Tissue-Engineered Bone Serves as a Target for Metastasis of Human Breast Cancer in a Mouse Model

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Abstract

The high frequency and mortality associated with breast cancer metastasis to bone has motivated efforts to elucidate tumor-stroma interactions in the bone microenvironment contributing to invasion and proliferation of metastatic cells. The development of engineered tissues has prompted the integration of engineered bone scaffolds into animal models as potential targets for metastatic spread. Silk scaffolds were coupled with bone morphogenetic protein-2 (BMP-2), seeded with bone marrow stromal cells (BMSC), and maintained in culture for 7 weeks, 4 weeks, and 1 day before s.c. implant in a mouse model of human breast cancer metastasis from the orthotopic site. Following injection of SUM1315 cells into mouse mammary fat pads, tumor burden of implanted tissues was observed only in 1-day scaffolds. Scaffold development and implantation was then reinitiated to identify the elements of the engineered bone that contribute to metastatic spread. Untreated scaffolds were compared with BMP-2–coupled, BMSC-seeded, or BMP-2/BMSC–combined treatment. Migration of SUM1315 cells was detected in four of four mice bearing scaffolds with BMP-2 treatment and with BMSC treatment, respectively, whereas only one of six mice of the BMP-2/BMSC combination showed evidence of metastatic spread. Histology confirmed active matrix modeling and stromal cell/fibroblast infiltration in scaffolds positive for the presence of metastasis. These results show the first successful integration of engineered tissues in a model system of human breast cancer metastasis. This novel platform now can be used in continued investigation of the bone environment and stem cell contributions to the process of breast cancer metastasis. [Cancer Res 2007;67(21):10304–8]

Introduction

Breast cancer is the leading cause of cancer-related death in American women, accounting for more than 200,000 new cases and 40,000 deaths annually (1). The difficulty in obtaining successful treatment (complete remission) results from the migration of cancer cells away from the primary tumor (2–5). As one of the few cancers that metastasizes to bone, breast cancer cells undergo a complex process involving migration away from the primary colony through the extracellular matrix into the blood or lymph followed by organ-specific adherence and extravasation into the stroma of the new tissue environment (5). The expression of identical and complementary bone proteins expressed on the surface of breast epithelial cells suggests a similarity in breast and bone stromal environments (6). This compatibility implicates a mutual selection of breast cancer and bone, emphasizing the contribution of the microenvironment to attracting, harboring, and sustaining skeletal breast cancer metastases.

To further understand the mechanisms of breast cancer metastasis, a mouse model has been developed that maintains both species- and organ-specific migration from the orthotopic site (7). With a nonobese diabetic/severe combined immunodeficient (NOD/SCID) mouse as host, implanted native human bone has remained viable and becomes an efficacious target for metastatic spread. The human breast cancer SUM1315 cell line showed the capacity to metastasize following mammary fat pad (MFP) injection, maintaining species specificity by migrating exclusively to the human bone implants and not to the mouse skeleton or peripheral organs (except for late-stage appearance in lung).

Despite the positive migration of the SUM1315 cell line to the implanted human bone fragments, the nature of the bone tissue itself renders it a difficult platform for analysis of invading cells or investigation of the stromal contribution to the metastatic process. Although morphologic analysis and histochemistry can be obtained, genetic analysis of metastatic tissues without contaminating bone tissue has proven a difficult obstacle to overcome. Variability of implanted bone may also affect the consistency of metastatic spread in the model due to differences in donated tissue (age, gender, and disease). Incorporation of a tissue-engineered alternative for native bone has the potential to overcome issues related to native bone in the mouse model.

Reemerging as a prominent matrix protein for scaffold development, extracted silk fibers from the silkworm Bombyx mori offer a mechanically robust and long-term degradable foundation for tissue engineering applications (8, 9). With the capacity to create various geometries ranging from nanofibers and films to sponges and gels, silk is readily adaptable to correspond to the overall morphology of developing tissue. Spongy scaffolds have been implemented in the in vitro and in vivo development of bone tissue by coupling bone morphogenetic protein-2 (BMP-2) to stimulate human bone marrow stromal cell (BMSC) differentiation along the osteogenic lineage (8, 10).

Incorporating engineered tissue in the model of breast cancer metastasis, we are afforded the potential of a controllable, reproducible, and functional bone implant that may be tailored to the study of the metastatic microenvironment. We hypothesize that tissue-engineered bone scaffolds will serve as target sites for metastatic spread within the mouse model of metastasis. Validation of this model system would offer a unique platform for the dissection of the components of the bone microenvironment contributing to metastatic spread.
Materials and Methods

Silk fibroin scaffolds. Cocoons of B. mori silkworm were supplied by M. Tsukada (Japan Institute for Sericulture). Cocoons were boiled for 20 min in an aqueous solution of 0.02 mol/L Na2CO3 and rinsed thoroughly with distilled water for sericin protein extraction. The extracted silk fibroin was then dissolved in a 9.3 mol/L solution of lithium bromide at 60°C for 4 h, yielding a 20% (w/v) fibroin solution, which was then dialyzed in distilled water for 2 days and lyophilized. The solid was then dissolved directly in hexafluoroisopropanol and sodium chloride (80 mesh particle size; Sigma-Aldrich) to generate 17 wt% fibroin cylindrical scaffolds, 5 mm diameter and 2 mm height in dimension, with 500- to 600-μm pores.

