CD47 Regulates Bone Mass and Tumor Metastasis to Bone

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Abstract

CD47, also called integrin-associated protein, plays a critical role in the innate immune response and is an atypical member of the immunoglobulin superfamily that interacts with and activates β3 integrins. β3 integrin−/− mice have defective platelet and osteoclast function and are protected from bone metastasis. The role of CD47 in skeletal homeostasis and bone metastasis has not been described. CD47−/− mice had increased bone mass and defective osteoclast function in vivo. Although the number of functional osteoclasts formed by differentiating CD47−/− bone marrow macrophages was decreased, high doses of RANKL rescued differentiation and function of CD47−/− osteoclasts ex vivo and rescued the osteoclast defect in CD47−/− mice. Inhibition of nitric oxide (NO) synthase, which is expressed at higher levels in CD47−/− osteoclasts, also rescued the osteoclast defect in CD47−/− cells. We then examined the consequences of this osteoclast defect in bone metastasis. In a model of tumor metastasis to bone, bone tumor burden was decreased in the CD47−/− mice compared with wild-type (WT) controls, with no decrease in s.c. tumor growth in CD47−/− mice. There was decreased tumor-associated bone destruction in the CD47−/− mice compared with WT controls, consistent with a defect in osteoclast function that was not rescued by the presence of tumor. Our data show that CD47 regulates osteoclastogenesis, in part, via regulation of NO production, and its disruption leads to a decrease in tumor bone metastasis. CD47 is a novel therapeutic target to strengthen bone mass and diminish metastatic tumor growth in bone. [Cancer Res 2009;69(7):3196–204]

Introduction

Bone metastases cause hypercalcemia, bone loss, fractures, and pain and are thus a significant cause of morbidity and mortality in cancer patients (1). Several tumor cell types (e.g., breast and prostate carcinomas and melanomas) metastasize to bone and lead to bone degradation via activation of bone-resorbing osteoclasts (1–4). Osteoclasts are formed from the fusion of monocytes/macrophages and are characterized by their large size, the presence of multiple nuclei, and positive staining for tartrate-resistant acid phosphatase (TRAP). Two cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL), are necessary for the survival and differentiation of macrophages into osteoclasts in vitro and in vivo (5). The osteoclast attaches to bone via the αvβ3 integrin through formation of the cytoskeletal organizations called actin rings. The resorbing osteoclast has a ruffled membrane and expresses cathepsin K, which is necessary for degrading the organic matrix of bone, and vascular proton pumps (H+-ATPases), which secrete acid and thus result in bone resorption by dissolving the inorganic matrix (5).

The presence of tumor cells in the bone microenvironment results in osteoclast and osteoblast recruitment and activation. This activation stimulates the release of growth factors from stromal cells as well as from the bone matrix, which further promote tumor growth in bone (1–4, 6). This is known as the vicious cycle of bone metastasis. We have previously shown that hyperfunctional osteoclasts are permissive for the growth of tumor cells that home to bone (7, 8). In addition, mice globally deficient in the β3 integrin, which show a profound defect in platelet and osteoclast function, are protected from bone metastasis and tumor-associated bone destruction (9–11).

CD47 (also called integrin-associated protein) was first isolated through its interaction with the αvβ3 integrin, which is prominently expressed on osteoclasts (12–15). Expressed on all vascular cells, CD47 is an atypical member of the immunoglobulin superfamily (16) with a single IgV domain, five transmembrane segments, and a cytoplasmic tail (17). CD47 associates in cis in the plane of the membrane with αvβ3 and other integrins (12–15) and can augment integrin function by this association (18). It has recently been discovered that CD47, when ligated by thrombospondin-1 (TSP1), inhibits nitric oxide (NO) signaling in vascular cells (19). In platelets, TSP1-CD47 signaling opposes the NO/cyclic guanosine 3′,5′-monophosphate (cGMP) inhibition of integrin activation (20), thus facilitating platelet aggregation. CD47 inhibition of NO signaling has also been shown in several other in vivo models, suggesting that limiting NO signaling is a primary function of the TSP1-CD47 system (19–26). Recent data indicate that the differentiation of bone marrow macrophages into osteoclast in vitro is sensitive to NO concentration, such that low levels of NO stimulate osteoclast formation, whereas increased NO inhibits formation of osteoclast (27). Therefore, CD47 may modulate NO signaling in osteoclast.

