

Human Placenta-Derived Adherent Cells Prevent Bone loss, Stimulate Bone formation, and Suppress Growth of Multiple Myeloma in Bone

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Abstract

Human placenta has emerged as a valuable source of transplantable cells of mesenchymal and hematopoietic origin for multiple cytotherapeutic purposes, including enhanced engraftment of hematopoietic stem cells, modulation of inflammation, bone repair, and cancer. Placenta-derived adherent cells (PDACs) are mesenchymal-like stem cells isolated from postpartum human placenta. Multiple myeloma is closely associated with induction of bone disease and large lytic lesions, which are often not repaired and are usually the sites of relapses. We evaluated the antimyeloma therapeutic potential, in vivo survival, and trafficking of PDACs in the severe combined immunodeficiency (SCID)-rab model of medullary myeloma-associated bone loss. Intrabone injection of PDACs into non-myelomatous and myelomatous implanted bone in SCIDrab mice promoted bone formation by stimulating endogenous osteoblastogenesis, and most PDACs disappeared from bone within 4 weeks. PDACs inhibitory effects on myeloma bone disease and tumor growth were dose-dependent and comparable with those of fetal human mesenchymal stem cells (MSCs). Intrabone, but not subcutaneous, engraftment of PDACs inhibited bone disease and tumor growth in SCID-rab mice. Intratumor injection of PDACs had no effect on subcutaneous growth of myeloma cells. A small number of intravenously injected PDACs trafficked into myelomatous bone. Myeloma cell growth rate in vitro was lower in coculture with PDACs than with MSCs from human fetal bone or myeloma patients. PDACs also promoted apoptosis in osteoclast precursors and inhibited their differentiation. This study suggests that altering the bone marrow microenvironment with PDAC cytotherapy attenuates growth of myeloma and that PDAC cytotherapy is a promising therapeutic approach for myeloma osteolysis.

Disclosure of Potential Conflicts of Interest

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Keywords

Myeloma; Bone disease; Cytotherapy; Osteoblasts; Placenta; Mesenchymal stem cells

Introduction

Multiple myeloma (MM) is a plasma cell malignancy characterized by accumulation of malignant plasma cells in the bone marrow. Myeloma cells often exhibit focal lesion growth patterns in bone and induce severe osteolytic bone disease in >80% of patients. The number of focal lesions correlates with clinical disease progression [1, 2]. Bisphosphonates [3], as well as novel agents such as lenalidomide and bortezomib, suppress MM bone disease by inhibiting osteoclast activity and/or by activating osteoblasts [4–6]; however, large lytic lesions are usually not repaired even after long-term remission, and relapses often occur in pre-existing lesions [1].

Recent studies suggest that MM bone disease is a reflection of osteoblast deactivation [7, 8]. Myeloma cells and micro-environmental cells in myelomatous bone produce osteoblastinactivating factors such as Wnt inhibitors (e.g., dickkopf-1 [DKK1], secreted frizzledrelated protein 2 [sFRP2], and frizzled-related protein-3 [FRZB]) and cytokines (e.g., hepatocyte growth factor [HGF], interleukin [IL]-7, and IL-3) [7, 9–14]. Inhibition of osteoblastogenesis tends to promote conditions that support osteoclastogenesis, resulting in "uncoupling" of bone resorption and bone formation and induction of typical osteolytic lesions in areas adjacent to tumor cells [15]. Mesenchymal stem cells (MSCs) from patients with MM possess abnormal genomic, phenotypic, and functional properties [16–18], which may contribute to impaired bone formation in this disease and to the ability of these MSCs to support and protect myeloma cells from spontaneous and drug-induced apoptosis [19].

Clinical and molecular characteristics of MM support the potential success of cytotherapy approaches for this disease. The clinical use of mesenchymal cell cytotherapy for various regenerative and inflammatory diseases and for enhancing engraftment of hematopoietic stem cells has been tested in clinical trials [20]. These adherent MSCs, often referred to as MSCs or multipotent mesenchymal stromal cells, are traditionally isolated from bone marrow and expanded in culture before use [21]. Despite concerns associated with their in vivo stemness characteristics (e.g., differentiation and self-renewal potential) and low engraftment rate, and the lack of knowledge on cellular and molecular mechanisms underlying their therapeutic effects [22, 23], these cells have been used successfully to treat patients with bone disease such as osteogenesis imperfecta [24] and may also be effective against MM bone disease.

Recently, human placenta has emerged as a valuable source of cells of mesenchymal and hematopoietic origin for multiple therapeutic purposes, including bone repair and enhanced engraftment of hematopoietic stem cells [25, 26]. In this study, we used placenta-derived adherent cells (PDACs), which were isolated from postpartum human placenta and expanded for clinical purposes (Celgene Cellular Therapeutics, Warren, NJ), to examine their effects on myeloma bone disease and tumor growth in the severe combined immunodeficient (SCID)–rab mouse model for myeloma [27]. In this model, primary myeloma cells are engrafted and grow restrictively in the implanted rabbit bone, producing typical disease manifestations, including induction of severe osteolytic bone disease, increased osteoclast activity, and suppression of osteoblastogenesis [5, 9]. We show here that PDACs effectively suppress MM-induced bone disease and tumor growth in bone by inhibiting osteoclastogenesis and stimulating endogenous osteoblastogenesis.

