by passing through a 4 Å molecular sieve column prior to use, respectively. Porous micro-aggregates of HA nanocrystals (Alfa Aesar) and TCP (tricalcium phosphate) powder (Fluka) were used as received. Human recombinant protein BMP-2/7 heterodimer and murine recombinant protein RANKL were purchased from R&D Systems. Tetracycline hydrochloride (>95%) and all histochemical reagents were purchased from Sigma.

Preparation and Mechanical Testing of Tetracycline-Containing FlexBone and pHEMA

Cylindrical FlexBone (50 wt% mineral content, 1:1 HA/TCP) and pHEMA hydrogel samples ($\Phi = 3.2$ or 4.8 mm) containing 0–5.0 wt% tetracycline were prepared using a recently developed protocol[4] (see Supplementary Information). Freshly prepared samples were used for tetracycline release kinetics studies and bacterial inhibition assays, whereas fully hydrated samples (subject to 24 h wash with water to remove residue monomer and radical initiators) were used for mechanical testing and cell culture studies.

The compressive behavior of FlexBone as a function of tetracycline content was analyzed in force-controlled mode using a Q800 Dynamic Mechanical Analyzer in water at 37°C. The controlled force cycle (0.03-10.0-0.03 N; 3.0 N/min) was repeated 10 times for each specimen ($n = 3$). Sample dimensions and testing parameters are described in Supplementary Information.

Tetracycline Release Kinetics from FlexBone versus from pHEMA

Each freshly prepared specimen ($\Phi = 4.8$ mm; $H = 5.0$ mm) was placed in MilliQ water at a 100:1 water-to-sample mass ratio without agitation and incubated at 37°C for 1 week. The release of tetracycline was monitored by UV-vis spectroscopy at 357.9 nm. Three specimens were examined for each composition. The quantification of tetracycline hydrochloride (TCH) release (cumulative percentage release) from FlexBone or pHEMA over time was calibrated by a standard tetracycline concentration-absorption curve.

Bacterial Inhibition by the Tetracycline Released from FlexBone

The antibiotic activity of the released tetracycline was evaluated by its ability to inhibit *E. coli* culture. Warm Luria Broth (LB) (25 g/L)-agar (15 g/L) solution was poured into P-150 cell culture dishes (35 mL/plate) and cooled to room temperature. The surfaces of the LB-agar plates were coated with 250 μL *E. coli* XL-2 solution ($OD_{600\ nm} = 0.256$) with glass beads and cultured at 37°C for 10 min before thin discs ($\Phi = 4.8$ mm; $H = 2.0$ mm) of FlexBone containing tetracycline were placed on the surface (six discs per plate). The *E. coli* culture was continued at 37°C, and the diameters of the clear zones developed surrounding the discs were monitored at 80 min, 160 min, 4, 8, 16, 21, 24, 28, 32, 40, and 48 h. Three specimens were examined for each time point. The diameters of the clear zones (average ± SD) as a function of time were plotted.

Loading of Recombinant Protein BMP-2/7 or RANKL to FlexBone and pHEMA

The recombinant protein solutions were reconstructed per vendor instructions. A 4 uL rhBMP-2 solution of predetermined concentration was applied to each 50 mu freeze-dried FlexBone carrier to yield the final loading doses of 20–80 ng/carrier. rmRANKL was loaded in a similar fashion to freeze-dried FlexBone or pHEMA control to give a 10–20 ng/carrier final loading dose.

Osteogenic Differentiation of Murine C2C12 Myoblastic Cells Induced by the rhBMP-2/7 Released from FlexBone

To establish the proper dose and optimal time required for rhBMP-2/7 to induce osteogenic differentiation of C2C12, cells ($5{,}000/cm^2$) were allowed to attach overnight in DMEM supplemented with 10% FBS (Hyclone) and 1% Pen-Strep before being switched to low mitogen media (DMEM, 5%; FBS, 1 mL/well; 24-well plate) supplemented with 20, 40, or 80 ng/mL BMP-2/7 ($n = 3$). The culture was continued for 2–5 days without media change or additional supplement of the growth factor before the cells were fixed and stained for ALP (alkaline phosphatase) using a Sigma Leukocyte Alkaline Phosphatase kit.