Although CD47 associates with αvβ3 and augments its function, β3−/− and CD47−/− mice have rather different phenotypes. β3−/− mice are a model of Glanzmann's thrombasthenia, displaying profound defects in platelet aggregation and clot retraction (10). In contrast, CD47−/− mice have normal bleeding times and a modest decrease in platelet numbers (28). These differences can be accounted by the fact that CD47 augments integrin signaling but is not essential for integrin-mediated adhesion (19). CD47 also has integrin-independent functions. For example, CD47-SIPRα interactions are important in innate immune reactions, negatively regulating phagocytosis in vivo. The presence of CD47 on hematopoietic cells limits their ability to

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cells provides them with a "self-signal" that inhibits their phagocytosis (16, 29). The SIRPα-CD47 interaction has also been postulated to be necessary for macrophage fusion, which is essential for the formation of osteoclasts (30, 31). Recently, Lundberg and colleagues (31) reported a defect in formation of osteoclasts from macrophages in the presence of blocking antibodies to CD47. Because CD47 is proposed to function in macrophage fusion, interacts with and activates avß3, and modulates NO signaling, we hypothesized that CD47−/− mice would have altered osteoclast differentiation and function. Here, we show that CD47−/− mice have a modest increase in bone volume due to defective osteoclast function in vivo. Furthermore, CD47−/− macrophages are defective in osteoclast formation in vitro. The osteoclast defect is rescued by increased administration of RANKL both in vitro and in vivo, and inhibition of NO synthase (NOS) rescues CD47−/− osteoclast differentiation in vitro. To test the role of CD47 in a pathophysiological context in which osteoclast function is central to disease pathogenesis, we evaluated bone metastasis and tumor-associated bone destruction in CD47−/− mice.

Materials and Methods

Cells. The B16-F10 C57BL/6 murine melanoma cell line was purchased from the American Type Culture Collection and modified to express firefly luciferase (B16-FL; ref. 32).

Animals. CD47−/− mice on a pure C57BL/6 background were housed under pathogen-free conditions according to the guidelines of the Division of Comparative Medicine, Washington University School of Medicine. The animal ethics committee approved all experiments. For generation of CD47−/− mice, CD47+/− females were crossed to CD47+/− males and the females were allowed to have two litters.

Micro-computed tomography. Tibias and femurs were suspended in agarose and the right proximal tibial and femoral metaphyses were analyzed under pathogen-free conditions according to the guidelines of the Division of Comparative Medicine, Washington University School of Medicine. The animal ethics committee approved all experiments. For generation of CD47−/− mice, CD47+/− females were crossed to CD47+/− males and the females were allowed to have two litters.

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Histology, bone histomorphometry, and longitudinal growth measurements. Mouse tibias were fixed in formalin and decalcified in 1% EDTA. Paraffin-embedded sections were stained with H&E and measured according to a standard protocol using Bioquant Osteo (Bioquant Research). Histology, bone histomorphometry, and longitudinal growth measurements. Mouse tibias were fixed in formalin and decalcified in 1% EDTA. Paraffin-embedded sections were stained with H&E and measured according to a standard protocol using Bioquant Osteo (Bioquant Research). Histology, bone histomorphometry, and longitudinal growth measurements. Mouse tibias were fixed in formalin and decalcified in 1% EDTA. Paraffin-embedded sections were stained with H&E and measured according to a standard protocol using Bioquant Osteo (Bioquant Research). Histology, bone histomorphometry, and longitudinal growth measurements. Mouse tibias were fixed in formalin and decalcified in 1% EDTA. Paraffin-embedded sections were stained with H&E and measured according to a standard protocol using Bioquant Osteo (Bioquant Research). Histology, bone histomorphometry, and longitudinal growth measurements. Mouse tibias were fixed in formalin and decalcified in 1% EDTA. Paraffin-embedded sections were stained with H&E and measured according to a standard protocol using Bioquant Osteo (Bioquant Research). Histology, bone histomorphometry, and longitudinal growth measurements. Mouse tibias were fixed in formalin and decalcified in 1% EDTA. Paraffin-embedded sections were stained with H&E and measured according to a standard protocol using Bioquant Osteo (Bioquant Research).
Results