Placenta-Derived Adherent Cell

PDACs were obtained from Celgene Cellular Therapeutics (Warren, NJ). These are human adherent mesenchymal-like cells isolated from postpartum human placenta. PDACs do not express hematopoietic markers (e.g., CD34, CD45, CD19, CD3) but express mesenchymal markers (e.g., CD200, CD105). Morphologic and phenotypic characteristics and culture conditions of similar types of cells have been recently described [28–30]. For in vivo experiments, cryopreserved PDACs were thawed and diluted in 10% dextran buffer. The final cell suspension buffer (diluent) contained 10% dextran, 0.9% sodium chloride, 2.5% dimethyl sulfoxide (DMSO), and 6% serum. Similar volumes and contents of the diluents, including DMSO, were used for treating indicated control groups. For experiments coculturing PDACs with myeloma cells or osteoclast precursors, PDACs were incubated in medium specified below ("Myeloma cell-PDAC/MSC cocultures"). For in vivo tracking, PDACs were stably infected with lentivirus containing luciferase/enhanced green fluorescent protein (EGFP) constructs as described [31]. Luciferase and EGFP expression was periodically monitored and detected in PDACs during their expansion in culture for several months (>10 passages).

Myeloma Cells and MSCs

Primary myeloma cells were obtained from heparinized bone marrow aspirates from patients with active MM during scheduled clinic visits. Signed Institutional Review Board-approved informed consent forms are kept on record. CD138-expressing myeloma cells were isolated and used for in vitro studies or global gene expression profiling as previously described [32, 33]. Myeloma cells used for the in vivo study were obtained from two patients. When myeloma cells were collected, patient 1 was previously treated and had stage IIIa MM, β 2M level of 19.6 mg/l, and myeloma cells-expressing IgG λ and molecular classification [33] in the proliferation, high-risk subgroup. Patient 2 was newly diagnosed with stage IIIb MM and had β 2M level of 34 mg/l and myeloma cells-expressing λ -free light chain and molecular classification in the MS, high-risk subgroup. Human myeloma cell lines (H929, ARP1, U266, and HLE) were cultured in RPMI1640 medium (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS) and 4 mM L-glutamine. Stroma-dependent myeloma cell lines (BN, JB, and DNC) were established in our institute and were maintained in coculture with MSCs as previously described [31]. Myeloma cells lines were stably infected with lentivirus-expressing luciferase and EGFP constructs as previously described [31].

MSCs from human fetal bone or from patient bone marrow samples were prepared as previously described [34]. Briefly, fetal fibula (Advanced Bioscience Resources, Alameda, CA) or patients' biopsies were crushed into small pieces and were cultured in Dulbecco's modified Eagle's medium, low glucose, supplemented with 10% FBS, and antibiotics (MSC medium). Half of the medium was replaced every 4–6 days, and adherent cells were allowed to reach 80% confluency before they were subcultured with trypsin-EDTA. Global gene expression profiling revealed that these MSCs did not express hematopoietic markers, such as CD38, CD45, or CD14.

Myelomatous SCID-Rab and SCID Mice

SCID-rab mice were prepared as previously described [27]. Briefly, 6- to 8-week-old CB.17/ Icr-SCID mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN), and pregnant New Zealand rabbits were obtained from Myrtle Rabbitry (Thompson Station, TN). The mice, female rabbits, and their offspring were housed and monitored in our animal facility. The Institutional Animal Care and Use Committee approved all experimental procedures

and protocols. The 4-week-old rabbits were deeply anesthetized with a high dose of phentobarbital sodium and euthanized by cervical dislocation. Each femur and tibia was cut into two pieces, with the proximal and distal ends kept closed. A bone was inserted subcutaneously in each mouse through a small (5 mm) incision. The incision was then closed with sterile surgical staples, and engraftment of the bones was allowed to take place for 6–8 weeks. For each experiment, myeloma cells (1 \times 10⁶ cells in 100 μ l phosphatebuffered saline [PBS]) were injected directly into the open end of the implanted rabbit bone. Mice were periodically bled from the tail vein, and changes in levels of circulating human Ig (hIg) of the M-protein isotype were used as an indicator of myeloma growth. Levels of circulating human κ and λ light chains were determined by enzyme-linked immunosorbent assay as previously described [35, 36]. Cytotherapy was initiated when Ig levels were $\geq 2 \mu g/$ ml in primary myeloma-bearing hosts and $\geq 0.1 \, \mu g/ml$ in H929-bearing hosts. Radiographs were taken with an AXR Minishot-100 beryllium source instrument (Associated X-Ray Imaging Corp., Haverhill, MA). Changes in bone mineral density (BMD) of the implanted rabbit bone were determined using a PIXImus DEXA (GE Medical Systems LUNAR, Madison, WI) [5].

For evaluating subcutaneous growth of the H929 myeloma cell line, myeloma cells (5×10^6 cells per mouse) were injected subcutaneously in SCID mice. PDAC treatment was initiated 4 weeks after injection of H929 myeloma cells. The effects of cytotherapy on subcutaneous myeloma burden were determined by measurements of κ light chain and tumor volume (length \times width² \times [1/2]).