The bioactivity of the released BMP-2/7 was evaluated using the C2C12 culture model. Upon cell attachment, a FlexBone carrier freshly loaded with 20, 40, or 80 ng BMP-2/7 was placed in the low mitogen media (1 mL/well; 24-well plate; $n = 3$). In the first subset of the experiment, FlexBone/BMP-2/7 carrier was retrieved after 4 days, and the cells were stained for ALP. The carrier retrieved from the first subset of experiments was immediately placed in a new set of fresh C2C12 cultures, which were maintained in low mitogen media for another 3.5 days before the cells were fixed and stained for ALP. In parallel to histochemical staining, ALP activity in cell lysates was quantified using a commercial kit (see Supplementary Information).

Osteoclastic Differentiation of Murine Macrophage RAW264.7 Cells Induced by the Murine Recombinant RANKL Released from FlexBone

The release of RANKL was evaluated by RANKL-induced osteoclastic differentiation of murine macrophage RAW264.7 cells over 6 days. RAW264.7 cells ($10{,}000/cm^2$) were seeded in a 24-well plate in α-MEM (1 mL/well) supplemented with 10% FBS and 1% Pen-Strep and allowed to attach overnight. One FlexBone or pHEMA carrier freshly loaded with 10 ng RANKL was then added to each well ($n = 3$), and the culture was continued for 6 days with media change every 2 days without additional supplement of RANKL. In the positive control well, 10 ng RANKL was supplemented directly in the culture media every 2 days. In the negative control well, 10 ng RANKL was supplemented directly without a carrier, and the medium was changed every 2 days without any additional RANKL supplement. The culture was terminated on day 6, when multinucleated cells were observed in the positive control well and in those containing the FlexBone carrier. FlexBone or pHEMA carriers were removed before the cells were stained for tartrate-resistant acid phosphatase (TRAP) activities using a Sigma TRAP kit. Both the number of multinucleated TRAP-positive cells and their average number of nuclei were determined, and the RANKL released in the media over time

was quantified using a RANKL Quantikine ELISA kit (R&D systems) as described in Supplementary Information.

RESULTS

Impact of Tetracycline Encapsulation on FlexBone Compressive Behavior and Microstructure

Tetracycline-containing FlexBone was prepared by crosslinking hydrogel monomer HEMA with 2% crosslinker EGDMA in the presence of 25 wt% nanocrystalline HA, 25wt% TCP, and 0–5.0 wt% tetracycline. The more soluble TCP was incorporated along with HA to enhance the potential in vivo resorption rate of the mineral component of FlexBone. Whereas the stiffness of freshly prepared FlexBone varied slightly as tetracycline content increased from 0 to 5.0 wt% (data not shown), no substantial difference in compressive behavior of water-equilibrated samples was detected (Fig. 1A). All specimens repeatedly recovered from unconfined compressive strains of >25% (up to 0.6 MPa stress) in water as indicated by the good overlaps among the 10 consecutive loading/unloading curves. This elastic property enabled convenient and secure press-fitting of FlexBone into a 5 mm rat femoral segmental defect (Fig. 1B). Excellent overlaps of the stress-strain curves were also observed across samples containing varying amounts of tetracycline (Fig. 1A), suggesting that the tetracycline tightly bound to the HA/TCP matrix (the majority that retained after the 24 h equilibration of FlexBone in water; Fig. 2A) had minimal impact on the compressive behavior. SEM micrographs confirmed that the incorporation of up to 5.0 wt% tetracycline did not alter the distribution of HA and TCP within the elastic pHEMA matrix (Fig. 1C,D). Further, the microstructures of all composites recovered after being subjected to 10 consecutive 0.6 MPa compressive loading/unloading cycles (representatively shown in Fig. 1E,F), supporting the robust structural integration of HA/TCP with the elastic polymer matrix.

