Biomaterials 51 (2015) 313–319

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Tissue engineering a surrogate niche for metastatic cancer cells

F. Philipp Seib [a, 1], Janice E. Berry [b], Yusuke Shiozawa [b], Russell S. Taichman [b], David L. Kaplan [c, *]

[a] Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, 161 Cathedral Street, Glasgow G4 0RE, UK
[b] Department of Periodontics and Oral Medicine, University of Michigan School of Dentistry, Ann Arbor, MI 48109, USA
[c] Tufts University, Department of Biomedical Engineering, 4 Colby Street Medford, MA 02155, USA

ARTICLE INFO

Article history:
Received 20 September 2014
Accepted 27 January 2015
Available online 4 March 2015

Keywords:
Bone
Silk
Cancer
Metastasis
Breast cancer

ABSTRACT

In breast and prostate cancer patients, the bone marrow is a preferred site of metastasis. We hypothesized that we could use tissue-engineering strategies to lure metastasizing cancer cells to tissue-engineered bone marrow. First, we generated highly porous 3D silk scaffolds that were biocompatible and amenable to bone morphogenetic protein 2 functionalization. Control and functionalized silk scaffolds were subcutaneously implanted in mice and bone marrow development was followed. Only functionalized scaffolds developed cancellous bone and red bone marrow, which appeared as early as two weeks post-implantation and further developed over the 16-week study period. This tissue-engineered bone marrow microenvironment could be readily manipulated *in situ* to understand the biology of bone metastasis. To test the ability of functionalized scaffolds to serve as a surrogate niche for metastasis, human breast cancer cells were injected into the mammary fat pads of mice. The treatment of animals with scaffolds had no significant effect on primary tumor growth. However, extensive metastasis was observed in functionalized scaffolds, and the highest levels for scaffolds that were *in situ* manipulated with receptor activator of nuclear factor kappa-B ligand (RANKL). We also applied this tissue-engineered bone marrow model in a prostate cancer and experimental metastasis setting. In summary, we were able to use tissue-engineered bone marrow to serve as a target or "trap" for metastasizing cancer cells.

1. Introduction

Metastasis is a highly complex process. In the case of breast and prostate cancers, hematogeneous metastasis is commonly encountered in the red bone marrow [1], and patient survival is poor once disseminated disease is diagnosed [2]; metastasis is responsible for 90% mortality of patients with solid tumors [3]. The lack of suitable *in vivo* tissue models has impeded clinical progress [4]. There are currently two main approaches for studying syngeneic or xenograft breast and prostate cancer bone metastasis in the orthotopic or experimental metastasis setting [5]. In the first, the host's skeleton serves as the site of metastasis and is commonly used to study osteotropism of cancer. In the second, fresh bone chips [6–9] or marrow [10] are used and implanted subcutaneously or in the mammary fat pad. While human fetal bone or marrow has been used in most cases [6,8], materials from discarded femoral heads [9] have also been used.

Tissue-engineering approaches for cancer research [11] have recently emerged as a potential third route for the study of bone metastasis. For example, microfabricated scaffolds seeded with human bone marrow stromal cells have been implanted in a window chamber model to permit intravital microscopy studies [12]. This microfabricated model generated a chimeric microenvironment, but the ability of this model to recapitulate native tissue remains to be established. Bone marrow stromal cells are clearly useful for driving osteogenesis and marrow formation [13]; however, bone morphogenetic proteins (BMPs) also have a robust clinical track record for the *de novo* formation of bone and marrow [14]. In particular, BMP-2 has been associated with bone development and maintenance in the adult skeleton [14,15]. *In vivo* tissue engineering of bone has been successful [16], but no attempts have yet been made to engineer a bone marrow microenvironment (BMM) that can be selectively manipulated. This manipulation of

* Corresponding author. Tel.: +1 617 627 3251; fax: +1 617 627 3231.
E-mail address: David.Kaplan@tufts.edu (D.L. Kaplan).
[1] Previous address: Tufts University, Department of Biomedical Engineering, 4 Colby Street, Medford, MA 02155, USA.

http://dx.doi.org/10.1016/j.biomaterials.2015.01.076
0142-9612/

the BMM would provide opportunities to ask fundamental questions about cancer metastasis to bones, and to explore the possibility that tissue-engineered bone could serve as a surrogate niche or "trap" for cancer metastasis. Several potential avenues are available for manipulating the BMM; chemokines were chosen in the present study.

In 1889, Stephen Paget established that breast cancer has preferred sites for metastasis (tissue tropism) [17], and recent studies have identified chemokines as potential regulators that dictate the actual organ metastasis of breast [18] and prostate [19] cancers (reviewed in Refs. [20,21]). For example, metastatic breast and prostate cancers "home" to bone by following gradients of stromal cell-derived factor 1 (SDF-1); this mechanism emulates the hematopoietic stem trafficking occurring during fetal development and following bone marrow transplantation [20].