Cytotherapy

The number of mice used for experiments varied (5–8 mice per group) as indicated for each experiment. In indicated experiments, PDAC expressing a luciferase/EGFP construct were used to allow imaging analyses. For intrabone injections, PDACs ($0.1-1 \times 10^6$ cells per mouse) or MSCs (1×10^6 cells per mouse) in 100 μ l of diluent were injected directly into the open end of the implanted rabbit bone in each SCID-rab mouse. For intratumor injections, PDACs (1×10^6 cells per mouse) were injected into the subcutaneous tumor areas in SCID mice (100μ l/injection); for intravenous (i.v.) injections, cells were injected into the tail veins in SCID-rab mice (200μ l/injection). For subcutaneous engraftment, PDACs were encapsulated using HyStem-C hydrogel Kit (Glycosan Biosystems, Salt Lake City, UT) and injected (5×10^6 cells per mouse) according to the manufacturer's instructions.

Live Animal and Ex Vivo Imaging

Luciferase-labeled cells were traced within mice by using live animal imaging and, at the end of the experiment, within the implanted bones and mouse tissues by using ex vivo imaging. For live imaging, mice were anesthetized with ketamine plus xylazine and injected intraperitoneally with D-luciferin firefly (150 mg/kg; Xenogen Corp., Alameda, CA). Luciferase activity was localized and quantified using an IVIS 200 imaging system (Xenogen) as previously described [31]. For ex vivo imaging, the implanted rabbit bone was cut in half, and murine organs were cut into small pieces and placed into a six-well plate. D-luciferin (100 μ l of 15 mg/ml diluted in PBS) at room temperature was added to each well; imaging analysis was performed with the IVIS 200 imaging system.

Immunohistochemistry and Histochemistry

Rabbit bones were fixed in 10% phosphate-buffered formalin for 24 hours. Rabbit bones were decalcified with 10% (wt/vol) EDTA, pH 7.0, and embedded in paraffin for sectioning. Sections (5 μ m) were deparafinized in xylene, rehydrated with ethanol, and rinsed in saline and then underwent antigen retrieval by microwave. After peroxidase quenching with 3%

hydrogen peroxide for 5 minutes and blocking with mouse serum to prevent nonspecific binding (Dako, Carpinteria, CA), sections were reacted with 5 μ g/ml of mouse antibovine osteocalcin monoclonal antibody and mouse IgG control antibody (QED Bioscience Inc., San Diego, CA); the assay was completed with the use of Dako's immunoperoxidase kit. Sections were lightly counterstained with hematoxylin [27, 34]. According to the manufacturer, the osteocalcin antibody cross-reacts with human and rabbit tissues but not with mouse tissues. Deparaffinized bone sections were stained with tartrate-resistant acid phosphatase (TRAP) with the use of an acid phosphatase kit (Sigma, St. Louis, MO) [37]. For cell quantification, osteocalcin-expressing osteoblasts and TRAP-positive multinucleate osteoclasts in four nonoverlapping, millimeter square areas were counted. Immunohistochemical staining for GFP was performed as previously described [34]. For image processing, an Olympus BH2 microscope (Olympus, Melville, NY) was used to obtain images with a SPOT 2 digital camera (Diagnostic Instruments Inc., Sterling Heights, MI). Adobe Photoshop, version 10 (Adobe Systems, San Jose, CA), was used to process the images.

Apoptosis and Differentiation of Osteoclast Precursors

Osteoclast precursors and bone-resorbing osteoclasts were prepared as previously described [38]. Briefly, human blood mononuclear cells were cultured in 24-well plates at 2.5×10^6 cells per millimeter in α -minimal essential medium supplemented with 10% FBS, RANKL (receptor activator for nuclear factor κ B ligand, 50 ng/ml; PeproTech Inc., Rocky Hill, NJ), macrophage colony-stimulating factor (25 ng/ml; PeproTech), and antibiotic cocktail (penicillin, streptomycin, and neomycin; Gibco, Grand Island, NY; osteoclast medium) for 3–4 days, at which time nonadherent cells were removed; the remaining adherent cells were used as osteoclast precursors [38]. Osteoclast medium was used for the entire study.

For coculture experiments, PDACs were cultured in the upper chamber of $1-\mu$ m Transwell inserts (noncontact conditions) and placed in 24-well plates containing osteoclast precursors in the bottom of the wells. This experimental setting precluded mixture of the two cell types, permitting analysis of apoptotic osteoclast precursors. For determining apoptosis or viability, the Transwell inserts were removed after 4 days, and osteoclast precursors were stained with annexin V or propidium iodide (PI; BioVision, Exton, PA). For investigating the effects of PDACs on osteoclast differentiation, osteoclast precursors were cultured alone or cocultured with PDACs in noncontact conditions; after 8 days, number of TRAP-expressing multinucleated osteoclasts were determined and analyzed as previously described [38].

Myeloma Cell–PDAC/MSC Cocultures

For assessing the effects of PDACs or MSCs on growth of six luciferase-expressing myeloma cells lines, PDACs or MSCs were cocultured with each of the myeloma cell lines (10⁴ cells per well for each cell type) in 96-well white plates in RPMI medium supplemented with 10% FBS for 7 days. Luciferase intensity was used to determine effects on growth as previously described [33].