In Vitro Release of Tetracycline from FlexBone versus pHEMA

The in vitro release of tetracycline from FlexBone versus pHEMA hydrogel in water as a function of time and tetracycline incorporation dose was monitored by visible spectroscopy at 357.9 nm over 1 week. As shown in Figure 3A, FlexBone released tetracycline in a sustained and dose-dependent manner. For instance, FlexBone containing 0.5 wt% tetracycline released 5.7% of the drug in the first 8 h and ~10% by 7 days whereas FlexBone containing 5.0 wt% tetracycline released 10% of the encapsulated drug in the first 8 h and reached ~20% cumulative release by 7 days. In contrast, unmineralized pHEMA hydrogel quickly released 30% of tetracycline in the first 8 h, reaching >60% release by day 7, regardless of the initial tetracycline encapsulation contents. The substantially slower and dose-dependent release of tetracycline from FlexBone is presumably due to the strong chelating interaction between tetracycline and the calcium mineral component of FlexBone. Such release characteristics would allow controls over both the quantity of early stage drug release (by adjusting the initial encapsulation content) and the sustainability of the release when delivery over a longer period is desired.

The antibiotic activity of the tetracycline released from FlexBone was examined by its ability to inhibit bacterial culture. As shown in Figure 2B, clear zones surrounding the FlexBone placed over the surface of the *E. coli* agar plate were developed by 8 h and were sustained throughout the 2 day bacterial culture.

In Vitro Release of BMP-2/7 from FlexBone

BMP-2/7 heterodimer, known for its more potent osteogenicity than either BMP-2 or BMP-7 homodimer,[16] was chosen as an osteogenic growth factor to promote the in vivo osteointegration of FlexBone upon implantation. BMP-2 induces osteogenic differentiation of C2C12 cells in a dose- and time-dependent fashion, with the expression of osteogenic markers ALP and osteocalcin in C2C12 culture stably established after 3 days of induction by 300 ng/mL rhBMP-2.[17] Using the same culture model, we showed that upon the supplement of a single dose of as low as 40 ng/mL BMP-2/7, osteogenic differentiation of C2C12 cells was induced in 2 days, with the expression of ALP peaking by day 4 (Fig. 3A–C). This observation confirmed the more

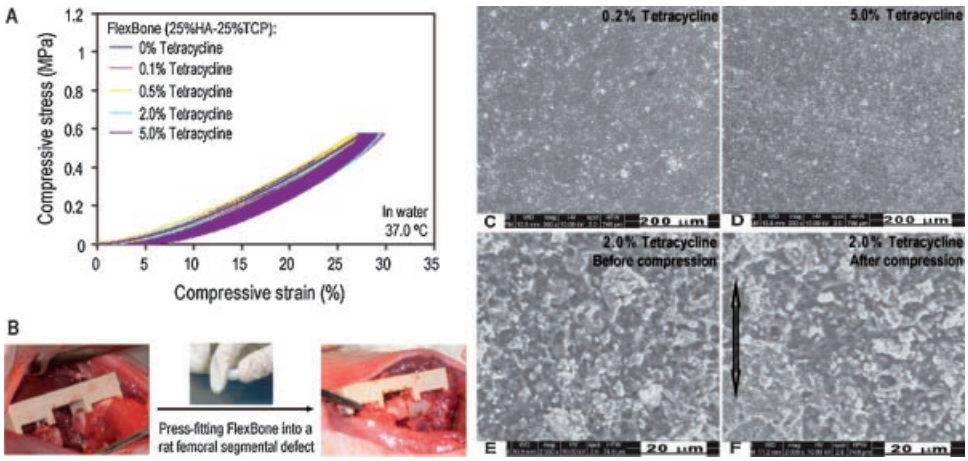

Figure 1. Tetracycline incorporation did not compromise the elasticity and structural integrity of FlexBone. (A) Unconfined compressive behavior of fully hydrated FlexBone as a function of tetracycline content. (B) Elasticity of a fully hydrated FlexBone facilitated its convenient and secure press-fitting into a femoral segmental defect. (C,D) SEM micrographs of FlexBone containing 0.2 and 5.0 wt% tetracycline. (E,F) SEM micrographs of the cross-sections of FlexBone (2.0 wt% tetracycline) before and after 10 consecutive loading-unloading cycles.