Bone colonization by metastatic cancer cells involves the hijacking of a multitude of signaling pathways [22]. For example, osteotropic cancers often induce osteoclast activity through receptor activator of nuclear factor kappa-B ligand (RANKL) signaling. Osteoclast activation in the BMM in turn liberates a myriad of growth factors and chemokines stored in the bone mineral matrix, thereby driving the recruitment of even more cancer cells to the bone [2,22]. Our current understanding of chemokine-mediated metastasis indicated SDF-1 and RANKL as appropriate choices for manipulation of the BMM in the present study.

In summary, this study examined the potential of BMP-2 functionalized scaffolds to support the *in vivo* development of bone and marrow and the subsequent ability of this tissue-engineered BMM to serve as a surrogate niche for metastatic cancer cells attracted by locally released chemokines.

2. Materials and methods

2.1. Preparation of silk scaffolds

Bombyx mori silk solution was prepared as described previously [23]. Briefly, cocoons were cut into 25-mm^2 pieces, boiled for 30 min in an aqueous solution of 20 mM Na_2CO_3, and then rinsed in distilled H_2O to remove sericin proteins. Extracted silk fibroin was subsequently air dried and then dissolved in 9.3 M LiBr solution at 60 °C for 4 h, yielding a 25% w/v solution. This solution was dialyzed against ddH_2O (molecular weight cut off 3500) for 48 h to remove the LiBr salt. The resulting aqueous silk fibroin solution was centrifuged twice at 9.700 g for 20 min to remove the small amount of silk aggregates that formed during processing. A salt-leach method was used where NaCl crystals were embedded within silk fibroin to generate highly porous silk scaffolds. First, the silk fibroin solution was diluted to 6% w/v with ddH_2O. Next, 4 g of NaCl crystals (500–600 μm) were added to 2 ml of this fibroin solution as porogens, and scaffolds were allowed to solidify for 24 h. Scaffolds were washed extensively in ddH_2O to leach out the NaCl to yield highly porous silk scaffolds. The size was optimized by generating scaffolds with a volume of either 125 mm^3 or 27 mm^3 and a constant 5 μg BMP-2 (human BMP-2, Wyeth, Andover, MA, USA) loading. For 125 mm^3 scaffolds, BMP-2 loading was optimized using BMP-2 concentrations between 0.5 and 10 μg. For all samples, the BMP-2 loading was performed by applying 30 μl of a 7% w/v silk solution containing the indicated amount of BMP-2. Scaffolds were air dried under a 0.2-m/s airflow at room temperature overnight. Where indicated, scaffolds were further modified by water annealing at room temperature for 8 h to induce β-sheets [23].

2.2. In vivo scaffold implantation

Animal studies were performed in accordance with the approved institutional protocols B2010–101 and PRO00004354 by the Institutional Animal Care and Use Committee (IACUC) of Tufts University and University of Michigan, respectively. Mice aged 6–10 weeks were purchased from Charles River Laboratories. For scaffold implantation studies, animals were anesthetized using isoflurane, shaved when necessary, and the surgical area was cleaned. As indicated, BMP-2 functionalized or control silk scaffolds were implanted subcutaneously at three different sites, namely the rotator cuff, lower abdomen, and upper thorax. As controls, 10 mg of demineralized human (0.125–0.850 mm particle size; Community Tissue Services, Dayton, OH, USA) or rat bone were added to a size 9 gelatin capsule (Torpac Inc., Fairfield, NJ, USA) and implanted as detailed above. Incisions were closed with a one-layer closure using skin clips. Animals were monitored daily over the course of 10 days, at which time the skin clips were removed. Scaffolds were removed at the indicated time points and processed for analysis as detailed below.

2.3. Cell culture

All cell lines were maintained in a humidified atmosphere of 5% CO_2 at 37 °C, and subconfluent cultures were routinely subcultured every 2–3 days. MDA-MB-231 B16F10, and PC3 cells were cultured in RPMI 1640 + 10% v/v FBS medium. For *in vivo* tumor studies, cells were harvested with trypsin and subsequently prepared as detailed below.