For testing the effects of cocultures on survival of primary myeloma cells from six patients, PDACs or fetal MSCs were cultured on the back of $3-\mu$ m Transwell inserts (10^5 cells per insert), and CD138-selected myeloma cells (5×10^5 cells per insert) were placed in the upper chambers of the inserts in 24-well plates for 7 days, as previously described. This coculture system permits limited contact between myeloma cells and their feeder cells and allows recovery of most myeloma cells. At the end of the experiment, myeloma cell viability was analyzed by trypan blue exclusion, and cell growth was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [34].

Statistical Analysis

All values are expressed as mean \pm SEM. Student's unpaired or paired *t* test was used when two groups (e.g., control vs. PDAC) were tested in vivo or in vitro. In case of comparing multiple groups (e.g., control, MSC, and PDAC), ANOVA (Dunnett Multiple Comparisons Test) was used to assess effects on parameters of bone disease and myeloma cell growth.

Results

PDACs Promote Bone Formation in Myelomatous and Nonmyelomatous Bone and Inhibit Myeloma Growth

The effects of PDACs on myeloma bone disease and tumor growth were examined in the SCID-rab model, using primary myeloma cells from two patients and the H929 myeloma cell line. In this animal model, growth of patient-derived myeloma cells is restricted to the implanted bone, but H929 myeloma cells are capable of also growing in murine organs (e.g., in mouse bone or subcutaneously). Ten SCID-rab mice were engrafted with primary myeloma cells from patient 1. On detection of tumor growth (3 weeks after injection of myeloma cells), vehicle or PDACs (1×10^6 cells per mouse) were injected directly into the implanted bones in SCID-rab mice (five mice per group). Response to treatment was examined 5 weeks after initiation of treatment. BMD of the implanted bone in vehicletreated hosts (controls) was $37\% \pm 2\%$ lower than pretreatment levels, but in the PDACtreated hosts it was 41% \pm 22% higher than pretreatment levels (p < .01, Fig. 1A). At the end of the experiments, implanted rabbit bones were removed and histologically analyzed; the two groups did not differ in the numbers of TRAP-expressing osteoclasts, but the numbers of osteocalcin-expressing osteoblasts were markedly higher in PDAC-injected bones than in vehicle-injected bones (Fig. 1B). PDAC treatment resulted in increased mass of myelomatous bones, which was visualized by x-ray radiographs (Fig. 1C), and in marked inhibition of myeloma cell growth, which was demonstrated by hIg enzyme-linked immunosorbent assay and analysis of tumor area in bone (Fig. 1D-1G).

To determine the effects of PDAC on BMD of non-myelomatous bone, vehicle or PDACs (1×10^6 cells per mouse) were injected directly into the implanted nonmyelomatous bones in SCID-rab mice. After injection of PDACs, but not vehicle, BMD of the implanted bone was markedly higher than pretreatment levels (Fig. 1H). These data indicate that directly injecting PDACs into myelomatous or nonmyelomatous bone resulted in increased local bone mass and that increased bone formation resulting from PDAC injection was associated with reduced myeloma burden.

PDACs Effects on Myeloma Bone Disease and Tumor Growth Are Dose-Dependent and Comparable with Those of Fetal MSCs

SCID-rab mice were engrafted with primary myeloma cells from patient 2; the myeloma cells did not grow in culture but were successfully passaged in the SCID-rab model. Treatment was initiated when myeloma growth was well-established and osteolytic lesions were evident (6 weeks after injection of myeloma cells). PDACs were injected directly (intralesional injection) into the implanted bones $(0.1-1 \times 10^6 \text{ PDACs}$ per bone, seven hosts per group) or subcutaneously using HyStem-C hydrogel carrier $(5 \times 10^6 \text{ PDACs}$ per mouse, six mice). Four weeks after treatment, no effects on BMD of the implanted bone or on myeloma growth were observed in response to subcutaneous injection or to intralesional injection of $0.1 \times 10^6 \text{ PDACs}$ (Fig. 2A). However, in mice that received intralesional injection of $0.5 \times 10^6 \text{ PDACs}$, bone loss was lower than in the control group (p < .05), and in mice that received intralesional injection of $1 \times 10^6 \text{ PDACs}$, BMD of the implanted bones increased from pretreatment levels (p < .01; Fig. 2A). The increased bone mass that resulted from injection of $1 \times 10^6 \text{ PDACs}$ was associated with reduced myeloma growth (p < .08;

Fig. 2B). Subcutaneous engraftment of high number of PDACs (see Fig. 4, below) had no effect on myeloma bone disease or tumor growth, suggesting that the presence of PDACs in or near a focal lesion is required for the effects on myeloma bone disease and tumor growth.

We also compared the effects of PDACs with those of human fetal MSCs on myeloma bone disease and tumor growth. Each cell type was injected $(1 \times 10^6 \text{ cells per mouse})$ directly into the implanted bones of SCID-rab mice 7 weeks after injection of myeloma cells from patient 2 (seven hosts per group). Treatment with PDACs and with MSCs resulted in increased BMD of the implanted bone from pretreatment levels, although the effect of PDACs was more profound (Fig. 2C). Treatment with PDACs and with MSCs significantly inhibited growth of myeloma cells in the SCID-rab model (Fig. 2D). These results suggest that, like fetal MSCs, PDACs have high bone anabolic activity in vivo.