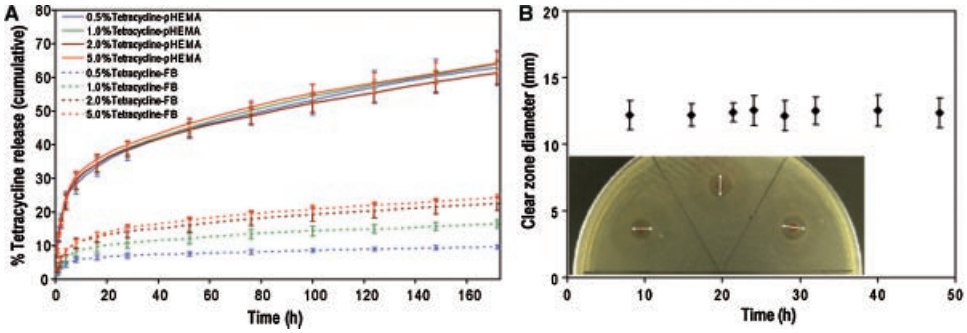

Figure 2. FlexBone released pre-encapsulated tetracycline in a dose-dependent and sustained manner with the retention of its bacterial inhibition activity. (A) Release kinetics of pre-encapsulated tetracycline from FlexBone and pHEMA hydrogel. (B) The development of clear zones in *E. coli* culture surrounding tetracycline-containing FlexBone samples over time.

potent osteogenicity of BMP-2/7. When the same dose of BMP-2/7 (40 ng) was absorbed on a 50 mg FlexBone and placed in C2C12 culture (one carrier/well), osteoblastic differentiation of C2C12 cells was again induced in 2 days and peaked at 4 days, but the detection of ALP activity was confined around the FlexBone carrier (Fig. 3D,E). This suggests that BMP-2/7 release from FlexBone was achieved in a highly localized fashion with the retention of its bioactivity, a property desired for local therapy via the delivery of protein therapeutics. In another subset of experiments, we demonstrated that FlexBone released BMP-2/7 in a sustained manner over 1 week, with sufficient amount of the 40 ng pre-encapsulated proteins released between day 4 and 7.5 to induce osteogenic differentiation of a fresh C2C12 culture (Fig. 3F). Quantification of the ALP activity in cell lysates (Fig. 3G) supported the histochemical observation. These data suggest that FlexBone is a suitable carrier for localized delivery of the osteogenic factor in a clinically relevant time frame (first 3–7 days) to expedite skeletal repair.[18,19]

In Vitro Release of RANKL from FlexBone

With the long term goal of modulating the in vivo remodeling of FlexBone via the delivery of RANKL, we investigated the release of RANKL using RANKL-induced osteoclastogenesis of murine macrophage RAW264.7[20] as culture model. To induce the osteoclast differentiation of RAW264.7 effectively, supplement of sufficient amount of RANKL over a 5 to 7 day period is required. Supplement of a single dose of 10 ng murine recombinant RANKL directly to the RAW267.4 culture was insufficient to induce osteoclast differentiation (Fig. 4D), while the continued supplement of 10 ng RANKL every other day led to the formation of TRAP-positive multinucleated (9 ± 5 nuclei) osteoclasts by day 6 (Fig. 4C). When the 50 mg FlexBone carrier preabsorbed with 10 ng RANKL was placed in culture, however, osteoclast differentiation was observed by day 6 without any additional supplement of RANKL (Fig. 4A,E), leading to the formation of TRAP-positive multinucleated (6 ± 2 nuclei) cells. This observation suggests that FlexBone could release RANKL in a sustained manner over 6 days. In contrast, when the unmineralized pHEMA hydrogel preabsorbed with the same amount of RANKL was placed in culture, little osteoclastogenesis was observed by day 6 (Fig. 4B,E). Quantification of the RANKL release (Fig. 4F) revealed that while the unmineralized hydrogel matrix rapidly released most of its absorbed RANKL (>98% release in the first 2 days), FlexBone still retained ~30% RANKL by day 6. The HA/TCP component of FlexBone likely played an important role in achieving the balance between sequestering and releasing RANKL over a 6 day period.

