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Systemic trafficking of macrophages induced by bone cement particles in nude mice

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ABSTRACT

Macrophages play an important role in the biological response to wear particles, which can result in periprosthetic osteolysis and implant loosening. In this study, we demonstrate that polymer particles induce systemic trafficking of macrophages by non-invasive in vivo imaging and immunohistochemistry. The distal femora of nude mice were injected with 10% (w/v) Simplex bone cement (BC) suspensions or saline (PBS). Reporter RAW264.7 macrophages which stably expressed the bioluminescent reporter gene *fluc*, and the fluorescence reporter gene *gfp*, were injected intravenously. Bioluminescence imaging was performed immediately and periodically at 2-day intervals until day 14. Compared to the non-operated contralateral femora, the bioluminescent signal of femora injected with BC suspension increased 4.7 ± 1.6 and 7.8 ± 2.9-fold at day 6 and 8, respectively. The same values for PBS group were 1.2 ± 0.2 and 1.4 ± 0.5, respectively. The increase of bioluminescence of the BC group was significantly greater than the PBS group at day 8 ($p < 0.05$) and day 6 ($p < 0.1$). Histological study confirmed the presence of reporter macrophages within the medullary canal of mice that received cement particles. Modulation of the signaling mechanisms that regulate systemic macrophage trafficking may provide a new strategy for mitigating the chronic inflammatory response and osteolysis associated with wear debris.

1. Introduction

Excessive production of wear particles from joint replacements is associated with periprosthetic osteolysis, which can lead to implant loosening [1–7]. Phagocytic cells engulf particulate debris and become activated; releasing proinflammatory cytokines, chemokines, degradative enzymes, reactive oxygen radicals and other substances which stimulate osteoclasts to undermine the prosthetic bed [8–14]. The key cell in the foreign body and chronic inflammatory response to wear particles is the macrophage [15–17]. Cells of the monocyte/macrophage lineage differentiate and maturate into phagocytic macrophages, foreign body giant cells and osteoclast precursors. These cells (in communication with stromal cells and other cell types) are primarily responsible for the cascade of events culminating in periprosthetic osteolysis. Despite ongoing research into the cellular and molecular processes associated with periprosthetic osteolysis, no in vivo studies have elucidated whether remote macrophages are stimulated to migrate to wear particles, or whether these events are a local phenomenon only. If macrophage recruitment to particles is a systemic phenomenon then novel strategies to mitigate these events may be potential targets for treatment.

We hypothesized that exogenous reporter macrophages introduced from a distant site would migrate and concentrate to an area in which phagocytosable polymer particles have been implanted. To examine this hypothesis, we use a model of femoral intramedullary polymer particle placement [18] in nude mice, a murine macrophage cell line transfected with a bioluminescent reporter gene, and sequential non-invasive imaging in vivo using bioluminescence.

2. Materials and methods

2.1. Animals and cells

8 to 11-week-old adult male nude mice (Charles River Laboratories, Inc., MA) were housed and fed in our Institution's Animal Facility. The murine macrophage cell line RAW264.7 was transfected with the lentiviral vector to express the bioluminescent optical reporter gene, firefly *luciferase* (*fluc*), and a fluorescence reporter gene, *green fluorescent protein* (*gfp*) [19].

2.2. Bone cement particles

Simplex® P bone cement (BC) powder (Howmedica Osteonics, Allendale, NJ) was used in the study. The BC powder is composed of 15% polymethylmethacrylate (PMMA), 10% barium sulphate, and 75% methylmethacrylate styrene copolymer. The

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particles vary from less than 1 μm in diameter to approximately 100 μm according to the manufacturer. The particles tested negative for endotoxin using a Limulus Amebocyte Lysate kit (BioWhittaker, Walkersville, MD). 10% (weight/volume) BC suspension was prepared in phosphate buffered saline (PBS, pH 7.4).