BMP-2 coupling to silk fibroin scaffolds. BMP-2 was covalently coupled to autoclaved scaffolds through carbodiimide cross-linking. Exposed carboxyl groups of aspartic and glutamic amino acids on silkworm cocoons were activated with an appropriate volume of 0.5 mg/mL 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.7 mg/mL N-hydroxysuccinimide (NHS) solution (Pierce) in MES buffer (pH 6.0) for 15 min at room temperature. Scaffolds were then rinsed extensively with MES to remove excess EDC/NHS and reacted with an appropriate volume of BMP-2 solution, allotting 20 μg BMP-2 per scaffold for 2 h at room temperature with gentle shaking. Scaffolds were then rinsed with PBS and stored at 4°C until seeding/implant.

BMSC culture expansion. All reagents for cell culture experiments were purchased from Invitrogen Corp., unless otherwise specified. Human BMSCs were isolated, expanded in culture, and stored as described previously (11). Briefly, unprocessed human whole bone marrow aspirates were obtained from donors <25 years of age (Clenotnic-Poietics), resuspended in DMEM supplemented with 10% fetal bovine serum (FBS), 0.1 mmol/L nonessential amino acids (NEAA), 100 units/mL penicillin and 100 mg/L streptomycin, and 1 mg/mL basic fibroblast growth factor, and plated at 8μL aspirate/cm² in tissue culture polystyrene. Nonadherent hematopoietic cells were removed with the culture medium during medium exchange after 7 days of culture. Culture medium was changed twice weekly thereafter. Primary BMSCs were detached at ~80% confluency using 0.25% trypsin/1 mL EDTA and replated at 5 × 10^5 cells/cm². Passage 1 BMSCs near confluency were trypsinized and subsequently frozen in 8% DMSO/10% FBS/DMEM for future use. Passage 1 BMSCs were defrosted, replated at 5 × 10^5 cells/cm² (passage 2), and trypsinized near confluency for use in matrix seeding.

SUM1315 cell line. The human breast cancer epithelial cell line SUM1315, expressing green fluorescent protein and luciferase, was maintained in subculture 2 weeks before BMP inclusions, with medium composed of F-12 medium with 1-glutamine supplement, 5% FBS, 50 units/mL penicillin, 50 μg/mL streptomycin, 5 μg/mL insulin, and 10 ng/mL epidermal growth factor (Sigma-Aldrich).

Silk scaffold cell seeding. Scaffolds were incubated overnight at 4°C in the culture medium used in BMSC expansion. Scaffolds were transferred to the bottom of individual 50 mL centrifuge tubes. Passage 2 BMSC were prepared in a suspension of 15 × 10^6 cells/mL in culture medium, applying 100 μL of suspension gently to each side of the cylindrical scaffold. Scaffolds were then incubated in the cell suspension for 30 min in a humidified incubator to facilitate primary cell seeding (cap loosened to allow gas exchange) with gentle agitation of the scaffold/solution at 15-min intervals. After primary seeding, an additional 4 mL of BMSC culture medium were added per tube and the tube was loosely recapped and stored at 37°C, 5% CO₂ overnight. For extended in vitro culture, scaffolds were transferred to individual wells of a 12-well culture plate and supplemented with osteogenic differentiation medium composed of DMEM, with 10% FBS, 0.1 mmol/L NEAA, 100 units/mL penicillin/streptomycin, 100 mmol/L dexamethasone, 0.05 mmol/L ascorbic acid-2-phosphate, and 10 mmol/L β-glycerophosphate.

Surgical implantation. All surgical procedures were approved through the Tufts University Department of Laboratory Animal Management (protocol 53-06) before experimentation. Human bone obtained from femurs removed from total hip replacement surgeries was cut into cylindrical fragments of ~5 mm diameter × 1 cm length dimension.
both collagen and calcium deposition directly correlating to the length of time in culture before implant (Fig. 1B).

In the determination of components contributing to metastatic spread, all scaffold treatments supported metastasis of SUM1315 as confirmed by luminescent imaging and histology (Figs. 2 and 3, respectively). BMP and BMSC scaffold groups had the highest incidence of metastatic spread, specifically four of four surviving animals in each group, whereas BMP/BMSC scaffolds supported metastasis in one of six. Signal intensity relating to the tumor burden was quantified at $1.18 \times 10^{-5} \pm 8.1 \times 10^{-4}$ pixels/second for all bone explants bearing SUM1315 spread and $8.36 \times 10^{-4} \pm 2.92 \times 10^{-4}$ pixels/second for all scaffolds. Of note, one animal in the BMSC scaffold group supported metastatic spread on the scaffold alone, as the native bone did not register a luminescent signal. Luminescent images of BMP explants were not obtained due to a mechanical malfunction, requiring histologic processing to identify metastatic spread. Untreated explants and respective native bone controls showed no evidence of metastasis in any of six surviving animals at the end of study. Xenogen images of the lungs and the site of scaffold/bone explant of mice bearing metastases were negative for luminescent signal, indicating that peripheral spread to other organs was not observed.