PDACs Inhibit Growth of H929 Myeloma Cells in Bone but Not in Subcutaneous Microenvironment

To further shed light on the antimyeloma mechanism of action of PDACs, the effects of PDACs on myeloma cells growing in the implanted bones in SCID-rab mice were compared with those on myeloma cells growing subcutaneously in SCID mice. H929 myeloma cells were used for this study because this cell line is capable of subcutaneous growth in SCID mice [39]. Myeloma growth was evident (based on hIg measurement) 4 weeks after injection of tumor cells into SCID-rab mice and SCID mice. At this stage, PDACs (1×10^{6} cells per mouse) were injected into the implanted bone or into the subcutaneous tumor (eight mice per group). In SCID-rab mice, BMD of the implanted bones in control mice was reduced 6.7% \pm 7% from pretreatment levels, but it was increased 32% \pm 11% (p < .04) from pretreatment levels in the group treated with PDACs (Fig. 3A). The bone anabolic effect of PDACs in myelomatous bones was observed on x-ray radiographs (Fig. 3B), and histological analysis of the myelomatous bones revealed reduced numbers of TRAPexpressing osteoclasts and increased numbers of osteocalcin-expressing osteoblasts in response to treatment with PDACs (Fig. 3C). Finally, PDAC treatment was also associated with reduced growth of H929 cells in the implanted bones, as assessed by hIg measurements (Fig. 3D). In contrast, injecting PDACs into subcutaneous tumors of H929 myeloma cells in SCID mice had no effect on growth of tumor cells, based on hIg and tumor-volume measurements (Fig. 3E, 3F). These data reveal a close association between the stimulatory effects of PDACs on bone formation and reduced myeloma burden, and they suggest that PDACs have neither stimulatory nor inhibitory effects on extramedullary myeloma.

PDACs Are Short-Lived in In Vivo and Mediate Their Effects Through a Trophic Mechanism

Nonmyelomatous SCID-rab mice and SCID-rab mice engrafted with myeloma cells from patient 1 were treated with PDAC infected with lentivirus containing luciferase/EGFP constructs to allow in vivo tracking. We previously showed that the number of luciferase-expressing cells correlates well with luciferase intensity in the SCID-rab model [5, 33]. One to five days after injecting the labeled PDACs into myelomatous (Fig. 4A) and nonmyelomatous (Fig. 4D) implanted bones, high levels of luciferase bioluminescence were detected in the implanted bones. Two weeks after injection, PDAC bioluminescence levels were substantially reduced; by 4 weeks, PDAC bioluminescence was undetectable in myelomatous bones (Fig. 4A), but live animal imaging detected residual cells in three of five nonmyelomatous hosts (Fig. 4D).

Sections of myelomatous bone were immunohistochemically stained for GFP 5 days and 5 weeks after engraftment of PDAC (at the end of the experiment), to assess the presence of PDAC (Fig. 4C). At 5 days, the presence of PDAC in bone was profound, whereas at 5 weeks, few GFP-expressing PDACs were detectable in the bone marrow. PDAC rarely

differentiated into osteogenic cells, such as osteoblasts and osteocytes (Fig. 4C). Live animal imaging of PDAC mixed in HyStem-C hydrogel carrier and injected subcutaneously also revealed gradual disappearance of PDACs, although these cells were still detected 8 weeks after engraftment (Fig. 4E), suggesting that the HyStem-C hydrogel carrier has a protective effect from the host environment. These results suggest that exogenous PDACs do not survive in bone for long period of time and that their stimulatory effects on bone formation are indirectly mediated through effects on endogenous osteogenic cells (i.e., a trophic mechanism [40]).

Trafficking of PDACs into Myelomatous Bone Following I.V. Injection

H929 myeloma cells were injected into the implanted bones in SCID-rab mice. When high tumor burden was established, luciferase/EGFP-expressing PDACs (1×10^6 cells per mouse) were injected intravenously into 18 SCID-rab mice. Live animal and ex vivo imaging were performed on days 1 (n = 5), 2 (n = 4), and 7 (n = 9) after PDAC injection. Live animal imaging showed PDAC in lungs on days 1 and 2, but not 7, after PDAC injection (Fig. 5A); ex vivo imaging detected PDAC in lungs on days 1, 2, and 7 but not in any other murine tissues (data not shown). Ex vivo imaging of the implanted myelomatous bones revealed PDAC in two of five bones on day 1, in four of four bones on day 2, and in four of nine bones on day 7 (Fig. 5A).

Immunohistochemical staining for GFP in myelomatous bone sections from days 1 and 7 detected few GFP-expressing PDACs in rabbit marrow areas infiltrated with myeloma cells (Fig. 5B). Furthermore, a single i.v. injection of PDACs was insufficient to affect myeloma bone disease or tumor growth in the experimental model (data not shown). These data indicate that most intravenously injected PDACs are trapped in lungs and disappear within 7 days and that few PDACs are capable of trafficking into myelomatous bones.

In Vitro, PDACs Induce Apoptosis of Osteoclast Precursors and Inhibit Formation of Osteoclasts

To study the effects of PDACs on apoptosis of osteoclast precursors, we used a noncontact coculture system, where osteoclast precursors were generated in a 24-well plate and PDACs were cultured in 1- μ m Transwell inserts (10⁴ cells per Trans-well) for 3 days. Coculturing osteoclast precursors with PDAC resulted in a significant increase in number of apoptotic (annexin positive) and nonviable (PI positive) osteoclast precursors (Fig. 6A, 6B). Using a similar 8-day coculture system, we investigated the effects of PDACs on osteoclast differentiation and found approximately 60% fewer mature osteoclasts (TRAP-expressing multinucleated cells) when osteoclast precursors were cocultured with PDACs than when they were cultured alone (p < .0002; Fig. 6C, 6D). Similar results were obtained when conditioned medium from PDACs was added to osteoclast precursors cultured alone (data not shown). These results suggest that PDACs produce soluble factors that inhibit osteoclastogenesis.

