A Cryptic Peptide Fragment of Type I Collagen Degradation acts as a Matrikine on Human Breast Tumor Cells, MDA-MB-231, to Upregulate Inflammatory Cytokines, MMPs and Osteoclastogenesis

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Introduction: The major site of metastasis of breast cancer cells is bone[1,2]. Bone metastases occur in 80% of patients with advanced disease and cause significant morbidity. The mechanisms of osteolysis and targeting of bone by the breast cancer cells are still unknown. The osteolysis that occurs in patients is mediated by factors that come from the tumor cells. The tumor cells provide a microenvironment to the osteoblasts and osteoclasts. Bone remodeling requires the degradation and turnover of type I collagen. Type I collagen is a triple helical fibrillar collagen that can only be cleaved by collagenases of the matrix metalloproteinase (MMP) family or by cathepsin K found only in osteoclasts[3]. The extracellular matrix plays an important role in tissue homeostasis. It regulates cellular differentiation, migration, and proliferation. Recently it has been shown that cleavage products of ECM proteins may have novel biological properties and have been termed matrikines[4]. Recent studies in our lab, have identified a type I collagen degradation fragment in the helical domain that can be generated by either MMPs or Cathepsin K cleavage of type I collagen. We tested the ability of this peptide fragment to stimulate human metastatic breast cancer cells, MDA-MB-231, to stimulate inflammatory cytokines, MMPs and osteoclastogenesis.

Materials and Methods: Peptide synthesis: A 24 amino acid synthetic peptide (CB4II) lacking RGD sequence from type I collagen was synthesized by FMOC chemistry.

Cell culture: Human breast cancer cells, MDA-MB-231 were cultured in the presence of DMEM + 10% FBS for 2 days. The cells were starved for 24h and then stimulated with 0.25, 2.5 or 25 μg/ml CB4II for 48h in serum-free media. 1 ng/ml rhIL-1β or 1 ng/ml rhTNF-α was used as positive control for up-regulation of MMPs. Rat monocyte/macrophages were flushed from the bone marrow of of tibias and femurs. Osteoclast formation was visualized by TRAP staining.

Real time PCR: Cells was extracted for RNA, converted to cDNA and subjected to quantitative relative real-time PCR using the ABI PRISM 7700 sequence detection system. Data was computed using the ddCt method.

Western blot & zymogram: MMP-1, and MMP-13 proteins from conditioned media were measured by Western blotting. Gelatinase activity was determined using gelatin zymograms.

Statistical analysis: Relative gene expression data was analyzed using the non-parametric approach of Friedman using SAS.. Differences were declared statistically significant when P<0.05, unless otherwise noted.

Results: Real time PCR analysis of MDA-MB-231 cells stimulated with 25 μg/ml CB4II (Fig.1), showed an upregulation of MMP-1, 2, 9, 13, 14 and were all upregulated significantly (p<0.05). PTHrP and SPARC have been shown to be associated with metastasis, proteolysis and present in bone and breast tumors, were also upregulated significantly (p<0.05).

Western blot of MDA-MB-231 condition media(Fig.2) showed a dose response stimulation of both MMP-1 and MMP-13 with CB4II. 25 μg/ml CB4II showed similar stimulation levels as 1 ng/ml rhIL-1β or 1 ng/ml rhTNF-α.

Zymography of MMP-2 and MMP-9 of MDA-MB-231 condition media was used to assay for gelatinase activity. Increasing CB4II stimulated both MMP-2 and MMP-9 activity. We also saw an increase in active MMP-2 with increasing CB4II concentration.

Osteoclast formation was measured by using purified Rat monocyte/macrophages. Adherent cells were plated and cultured in the presence of MDA-MB-231 conditioned media which were grown in the presence or absence of 25 μg/ml CB4II. Monocyte/macrophage cultures formed large multi-nucleated TRAP positive osteoclasts only in CB4II stimulated conditioned media suggesting CB4II stimulated osteoclast forming cytokines in MDA-MB-231 cells.

Discussion: We have shown that a peptide sequence in the CB4 region of type I collagen can stimulate MMP production in MDA-MB-231 cells. This observation has led us to hypothesize that type I collagen fragment, CB4II, may have several functions in breast tumor metastasis to bone and osteolysis. Bone is an active remodeling tissue and accounts for the major of type I collagen turnover in the body. We believe that these fragments may be chemotactic to breast tumor cells. Once the breast tumor cells metastasize to the bone, the tumor cells secrete inflammatory chemokines and cytokines such as PTHrP receptor activator of nuclear factor kappa B (RANK) and Runx2 that stimulate osteoblasts and osteoclasts formation. These events result in increase osteolysis due to increase protease production and osteoclasts formation. The increase osteolysis of bone leads to increase of type I collagen fragments that signal back to the tumor cells, osteoblasts and osteoclasts perpetuating this cascade. Further studies looking at in vivo generation of this matrikine in breast tumor patients could demonstrate this novel pathway in tumor mediated bone osteolysis.