2.4. Tumor models

To examine the potential of tissue-engineered bone to serve as a surrogate niche to cancer metastasis, a syngeneic experimental tumor model employing C57BL/6 mice and B16F10 cancer cells was used [24]. Prior to tumor cell injection, a 125-mm^3 scaffold functionalized with 5 μg BMP-2 was implanted over the rotator cuff in mice and allowed to integrate for more than 4 weeks. On the day of tumor induction, B16F10 cells were washed and harvested with trypsin-EDTA, blocked with complete medium, and pelleted. The pellet was subsequently washed twice with PBS, and cells were resuspended in PBS at a concentration of 10^5 cells/ml and kept on ice. Mice were shaved, cleaned and the landmarks palpitated to facilitate the intracardiac injection of cells into the left ventricle. The spontaneous pulsatile entrance of bright red oxygenated blood into the transparent needle hub indicated proper positioning of the needle. A dose of 10^4 B16F10 cells in 100 μl was administered over 30 s into the left ventricle with a 27-gauge needle. Within 18 days of B16F10 injection, animals were euthanized.

For studies that examined the potential of the scaffolds to serve as a surrogate niche for breast cancer metastasis, a human xenograft model was used. Breast tumors were induced by inoculating MDA-MB-231 derived tumor cells that metastasized following orthotopic injection in mice [25]. Cells were genetically modified to carry the firefly luciferase gene to allow *in vivo* bioluminescence imaging [25]. Analogous to the syngeneic tumor studies, scaffolds were implanted over the rotator cuff in female NOD/SCID mice (NOD.CB17-$Prkd^{scid}$/NcrCrl), 6–10 weeks in age, and allowed to integrate ≥8 weeks. Next, a total of 5×10^5 cells in 20 μl Matrigel (BD Biosciences, Bedford, MA, USA) was injected bilaterally into the 4th or 5th mammary fat pad using a Hamilton syringe equipped with a 22-gauge needle. To manipulate the microenvironment of the tissue-engineered bone, osmotic minipumps (Durect Corporation, Cupertino, CA, USA) were used. Pumps with a nominal pumping rate of 0.11 μl/h over 4 weeks were fitted with an infusion catheter and filled with SDF-1 (100 μg/ml), RANKL (100 μg/ml), or PBS according to the manufacturer's instruction; human SDF-1 alpha (catalogue number 100-20) and mouse RANKL (catalogue number 200-04) were purchased from Shenandoah Biotechnology, Warwick, PA, USA. Twelve days after tumor inoculation, pumps were implanted s.c. and the catheter was implanted into the scaffold. Disease progression was monitored weekly with intraperitoneal injections of D-luciferin (Molecular Probes, Eugene, OR, USA), followed by measuring tumor cell-associated bioluminescence using the Xenogen IVIS 200 imaging system and Living Image Software 4.2 (Caliper Life Sciences, Hopkinton USA). At the study endpoint at 6 weeks post-tumor induction, scaffolds, brain, lung, liver, and bones were examined for metastasis by dissecting them from the carcass. Tibia and femur from hind legs were harvested and dissected free from muscle and tendons to serve as bone samples. Organs were imaged at maximum sensitivity to detect metastatic cancer cells. Tissues were scored for the presence or absence of metastasis. Primary tumors were dissected and weighed.

For prostate cancer studies, one scaffold was implanted on the back of male athymic nude mice (Athymic Nude-*Foxn1*^{nu}), 6–10 weeks in age, and allowed to integrate >6 weeks. PC3 cells were transduced with GFP-luciferase lentivirus to allow for bioluminescence imaging of tumor growth (via luciferase) and localization of cells in tissue sections (via GFP). Next, a total of 1×10^5 cells in 10 μl growth medium was injected into the ossified scaffold with a 30-gauge needle. At the time of cell injection, a single pump with either SDF-1 or PBS was implanted as detailed above.

2.5. Histology and X-ray microtomography

X-ray microtomography was performed on formalin-fixed tissues in 70% v/v ethanol. Measurements were carried out with an HMX ST 225 X-ray tube equipped with a molybdenum target and a 2000 × 2000 pixel detector (Nikon Metrology, Leuven, Belgium). Projections were recorded over 360°, and dataset voxel sizes were typically 10 μm isotropic. The dataset was reconstruction with the CTPro 3D software package (Nikon Metrology) in the absence of noise reduction or binning. Images were rendered using VGStudio MAX version 2.2 (Volume Graphics, Heidelberg, Germany).

Bones and scaffolds were prepared for histology by fixing them for 24 h in buffered formalin and subsequently demineralizing them for 21 days at 4 °C with a 10% w/v EDTA solution at pH 7.4. Next, samples were tissue processed and paraffin embedded as detailed previously [26]. For all histology samples, at least two level sections were cut to ensure representative images. For immunofluorescence images to detect human cells grown in scaffolds, anti-human HLA-ABC antibody (BioLegend) was conjugated using the Zenon Alexa Fluor488 mouse IgG labeling kit (Invitrogen, San Diego, CA). Seven μm thick paraffin sections were blocked with Image-iT FX signal enhancer (Invitrogen) for 30 min before fluorescence-labeled and primary antibodies were applied for 2 h at room temperature in the dark.