Myeloma Cell Survival Is Lower in Coculture with PDACs Than in Coculture with MSCs

To assess the effects of PDACs and MSCs (from human fetal bone and from bone marrow of patients with MM) on survival and growth of myeloma cell lines, we used a panel of luciferase-expressing myeloma cell lines, which were cocultured with PDACs or MSCs for 7 days in conditions that allow cell-cell contact. Mesenchymal cells (e.g., MSC) are long known to support myeloma cell survival in vitro [19, 41]. We found that, in contrast to MSCs, PDACs moderately supported growth of myeloma cells (Fig. 7A). The growth of myeloma cells (determined by measuring luciferase activity) [31] was consistently and significantly lower (p < .01) in coculture with PDACs than in coculture with similar number of MSC generated from fetal bone or bone marrow of patients with MM (Fig. 7B).

PDACs or MSCs were cultured on the backside of 24-well Transwell membranes, and myeloma plasma cells were cultured in the upper chambers of the Transwell inserts [34]. This approach allows close interactions between the cell types and facilitates collection of myeloma cells at the end of the experiment. Myeloma plasma cells (5×10^5 cells per well) from six patients were cocultured with PDACs or MSCs (10^5 cells per well) for 7 days. Myeloma cell viability (determined by trypan blue exclusion; Fig. 7B) and overall growth (using MTT assay; data not shown) in coculture with PDACs were similar to those with MSCs, with a trend toward increased myeloma cell apoptosis in coculture with PDACs (Fig. 7C). These results suggest that, in comparison with coculture with MSCs, coculture with PDACs results in reduced growth of rapidly growing myeloma cell lines, but no significant change is seen in survival of primary myeloma cells, which often remain quiescent in coculture with mesenchymal cells or spontaneously undergo apoptosis in vitro.

DISCUSSION

In this study, we demonstrated that injecting PDACs into myelomatous osteolytic bone lesions effectively prevented bone loss and promoted bone formation by inhibiting osteoclast formation and stimulating differentiation of the host's osteoblasts. Intralesional PDAC cytotherapy also resulted in inhibiting growth of H929 myeloma cells and primary myeloma cells categorized by global gene expression classification [31, 32] as high risk. Furthermore, PDACs had no effect on subcutaneous growth of H929 myeloma cells in SCID mice, and PDACs did not confer a growth advantage to myeloma cells cocultured with PDACs or the supportive MSCs [41]. Along with the reported potential for PDACs to enhance engraftment of hematopoietic stem cells [26], our results provide proof-of-concept that PDAC cytotherapy is a promising approach for treating myeloma bone disease and controlling myeloma progression.

Our study revealed that, like MSC [40], exogenous PDACs were not detectable in vivo for long periods of time; the majority of these cells disappeared 3-5 weeks after injection. Clinically, this phenomenon might be advantageous because it limits the duration of the intervention. Biologically, these results indicate that, like MSCs, PDACs can be expanded and undergo many passages in vitro, but in vivo they lack typical stem cell properties such as self-renewal potential and differentiation into mesenchymal lineages (e.g., osteoblasts). Rather, these cells stimulate bone metabolism by acting as bystander cells that increase endogenous osteoblastogenesis and inhibit osteoclastogenesis, presumably by producing soluble factors and by cell-cell interactions. Interestingly, the ability of PDACs to increase bone mass and subsequently reduce myeloma burden seem to be sustained even when most PDACs have already disappeared, consistent with similar "touch-and-go" mechanisms implied for MSCs [22].

Despite a safety concern that mesenchymal cells, rich with cytokines and chemokines, may stimulate tumor growth [42], our findings revealed that growth rates of myeloma cell lines were lower when cocultured with PDACs than with MSCs from fetal bone or MM patients. In vivo, PDACs and MSCs suppressed myeloma growth in bone, but injection of PDACs into subcutaneous tumors had no effect on growth of myeloma cells. Antitumor effects of bone marrow MSCs were recently demonstrated in Kaposi's sarcoma, a highly inflammatory angiogenic malignancy, and were attributed to contact-dependent inhibition of Akt activity in a xenograft model and coculture setting [43]. The mechanisms by which PDACs inhibit myeloma cell growth in bones can only be partially delineated from our study. We and others have demonstrated that inhibition of bone resorption and stimulation of bone formation by pharmaceutical and osteoblast-activating agents or MSC cytotherapy is associated with inhibition of myeloma burden in experimental models [9, 37, 39, 44, 45].

PDACs are likely to affect osteoclastogenesis and osteoblastogenesis: two unregulated physiological processes in myelomatous bone [46]. Our in vitro study demonstrated that PDACs directly inhibit osteoclast formation. Osteoblastogenesis is impaired in MM, particularly as a consequence of suppression of Wnt signaling in bone through production of Wnt inhibitors such as DKK1 [10]. PDACs may help restore critical signaling pathways associated with osteoblast deactivation in MM, resulting in increased bone formation.