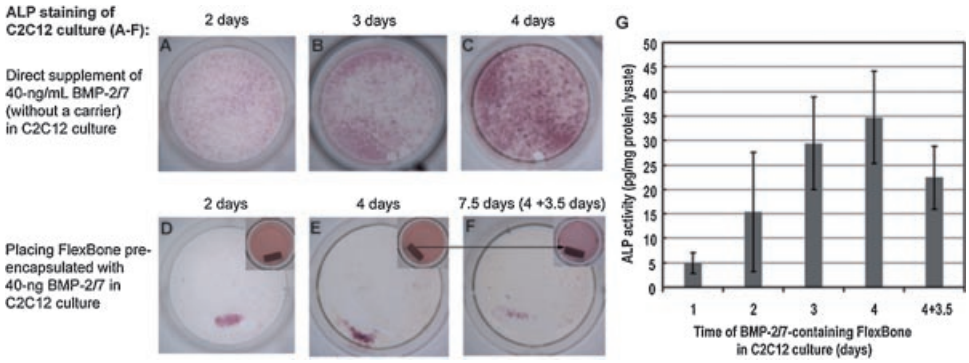

Figure 3. FlexBone released rhBMP-2/7 over a 7 day period and induced localized osteogenic differentiation of C2C12 cells. (A–C) ALP staining 2–4 days after direct supplement of 40 ng/mL BMP-2/7. (D–F) ALP staining 2 and 4 days after placing a 50 mg FlexBone preabsorbed with 40 ng BMP-2/7 in culture showing localized ALP activity near where the FlexBone carriers were placed (insets). (F) ALP staining 3.5 days after placing the carrier retrieved from (E) in a fresh culture. (G) Quantification of the ALP activity ($n = 3$) in lysate protein of C2C12 cultures stimulated by a FlexBone carrier (preabsorbed with 40 ng BMP-2/7). The "4 + 3.5 day" data correspond to the ALP content in the culture described in (F).

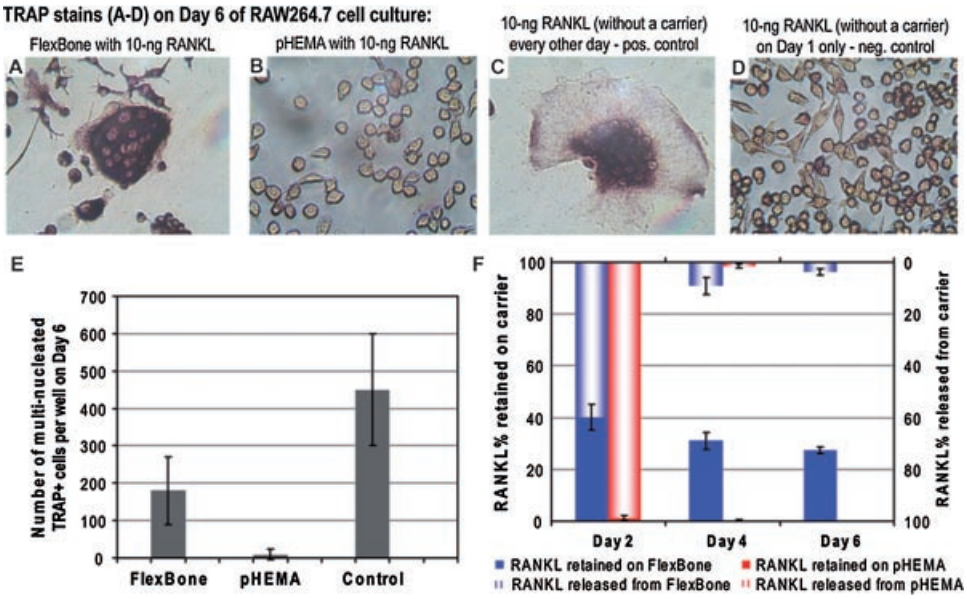

Figure 4. FlexBone released rmRANKL over a 6 day period and induced osteoclastogenesis of RAW264.7 cells. TRAP staining 6 days after: (A) placing a FlexBone carrier preabsorbed with 10 ng RANKL; (B) placing a pHEMA carrier preabsorbed with 10 ng RANKL; (C) supplementing 10 ng RANKL (without a carrier) every other day for 6 days; or (D) a one-time supplement of 10 ng RANKL (without a carrier) in culture. (E) Number of multinucleated (>3 nuclei) TRAP-positive cells per well on day 6 of the culture stimulated by FlexBone (10 ng RANKL), pHEMA (10 ng RANKL), or continuous RANKL (10 ng every other day without a carrier: positive control). (F) ELISA quantification of RANKL released from FlexBone versus pHEMA carriers ($n = 3$) over time. Media were changed on day 2, day 4, and day 6.