CD47<sup>−/−</sup> mice have increased bone volume due to dysfunctional osteoclasts. CD47 interacts with and activates β3 integrins. β3<sup>−/−</sup> mice display osteopetrosis due to osteoclast dysfunction. Therefore, we hypothesized that CD47<sup>−/−</sup> mice might have an osteoclast defect. To test this hypothesis, μCT analysis on tibias from 8-week-old WT and CD47<sup>−/−</sup> littermates was performed. We observed an increase in trabecular BV/TV in CD47<sup>−/−</sup> mice compared with WT mice (Fig. 1A and B). There was no change in trabecular number, but the trabecular thickness was increased in

![Graph showing increased trabecular bone volume in CD47<sup>−/−</sup> mice](image)

**Figure 1.** CD47<sup>−/−</sup> mice have increased bone volume due to dysfunctional osteoclasts. A, WT and CD47<sup>−/−</sup> tibias were subjected to μCT analysis of bone parameters (WT, n = 5; CD47<sup>−/−</sup>, n = 5). Trabecular BV/TV by μCT (P = 0.04); trabecular number by μCT (P = 0.22); trabecular thickness by μCT (P = 0.04). B, representative images of three-dimensional μCT reconstruction of WT and CD47<sup>−/−</sup> tibias (left) and lumbar vertebral bodies of WT and CD47<sup>−/−</sup> mice stained with von Kossa (right). C, a, representative histologic TRAP-stained tibial sections. B, BV/TV in tibias by histomorphometry (P = 0.02). c, osteoclast perimeter/trabecular bone perimeter (P = 0.23). D, collagen breakdown products (CTX) were measured in the serum of starved WT and CD47<sup>−/−</sup> mice (P = 0.03).
CD47−/− tibias (Fig. 1A and B). Von Kossa staining of the lumbar vertebral bodies, a marker for mineralized matrix, confirmed an increase in bone volume and trabecular thickness in CD47−/− mice (Fig. 1B). The increase in trabecular bone volume (BV/TV) was also confirmed by histomorphometry on histologic sections (Fig. 1C, a and b). We did not observe a difference in longitudinal growth of CD47−/− mice as measured by femur length and whole body length (Supplementary Fig. S1). Interestingly, we also observed that osteoclast perimeter/total trabecular bone perimeter was not changed in CD47−/− mice in vivo (Fig. 1C, c). To confirm that this increase in BV/TV was due to dysfunctional osteoclasts, we measured serum CTX, the COOH-terminal telopeptide of collagen type I that is cleaved on bone resorption by osteoclasts. We observed a decrease in CTX activity (Fig. 1D), which along with an increase in trabecular bone volume suggests that the osteoclast activity was decreased in CD47−/− mice in vivo (Fig. 1E).