Similar to MSCs [22], PDACs may also exert immunomodulatory properties, mostly immunosuppressive effects in vivo. Our study used SCID mice that lack T and B lymphocytes but still harbor other immune cells such as NK cells and macrophages, which may be responsible for the rapid clearance of PDACs. The consequences of the interactions of PDACs with host macrophages on the properties, survival, and function of both cell types are yet to be determined.

Our study strongly suggests that intralesional PDAC cytotherapy will help recover large osteolytic lesions in patients with MM and suppress myeloma growth in these lesions. This is of particular interest because, as we have reported, relapses occur in pre-existing lesions in most patients with focal myeloma lesions, suggesting that these lesions may harbor "dormant myeloma cells" [1]. The consequences of systemic injection of PDACs on myeloma bone disease and tumor growth are still under investigation. Our experimental data indicate that the majority of PDACs are trapped in the lungs after i.v. administration, but a few of these cells do traffic into implanted myelomatous bones in SCID-rab mice, particularly, when myeloma burden is well established. These results suggest that PDACs are attracted by chemokines produced by myeloma cells or by conditions induced as a consequence of myeloma growth in bone (e.g., wound-like conditions). MSCs were previously reported to home to tumor sites [47], and although various chemokines and their receptors have been implicated in this process, the common notion is that a complex set of factors attract MSCs to tumor sites [42]. An ongoing study is underway to develop approaches to improve trafficking of PDACs or MSCs to myelomatous bone, increasing their systemic therapeutic potential during active disease or maintenance therapy.

CONCLUSION

This study demonstrated that PDACs effectively promote bone formation and inhibit bone disease and growth of highly aggressive myeloma cells in bone with a "touch-and-go" mechanism of action.

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Figure 1.

Intrabone injection of PDAC promotes bone formation in myelomatous and nonmyelomatous bones and inhibits growth of myeloma cells. Nonmyelomatous severe combined immunodeficient (SCID)-rab mice or SCID-rab mice engrafted with myeloma cells from patient 1 were injected with PDAC (1×10^6 cells/bone) or control vehicle (five mice per group). (A): Changes from pretreatment levels of BMD of the implanted myelomatous bone. (B): Number of TRAP-expressing osteoclasts and osteocalcinexpressing osteoblasts in the myelomatous bone sections at the end of the experiment (5 weeks). (C): X-ray radiographs of the myelomatous bones from 10 mice before treatment and at the end of the experiment (final). (D): Human Ig levels from myeloma-engrafted SCID-rab mice prior to treatment and 2 and 4 weeks after treatment. (E): Histomorphometric analysis of tumor area in the implanted myelomatous bone. (F, G): Representative H&E staining of myelomatous bones from control (F) and PDAC-treated (G) myeloma-engrafted SCID-rab mice (MM, OC, OB). Note that PDAC-treated bone (G) exhibited reduced myeloma cell infiltration, increased osteoblast numbers along the bone surface, and normal marrow structure, as compared with control bone (F). (H): Changes in BMD from pretreatment levels of the implanted nonmyelomatous bone in SCID-rab mice not engrafted with myeloma cells. See "Materials and Methods" section for the acquisition and processing of images. Abbreviations: BMD, bone mineral density; CONT, control vehicle; MM, myeloma; OB, osteoblasts; OC, osteoclasts; PDAC, placenta-derived adherent cell; pre-Rx, prior to treatment.



Figure 2.

Effects of PDAC on myeloma bone disease and tumor growth are dose-dependent and comparable with those of fetal MSC. Severe combined immunodeficient (SCID)–rab mice engrafted with myeloma cells from patient 2. (**A**, **B**): On establishment of high tumor burden, hosts were intralesionally injected with vehicle (CONT) or with PDAC (0.1, 0.5, or 1×10^6) or they were subcutaneously engrafted with PDAC (5×10^6) by using HyStem-C hydrogel carrier (six to seven mice per group). (**A**): Changes from pretreatment levels of BMD of the implanted myelomatous bone and (**B**) changes in human Ig (hIg) prior to treatment and 2 and 4 weeks after treatment. (**C**, **D**): Hosts were intralesionally injected with vehicle, PDAC (1×10^6), or MSC (1×10^6); (**C**) changes in BMD from pretreatment levels of the implanted myelomatous bone and (**D**) changes in hIg prior to treatment and at the end of the experiment (final). Abbreviations: BMD, bone mineral density; CONT, control vehicle; IL, intralesional; MSC, mesenchymal stem cell; PDAC, placenta-derived adherent cell; pre-Rx, prior to treatment; SC, subcutaneous.



Figure 3.

PDACs inhibit growth of H929 myeloma cells in bone but not in subcutaneous microenvironment. H929 myeloma cells were engrafted in the implanted bone in Severe combined immunodeficient (SCID)–rab mice (A-D) or injected subcutaneously in SCID mice (E, F). On establishment of myeloma, vehicle (CONT) or PDACs (1×10^6 cells per mouse) were injected into the implanted bone or subcutaneous tumor (eight mice per group). In SCID-rab mice engrafted with myeloma, (A) changes in BMD of the implanted bone; (B) representative x-ray radiographs of the myelomatous bones from three of eight mice from each group, taken before treatment and at the end of the experiment (final); (C) numbers of tartrate-resistant acid phosphatase-expressing osteoclasts and osteocalcin-expressing osteoblasts in the myelomatous bone sections; and (D) human Ig (hIg) levels before treatment and at the end of the experiment and (F) subcutaneous tumor volume. Abbreviations: BMD, bone mineral density; CONT, control vehicle; PDAC, placenta-derived adherent cell; pre-Rx, prior to treatment.