Stromal cells and fibroblasts were the most abundant cell types present in all scaffolds supporting metastatic spread, actively

Figure 1. Luminescent and histologic images of scaffold explants. A, composite luminescent image of 1-d explants with metastatic spread of SUM1315 from the orthotopic site-engineered bone scaffolds (left) and native bone (right). B, scaffold cross-sections of BMSC stained with von Kossa and trichrome, respectively. Visible increases of calcium (brown, left column) and collagen type I (blue, right column) are evident with increasing in vitro culture before implant. In all panels, the remaining silk scaffold stains pink and can be identified as the framework of a porous cross-section. D0, 1 d; W4, 4 wk; W7, 7 wk. Bar, 250 μm.
remodeling the silk matrix, and depositing collagen as observed in trichrome-stained sections for all groups (Fig. 3). Conversely, the BMP/BMSC scaffolds that did not support metastases seemed necrotic with lymphoid cells in place of fibroblasts and stromal cell remodeling. Significant calcium deposition was observed only in the BMP/BMSC explant in regions that previously contained the silk scaffold. Cytokeratin 5/6 immunostaining confirmed the peripheral invasion of SUM1315 into engineered scaffolds, with the absence of positive stain in native bone controls.

Discussion

By integrating research approaches from the disciplines of tissue engineering and cancer biology, we have identified a functional and reproducible engineered bone scaffold capable of supporting the metastatic spread of breast cancer from the orthotopic site. This novel model system holds promise for advancing the study of the contribution of the bone microenvironment to metastasis. Our findings parallel the spontaneous migration of the SUM1315 human breast cancer cell line to native human bone observed in our mouse model of metastasis. This new animal model offers a novel approach to characterizing the metastatic microenvironment of bone in breast cancer and other models of disease.

In the development of a disease model, engineered tissues offer a unique platform that undergo the development and remodeling characteristic of native tissue while permitting environmental control. Both the structure and porosity inherent in the silk scaffold preparation predispose seeded cells to osteogenic differentiation, characterized by calcium deposition and the up-regulation of bone-specific markers (12). Advanced bone development was attained in 7-week cultures, with progressively immature states at 4 weeks and 1 day, respectively. Although 7 weeks in in vitro culture produced the most mature bone environment, the results observed with these scaffolds contradicted our initial assumption that the most developed bone environment would yield the greatest metastatic spread. Instead, the more primitive bone environments were more conducive to breast cancer cell invasion. Previous work by Lyden et al. has implicated stem cells in the environmental preparation and signaling before breast cancer cell invasion in bone, supporting our findings of metastasis in the most actively regenerating scaffold (13). Perhaps the relatively rich proportion of stem cells in immature scaffolds contributes to enhanced metastasis. Characterization of the 1-day scaffolds will be emphasized in future studies to identify the means by which the s.c. environment affects cell differentiation and the subsequent development of a metastatic niche.

Figure 2. Luminescent and histologic images of scaffold explants. A, composite luminescent image of scaffolds from BMSC and BMSC/BMP treatment groups bearing metastatic spread-engineered bone (left) and native bone (right). B, scaffold cross-sections of labeling cytokeratin 5 and 6 and collagen type I, respectively. The brown stain (left column) confirms the presence of invading SUM1315 cells at the scaffold perimeter. Bar, 100 μm. The predominance of stromal cells and fibroblasts within the scaffold has produced abundant collagen type I visible in the trichrome images (right column). Bar, 250 μm.
In the attempt to identify a specific component responsible for the initiation of metastasis to the engineered scaffold, we instead found three distinct environments capable of supporting SUM1315 cell invasion. Individually treating the scaffolds with either BMP-2 or BMSC induced metastasis at a remarkable frequency, maintaining a primitive yet actively remodeling bone environment. Distant changes in the premetastatic "soil" environment have been implicated in the migration of breast cancer cells to distant sites (13, 14). Given its capacity to recruit a blood supply, the direct addition of BMSC to the silk scaffold may equip the implant with externally derived components that trigger metastasis. A similar metastatic niche may be achieved through BMP-2 scaffold treatment, given its capacity to attract and select stem cells for bone development and maturation (15). The importance of both the balance and timing of environmental cues is reflected by BMP/BMSC scaffolds and their reduced frequency in fostering metastatic spread.

Combining a mouse model of breast cancer metastasis with tissue-engineered bone has enabled us to demonstrate, for the first time, that engineered scaffolds are capable of functioning as homing sites for metastatic spread. This novel platform will enable future study of bone stromal factors essential for or contributing to metastasis. Moreover, our findings suggest that engineered tissues can be integrated as advantageous components in emerging models of disease.

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Figure 3. Nonmetastatic BMP/BMSC scaffolds in cross-section with trichrome staining. In contrast to scaffolds positive for metastatic spread, negative groups showed evidence of necrosis with the infiltration of lymphoid cells and the absence of fibroblast/stromal cell infiltration and remodeling. Bar, 500 μm.

References

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