CD47−/− osteoclast defect can be rescued by high doses of RANKL in vitro. To investigate if the osteoclast defect is cell autonomous in CD47−/− mice, we first determined if macrophages or osteoclast precursors were decreased in CD47−/− bone marrow. We stained WT and CD47−/− whole bone marrow with CD47-FITC as control and F4/80 as a macrophage marker and carried out flow cytometry. We did not observe a significant difference in F4/80+ cells in CD47−/− bone marrow compared with WT controls (Fig. 2A). To test the differentiation of macrophages to osteoclasts, we cultured whole bone marrow in M-CSF alone for 3 days to enrich for macrophages. An equal number of macrophages were then cultured in the presence of M-CSF and RANKL to promote differentiation into osteoclasts for 7 days. At a dose of 50 ng/mL of RANKL, we observed induction of CD47 transcript levels over the course of osteoclast differentiation in WT but not in CD47−/− cells (Fig. 2B). We observed that WT macrophages produced large, multinucleated, TRAP-positive osteoclasts by day 5, but only a few osteoclasts formed from CD47−/− macrophages. When WT and CD47−/− macrophages were plated onto bovine bone slices, there was a decrease in the number of multinucleated CD47−/− osteoclasts with multiple actin rings visualized by phalloidin staining (Fig. 2C). However, when the dose of RANKL was increased 2-fold to 100 ng/mL, the osteoclast differentiation defect was largely rescued in CD47−/− cells. This was reflected in equivalent numbers of osteoclasts with multiple actin rings formed on bovine bone from macrophages of both genotypes (Fig. 2C). To determine
The osteoclast dysfunction in CD47<sup>−/−</sup> mice was rescued by in vivo RANKL injections. To determine if RANKL could rescue CD47<sup>−/−</sup> osteoclast function in vivo as was observed in vitro, we injected 100 µg RANKL subperiostally into the midline calvaria of 8-week-old WT and CD47<sup>−/−</sup> mice. We measured serum CTX before and after RANKL injections in these mice. Before RANKL injection, CD47<sup>−/−</sup> mice had lower serum CTX as we have shown before (Fig. 3A). However, after RANKL administration, osteoclast activation was not significantly different between WT and CD47<sup>−/−</sup> mice as measured by serum CTX (Fig. 3A). Furthermore, direct visualization of osteoclast recruitment by TRAP staining on histologic sections of calvarial bone in WT and CD47<sup>−/−</sup> mice confirmed the CTX results (Fig. 3B). We conclude that a high dose of RANKL rescued the cell-autonomous osteoclast defect in CD47<sup>−/−</sup> mice.

NOS inhibition restores osteoclast differentiation in CD47<sup>−/−</sup> cells. Several tissues from CD47<sup>−/−</sup> mice have increased NO levels (36). We differentiated WT and CD47<sup>−/−</sup> macrophages into osteoclasts in the presence of M-CSF and RANKL. We observed that iNOS expression levels were higher in CD47<sup>−/−</sup> osteoclast cultures compared with WT controls (Fig. 4A). It has been previously shown that increased levels of NO lead to a block in osteoclast differentiation (27). We hypothesized that this increase in NO levels is responsible for the inhibition of osteoclast differentiation in CD47<sup>−/−</sup> cells. We differentiated WT and CD47<sup>−/−</sup> macrophages into osteoclasts in the presence of L-NAME, a pan-NOS inhibitor. In CD47<sup>−/−</sup> cells, we observed a dose-dependent rescue of osteoclast differentiation with L-NAME administration. We observed a biphasic effect of L-NAME on WT cells, where, at a low dose of L-NAME, there was modest enhancement of osteoclast formation and, at a high dose of L-NAME, there was inhibition of osteoclast differentiation (Fig. 4B). The osteoclast inhibitory dose of L-NAME on WT cells was enhancing in CD47<sup>−/−</sup> cells. Taken together, these data support that the increased levels of iNOS in CD47<sup>−/−</sup> cells negatively affect osteoclast formation.