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Figure 4.

PDACs injected intralesionally gradually disappear from bone within 2–4 weeks. Luciferase/GFP-expressing PDACs were analyzed by live animal imaging and immunohistochemistry. (**A**, **B**): Myelomatous severe combined immunodeficient (SCID)– rab mice were engrafted with myeloma cells from patient 1 and were intralesionally treated with luciferase/enhanced green fluorescent protein (EGFP)–expressing PDACs, which were examined by (**A**) live animal imaging and (**B**) bioluminescence analysis. (**C**) Myelomatous bone sections were immunohistochemically stained for EGFP 5 days and 5 weeks after injection of PDACs; note that clusters of PDAC were visible at day 5, whereas at 5 weeks, PDACs were occasionally detected with fibroblastoid-like and occasionally osteoblast-like, morphologies. (**D**): Nonmyelomatous SCID-rab mice were injected (via intrabone route) with PDACs and were examined by live animal imaging. (**E**): Hosts were subcutaneously injected with 5×10^6 PDACs (using HyStem-C hydrogel carrier) and examined by live animal imaging. See "Materials and Methods" section for the acquisition and processing of images. Abbreviations: OB, osteoblasts; PDAC, placenta-derived adherent cell.



Figure 5.

PDACs are capable of trafficking to myelomatous bone. Luciferase/enhanced green fluorescent protein (EGFP)–expressing PDACs were intravenously injected into SCID–rab mice engrafted with H929 myeloma cells; at indicated times, labeled PDACs were examined by live animal and ex vivo imaging and by immunohistochemical analysis for GFP. (**A**): Live animal imaging and ex vivo imaging of implanted bones in individual mice. Bioluminescence activity was detected in implanted bones of 10 of 18 mice (numbers 2, 5, 6, 7, 8, 9, 12, 13, 15, and 18). (**B**): Sections of implanted myelomatous bones were immunohistochemically stained for GFP, which detected PDACs in these bones 1 and 7 days after they were injected. See "Materials and Methods" section for the acquisition and processing of images. Abbreviations: MM, multiple myeloma; PDAC, placenta-derived adherent cell.



Figure 6.

PDACs induce apoptosis in osteoclast precursors and inhibit osteoclast formation. Osteoclast precursors were cultured alone (CONT) or cocultured with PDACs in noncontact conditions in osteoclast medium. (**A**): Numbers of apoptotic (annexin V positive) or nonviable (PI positive) osteoclast precursors after 4 days of culture. (**B**): Representative photos demonstrating the effects of PDACs on osteoclast apoptosis (top panel) and viability (middle panel). Total numbers of TRAP-expressing osteoclast precursors are visualized on the bottom panel. (**C**): Numbers of multi-nucleated and TRAP-expressing osteoclasts after 8 days of culture. (**D**): Representative photos demonstrating the effects of PDACs on OC differentiation. See "Materials and Methods" section for the acquisition and processing of images. Abbreviations: CONT, control vehicle; OC, osteoclast; PDAC, placenta-derived adherent cell; PI, propidium iodide; TRAP, tartrate-resistant acid phosphatase.



Figure 7.

Survival of myeloma cells is poorer in coculture with PDACs than with MSCs. (A): The luciferase-expressing myeloma cell lines U266 and JB were cultured in a 96-well plate alone (CONT; 10^4 cells per well) or were cocultured for 7 days with PDACs or fetal MSCs (10^4 cells per well, for each cell type) and then subjected to bioluminescence quantification for measurement of growth rate [31]. Note that MSCs and PDACs supported growth of myeloma cells but the effect of MSCs was more profound. Indicated p values are compared with CONT group. (B): Similar to (A), a panel of seven luciferase-expressing myeloma cell lines (BN, JB, ARP1, U266, DNC, HLE, H929; 10⁴ cells per well) were cocultured for 7 days with fetal MSCs, Pt. MSCs, or PDACs and then subjected to bioluminescence quantification. Results are expressed as fold of myeloma cell growth in coculture with fetal MSCs. Note reduced growth of myeloma cell lines in coculture with PDACs (p < .01, comparing all tested cell lines using analysis of variance). (C): CD138-selected myeloma cells (5 \times 10⁵ cells per well) from six patients were cocultured for 7 days in a 24-well plate in conditions allowing limited cell-cell contact with fetal MSCs or PDACs (10^5 cells per well, for each cell type) and then subjected to trypan blue analysis. Survival of myeloma cells was slightly, but not significantly, lower in coculture with PDACs than with fetal MSCs. (D): Annexin V/PI analysis of CD138-selected myeloma cells (from patient D) revealed a higher percentage of apoptotic myeloma cells in coculture with PDACs than with fetal MSCs. Abbreviations: CONT, control vehicle; MSC, mesenchymal stem cell; PDAC, placenta-derived adherent cell; Pt. MSC, patient mesenchymal stem cell.