MIP-1δ: A Potential Mediator of Osteolysis in Renal Cell Carcinoma Bone Metastasis
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INTRODUCTION: Approximately 30,000 individuals develop renal cell carcinoma (RCC) each year in the United States, 20-40% of which develop metastasis to the bone. Patients with RCC bone metastasis (RBM) have a dismal prognosis with less than 10% surviving 5 years. RCC bone metastases are characterized by extensive osteolysis leading to severe bone pain and pathological fractures. Unlike breast cancer, there is a paucity of information describing the mechanisms of RCC tumor growth within the bone and subsequent osteolysis. A better understanding of the biology of how metastatic RCC cells interact with the bone microenvironment is vital to the development of more effective therapeutics. Here, we report the discovery of a novel osteoclast (OCL) stimulatory factor secreted by RBM.

METHODS: ELISA-MIP-1δ levels were analyzed using Quantikine MIP-10 enzyme immunoassay kit (R&D Systems). Western blotting- Equal amounts of protein from cell lysates were resolved using 12% SDS-PAGE and protein was transferred to ECL nitrocellulose membranes, which were probed with antibodies of interest. Binding was revealed using enhanced chemiluminescence. Migration Assay-Cells were added to the upper chamber of transwells containing MIP-1δ in the lower chamber. Transwell units were incubated at 37°C for 3 hours and migrated cells on transwell membranes were stained with toluidine blue and counted by light microscopy. Calvarial Bone Resorption Assay-Calvaria of neonatal mice were removed, washed with PBS, and incubated in uMEM containing experimental factors. Media were replaced every 3 days and bone resorption was assessed by measuring released calcium using a calcium assay kit. OCL Differentiation Assay-Murine BM-MNC and RAW 264.7 cells were incubated for 3 days in complete media with or without MIP-1δ. Media was removed and complete media with or without RANKL (50 ng/ml) was added. To generate M-CSF-dependent BMN, BM-MNC were treated with 10 ng/ml M-CSF for 3 days. BM-MNC were then treated with a suboptimal dose of RANKL (5 ng/ml) with or without MIP-1δ (0.1 pg/ml). OCL formation was determined by positive TRAP staining and the presence of ≥ 3 nuclei under light microscopy. To determine the fusion index, the number of nuclei/OCL was counted in 20 random fields at 100x magnification. OCL Activity Assay-Cells were cultured in an Osteoassay human bone plate and differentiated under the conditions above. Levels of type I collagen helical peptide released into the supernatant were then measured by Metra Helical Peptide ELISA kit as a measure of OCL activity.

RESULTS: Through microarray analysis, we found expression of the chemokine, macrophage inflammatory protein-1δ (MIP-1δ), to be increased in RBM versus patient-matched primary RCC tissues, and confirmed this finding by quantitative (q)RT-PCR and ELISA (p < 0.05) (Figure 1). Further, MIP-1δ expression in RBM tissues was significantly (p < 0.001) higher than in human bone marrow, suggesting a potential alteration of the bone microenvironment. The receptors for MIP-1δ, CCR1 and CCR3, were expressed in both osteoclast (OCL) precursors and mature, bone-resorbing OCL, as demonstrated by qRT-PCR and Western analysis.

In functional studies MIP-18 stimulated chemotactic recruitment of two OCL precursor cell types: murine bone marrow mononuclear cells (BM-MNC) and RAW 264.7 cells. Further, MIP-1δ treatment of murine calvaria caused increased bone resorption as determined by measurement of released calcium. Correspondingly, MIP-1δ significantly (p < 0.01) enhanced RANKL-induced OCL formation in both BM-MNC and RAW 264.7 cells in a dose-dependent fashion (Figure 2). Further, OCL formed in BM-MNC and RAW 264.7 cultures treated with MIP-1δ had a significantly (p < 0.001) higher fusion index (13.4 and 14.2 nuclei per OCL, respectively) relative to those treated with RANKL alone (8.3 and 7.8 nuclei per OCL) (Figure 2). Consistent with this finding, combined stimulation of OCL precursors with MIP-1δ and RANKL significantly increased the degradation of human bone fragments in an Osteoassay human bone plate as evidenced by release of type I collagen helical peptide.

To further test the ability of MIP-1δ to enhance OCL differentiation, we examined its effect in combination with a suboptimal dose of RANKL (5 ng/ml). Consistent with our previous findings, MIP-1δ significantly (p < 0.05) potentiated OCL differentiation in the presence of sub-optimal levels of RANKL in M-CSF-dependent bone marrow macrophages (BMM) in addition to BM-MNC and RAW 264.7 cells. (Figure 3). The results observed in both BMM and RAW 264.7, both of which lack stromal elements; suggest that MIP-1δ is exerting its effects directly through stimulation of OCL precursors.

To begin to examine the potential mechanism whereby MIP-1δ enhances OCL differentiation, we tested the ability of MIP-1δ to activate key signaling pathways involved in OCL formation including p38- MAPK, ERK, AKT, JNK1, NF-κB, and PLCγ2. Although MIP-1δ did not stimulate p38MAPK, ERK, AKT, or JNK1; the NF-κB (p65) and PLCγ2 signaling pathways were activated by MIP-1δ in BMM and RAW 264.7, suggesting their potential involvement.

DISCUSSION: This study provides evidence that MIP-1δ secretion by RBM may contribute to RBM-induced osteolysis by stimulating recruitment and differentiation of OCL precursors into active OCL, suggesting that MIP-1δ may serve as a novel therapeutic target. In addition, MIP-1δ may also be useful as a prognostic indicator. In two of five primary RCC tissues tested, MIP-1δ was expressed at similar levels to that observed in RBM tissues (Figure 1), suggesting a possible clinical correlation between MIP-1δ expression in the primary tumor and the development of bone metastasis. Taken together, these data warrant further investigation into the biological role of MIP-1δ in RBM establishment and osteolysis, and its clinical potential as a prognostic indicator and therapeutic target.

Figure 1: Levels of MIP-1δ protein in RBM, RCC, and BM by ELISA.

Figure 2: (Top) Effect of MIP-1δ on RANKL-induced OCL formation in BM-MNC. Significant differences displayed are relative to RANKL treatment alone. (Right) OCL were identified by positive TRAP staining and ≥ 3 nuclei. Nuclei/OCL were counted in 20 random fields at 100X.

Figure 3: MIP-1δ enhances OCL differentiation stimulated by suboptimal doses of RANKL (5 ng/ml). OCL were identified by positive TRAP staining and the presence of > 3 nuclei. Significant differences displayed are relative to RANKL treatment alone.