DISCUSSION

Synthetic bone substitutes that possess the structural and biochemical microenvironment emulating that of natural bone and exhibit good surgical handling characteristics are highly desired in orthopedic care. We recently developed FlexBone, an elastomeric hydrogel-mineral composite with a high content of nanocrystalline HA/TCP, as a new generation of synthetic bone substitute.[4] FlexBone exhibited excellent structural integration of the organic and inorganic components and withstood >25% compressive stains repeatedly in an aqueous environment at body temperature (Fig. 1A). These properties make FlexBone suitable as a structural support and an osteoconductive conduit for the repair of volumetric bony defects.

We demonstrated in this study that FlexBone could serve as an effective carrier for the encapsulation and localized delivery of therapeutic agents in vitro. By direct encapsulation during the crosslinking of the hydrogel, tetracycline (0–5.0 wt%) was incorporated in FlexBone without compromising the structural integrity and compressive behavior of the organic-inorganic composite. The high affinity of tetracycline for the calcified matrix enabled its release from FlexBone in a dose-dependent, sustained manner (e.g., 20% release within 1 week), in contrast to the more rapid release of tetracycline from unmineralized pHEMA hydrogel (e.g., 30% release within the first 8 h; Fig. 2). Such characteristics would not only allow manipulation of the quantity of early-stage (first 24 h) drug release by adjusting the initial drug encapsulation content, but would also enable sustained release beyond 1 week when longer-term delivery of the therapeutics is desired. Although tetracycline was chosen for its broad orthopedic implications beyond its antibiotic activity, our encapsulation strategy can be extended for delivery of more potent antibiotics such as tobramycin and gentamicin.

The excellent structural integration between the mineral components and the hydrophilic hydrogel matrix of FlexBone also enabled the retention and localized and sustained release of protein-based therapeutics. Taking advantage of the hydrophilic nature of the organic matrix of FlexBone, recombinant protein solutions of BMP-2/7 and RANKL were conveniently loaded to the freeze-dried composite. Localized release of the osteogenic BMP-2/7 and osteoclastogenic RANKL from FlexBone was achieved over 1 week, a time frame within which the effects of these molecules in promoting early osteointegration and graft healing are the greatest.[18,19] Such retention/release properties reduced the required minimal loading doses of these proteins. A single dose as low as 40 ng BMP-2/7 or 10 ng RANKL per 50 mg FlexBone carrier (comparable dimension of a rat critical femoral defect, shown in Fig. 1B) effectively induced osteogenic differentiation of C2C12 and osteoclastic differentiation of RAW264.7 in culture, respectively. This represents a major improvement over most existing synthetic carriers for the delivery of these recombinant proteins. For instance, a recently reported rhBMP-2 delivery system based on bulk TCP-chitosan scaffold required loading doses three orders of magnitude higher than ours (e.g., 50 μg BMP-2 every 60 mg scaffold) to induce osteogenic differentiation of C2C12 cells.[21] Another RANKL-coated brushite cement system designed to stimulate bone remodeling required a minimum of 600–800 ng RANKL in every 40 mg cement to induce osteoclastogenesis of RAW264.7 under similar culture conditions.[22] Scaffold-based delivery of protein therapeutics could significantly benefit from the reduction of minimal loading doses, from both clinical outcome (minimizing systemic side effects) and cost perspectives. The minimal loading doses of BMP-2/7 and RANKL determined in our study provide a rational starting point for subsequent evaluations of these therapeutic agents in expediting the repair and

remodeling of critical-sized bony defects using FlexBone as an osteoconductive carrier.

In summary, FlexBone exhibits key features of a viable synthetic bone substitute, including bonelike structural compositions (high osteoconductive mineral content), useful surgical handling characteristics (elasticity and the ability to withstand moderate compressive loads), and a tunable biochemical microenvironment (capacity for sustained and localized delivery of therapeutic agents). These properties provide an exciting opportunity for the treatment of hard-to-heal bony defects using FlexBone both as an effective delivery carrier for bioactive factors and a robust osteoconductive framework for expedited osteointegration.

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