2.3. Surgical procedure

Institutional Guidelines for the care and use of laboratory animals were strictly followed. All the operations were done using general anesthesia using a mask (3% isoflurane in 100% oxygen). We used the transpatellar tendon approach for distal femoral medullary cavity injection [20]. Briefly, the patellar tendon was exposed through a 5 mm lateral skin incision, and then the lateral aspect of the femoral shaft was exposed by another 5 mm incision over the distal quadriceps. The intramedullary injection (10 μl) was performed through the patellar tendon into the inter-condylar region of the femur with a 5 mm insertion of the needle guided by palpation of the lateral femoral shaft. The quadriceps-patellar complex was repaired with suture after injection. The incisions on the skin were closed by surgical adhesive glue and suture. Buprenorphine (Ben Venue Laboratories, Bedford, OH) at 0.1 mg/kg was given subcutaneously immediately and 4 h later post-operatively for pain control.

In addition to the BC suspension treatments, additional mouse limbs were used as negative controls (no injection, or injection of PBS only) and positive controls (injection of lipopolysaccharide from *Escherichia coli* O127:B8 at a concentration of 1 μg/g bodyweight in PBS, purchased from Sigma, Saint Louis, MO).

2.4. Bioluminescence imaging

For in vivo surveillance of the trafficking of macrophages, 7 days post-operation, macrophages (5×10^5 cell) suspended in 0.1 ml Hanks' balanced salt solution (HBSS, Invitrogen, Carlsbad, CA) were injected intravenously via a syringe and needle (25 gage) into the lateral tail vein of mice. Fifteen minutes after intraperitoneal administration of D-luciferin (3 mg/mouse, Biosynth International), 5-min images were taken with an in vivo imaging system (IVIS) employing a cooled charge-coupled device camera (Caliper LifeSciences, Hopkinton, MA). Prone and lateral images were obtained from each animal at each time point to better determine the origin of photon emission. Animals were imaged at 2-day intervals' post-macrophage injection. Bioluminescence images were quantified by drawing uniformly sized regions of interest (ROIs) throughout the whole experiment, over the thigh on the lateral images of the mice, and the data were collected as to photon/cm^2/s/sr.

2.5. Histology and immunohistology

Femora were collected at day 0 immediately after particle injection (6 femora) and 3 weeks after completion of the imaging experiment (38 femora). Frozen sections were cut using a cryostat (Cambridge Instruments, Buffalo, NY). Polarized light microscope (Nikon E1000 M, Japan) was used to confirm the existence of BC particles in the femoral medullary canal.

Mouse anti-GFP monoclonal antibody (Chemicon International, Temecula, CA) was used to detect exogenous macrophages tagged with GFP. Rat anti-mouse macrophage/monocyte monoclonal antibody (MOMA-2, Chemicon International, Temecula, CA) was used to detect macrophages. The secondary antibody used was Alexa Fluor 488 (or 594) conjugated goat anti-mouse (or rat) IgG (Invitrogen, Carlsbad, CA). Briefly, neutral buffered formaldehyde (10%, pH 7.4) fixed frozen sections were blocked by Image-iT FX signal Enhancer (Molecular Probes, Eugene, OR). Mouse anti-GFP monoclonal antibody and rat anti-MOMA-2 monoclonal antibody were incubated at room temperature for 3 h, respectively. Then the sections were incubated with Alexa Fluor 488/594 conjugated goat anti-mouse/rat IgG (Invitrogen, Carlsbad, CA) for 1 h at room temperature in the dark. DAPI containing ProLong Gold antifade reagent (Molecular Probes, Eugene, OR) was used for nuclear staining and slide mounting.

2.6. Statistical methods

The non-parametric Mann–Whitney *U* test was used for statistical analyses between groups and the signed rank test was used to compare right and left limbs in the same animals.