CD47<sup>−/−</sup> mice have decreased tumor burden and bone loss in an intracardiac metastasis model. The data above indicate
that, at high doses of RANKL, CD47 was not necessary for osteoclast differentiation and function. RANKL is produced from T cells and osteoblasts during inflammatory conditions such as arthritis and bone metastasis. RANKL is not expressed by B16-FL cells (data not shown). We thus examined bone metastasis and osteolysis in CD47−/− mice to determine if this local increase in RANKL is able to rescue the CD47−/− osteoclasts in this pathophysiologic context. We evaluated bone metastasis in WT and CD47−/− mice using murine melanoma B16-F10 cells engineered to express firefly luciferase (B16-FL). We measured bone tumor burden by real-time bioluminescence on days 7, 10, and 12 after intracardiac B16-FL injection, a route of administration that allows for bone metastasis rather than lung infiltration of injected cells (9, 32). There was a significant decrease in tumor burden in the femoral/tibial bones and the mandible of CD47−/− mice compared with WT mice (Fig. 5B and C) as measured by bioluminescence and confirmed by histomorphometric measurement of tumor volume in histologic sections at day 12 after B16-FL inoculation (Supplementary Fig. S2; Fig. 5B and C). Tumor cells are known to secrete factors that activate osteoclasts to degrade bone and induce osteolysis, which is a significant outcome of bone metastases (1–4, 6). Trabecular bone volume was measured in
WT and CD47−/− mice injected with B16-FL or saline. Whereas there was significant bone loss in B16-FL in WT mice, there was no bone loss in CD47−/− bones injected with B16-FL, consistent with the osteoclast dysfunction in CD47−/− mice (Fig. 5B and C). We did not observe a difference in osteoclast perimeter/trabecular bone perimeter in tumor-bearing CD47−/− bones (Fig. 5D). Thus, we conclude that CD47−/− mice had decreased bone tumor burden and tumor-associated bone destruction, and the local increase in RANKL levels in vivo was not sufficient to rescue the osteoclast defect in CD47−/− mice.

Figure 6. CD47−/− mice have decreased tumor burden and bone loss in an intratibial metastasis model but not in a s.c. model. A, B16-FL cells were injected directly into the tibia of WT (n = 6) and CD47−/− (n = 5) mice. Tumor burden in the tibia was measured by bioluminescence imaging at days 7 and 9 after B16-FL injection (P = 0.06). Representative bioluminescence images are shown. B, representative images of tibial histologic bone sections at day 9. Tumor volume/total volume in the tibias of WT (n = 6) and CD47−/− (n = 5) mice at day 12 was measured by histomorphometric analysis (P = 0.03). Three independent experiments showed similar results. C, B16-FL cells were injected s.c. into the hind flank of mice, and tumor burden was measured by bioluminescence imaging at days 5, 7, 10, and 14 after B16-FL injection.

To confirm that the decrease in bone tumor burden in CD47−/− mice was specific to CD47 function in the bone microenvironment and not a result of intervention by the immune system or tumor-associated angiogenesis in the CD47−/− mice, we measured local tumor burden in s.c. injected B16-FL cells. We observed no change in s.c. tumor burden as measured by bioluminescence on days 5, 7, 10, and 14, at which point the experiment was terminated due to necrotic tumors (Fig. 6C). We conclude that the decrease in tumor burden in CD47−/− mice was specific to the bone compartment and not due to compromised immunity or platelet interactions.

Discussion

We show here that CD47−/− mice exhibited mild osteopetrosis and decreased osteoclast function in vivo and in vitro. In vitro, CD47−/− macrophages did not form as many multinucleated spread osteoclasts as WT cells. Bone resorption as measured by pit staining was decreased in CD47−/− cells in vitro. Interestingly, this osteoclast defect was rescued by delivery of high doses of RANKL both in vitro and in vivo, suggesting that CD47 may cooperate with RANKL during osteoclast development/function. We observed that iNOS levels were increased in CD47−/− osteoclasts and inhibition of NOS rescued the osteoclast differentiation defect in CD47−/− cells. We also observed decreased tumor burden and osteolysis in CD47−/− bone after intracardiac or intratibial delivery of tumor cells.

The first role known for CD47 was based on its physical and functional interaction with αvβ3 (37). It is well known that the...
integri αvβ3 plays a critical role not only in osteoclast function (11) but also in the differentiation of macrophages into osteoclast (38). CD47 has been shown to augment αvβ3 function by physical association, leading to increased integrin clustering and therefore increases in avidity. Furthermore, CD47 signals through Goi to activate αvβ3 via an “inside-out” signaling pathway analogous to that used by G protein–coupled receptors on platelets to activate αIIbβ3 via activation of the small G protein Rap1 (20). In platelets, NO-stimulated cGMP levels oppose Rap1b activation. CD47 acts to prevent this inhibitory effect of NO, thus allowing more GTP loading of Rap1b, resulting in αIIbβ3 activation (20). It is likely that CD47 in osteoclasts can activate αvβ3 in a similar fashion.

Although CD47 could be playing a role in osteoclast differentiation and function by augmenting αvβ3 signaling, it could also exert its effects on osteoclasts in a β3-independent manner. The defect in osteoclast differentiation can be overcome by addition of high doses of M-CSF to cultures by increasing expression of c-fms (39). In contrast, we observed that exogenous RANKL administration could rescue the osteoclast defect in CD47−/− mice in vivo and in vitro, suggesting that CD47 might play a role in osteoclastogenesis by enhancing RANKL signaling. It is possible that CD47 and RANKL might stimulate osteoclasts in parallel pathways that merge at a common signaling molecule. Our data suggest that the NO/cGMP pathway might be the link between CD47 and RANKL signaling. There is a growing literature on the role of NO in osteoclast differentiation and function. Often, the proposed “Goldilocks effect” comes into play in NO regulation. NO has been reported to both promote and inhibit osteoclast differentiation/survival (40, 42, 43). Low signaling levels of NO derived from eNOS (NOSIII) and/or nNOS (NOSI) are most likely needed to support osteoclast differentiation, whereas higher levels, such as those produced by iNOS, become inhibitory. We found that iNOS was significantly induced in CD47−/− osteoclasts and inhibition with a NOS inhibitor, L-NAME, rescued CD47−/− osteoclast differentiation (Fig. 4B). Many signaling effects of NO are mediated via stimulation of soluble guanylate cyclase, production of cGMP, and activation of protein kinase G. However, there are effects of NO that are accomplished by its direct interaction with protein thiols (S-nitrosylation) to modify their enzymatic activity (44, 45). Src, a kinase downstream of RANK signaling known to mediate via stimulation of soluble guanylate cyclase, production of cGMP, and activation of protein kinase G. However, there are effects of NO that are accomplished by its direct interaction with protein thiols (S-nitrosylation) to modify their enzymatic activity (44, 45). Src, a kinase downstream of RANK signaling known to

is suggested by our finding that only a 2-fold increase in RANKL concentration in vitro permits formation of CD47−/− osteoclasts that are as functional as WT osteoclasts.

The second major finding of our study is that the role of CD47 in osteoclast differentiation/function has a significant effect on the interaction of tumor cells within the bone microenvironment. The decreased osteoclast function in CD47−/− mice leads to a decrease in bone tumor burden and osteolysis, consistent with the model of the vicious cycle of bone metastasis (1–4). Notably, the increase in bone volume in CD47−/− was only ~4% compared with WT mice, not as profound a change as observed in the osteopetrotic β3−/− mice (11). It is interesting that the relatively modest, resuable decrease in osteoclast function in CD47−/− mice in vivo could result in a more pronounced decrease in tumor burden and osteolysis. These results suggest that the relationship between the osteoclast and the tumor cell with regard to the vicious cycle of bone metastasis is not one of direct proportionality. The complex signaling pathways involved in the relationship between tumor cells and bone cells do not involve amplification steps. Both Src kinase–initiated pathways and the NO/cGMP pathways implicated here are classic examples of signal amplifiers.

One of the major complications of currently available therapies for bone metastasis, such as bisphosphonates, is their harsh effect on bone, leading to conditions such as suppressed bone turnover or osteonecrosis of the jaw (48, 49). We observed that CD47−/− mice displayed a mild increase in bone volume and a decrease in tumor burden in bone. Our results suggest the potential for manipulating CD47 signaling as a novel approach to treatment of osteoporosis in the general population and to treatment of bone metastasis in cancer patients without causing severe complications to the skeleton.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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