3. Results

3.1. Polarized light microscopy of frozen section of femora

To demonstrate the presence of the BC particles in the femoral medullary canal of experimental animals, femora of selected mice were harvested immediately after injection. Frozen sections were stained with hematoxylin and eosin (H&E). The adopted histological protocol utilized reagents that precluded particle disruption and dissolution during preparation of the slides. Polarized light microscopy was used to observe the birefringence of the BC particles. As shown in Fig. 1, bright white spots indicated the presence of cement particles within the medullary space of the femur, indicating successful particle injection.

3.2. Imaging and bioluminescent signals of nude mice in BC and control group

We injected lipopolysaccharide (LPS) into the femoral medullary cavities of nude mice ($n = 10$) to ensure that there was a positive response of the tagged macrophages to endotoxin. Compared to PBS injected femora, LPS injected femora had a stronger bioluminescent signal on day 6 onwards, as shown in Fig. 2. This demonstrated that the tagged macrophages could be induced to migrate and proliferate to an area containing endotoxin in vivo in this animal model.

Femora of 12 nude mice were injected with a 10% (w/v) BC suspension unilaterally and eight nude mice were injected with the carrier PBS alone. The pulmonary bioluminescent signal immediately after injection indicated a successful intravenous injection of tagged macrophages (Fig. 3, day 0). One of the 12 mice in the BC group was excluded because of the lack of a pulmonary signal. As shown in Fig. 3 in a typical experimental animal, a strong signal was seen in the lungs on day 0. From day 6 onwards, a strong

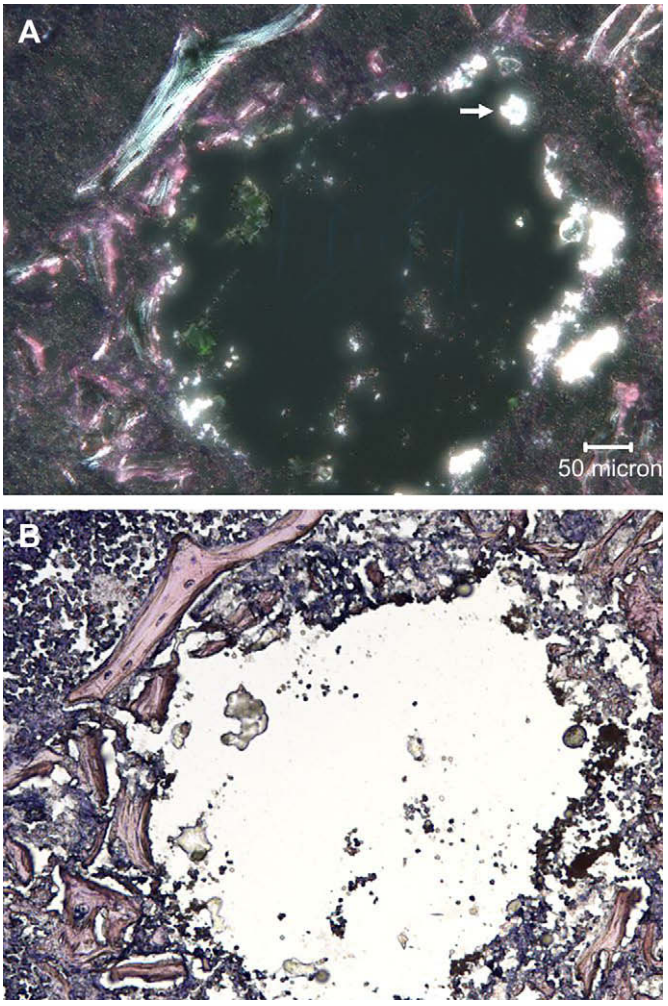

Fig. 1. Polarized light microscopy of an H&E stained section of a mouse femur injected with 10% BC particles at day 0, and sacrificed immediately post-operatively. The irregular bright white spots (arrows) are areas of birefringent BC particles, which are in the medullary injection area. The multi-colored birefringent strands are cortical and trabecular fragments containing collagen fibers. The scale bar equals to 50 μm. (The full colour images can be found in the on-line version.)