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The proteolytic enzyme meprin has been associated with a variety of human cancer cell lines, including the breast cancer cell lines MCF-7 and SK-BR-3 (Matters and Bond, 1999). The current project has focused on the effect of meprin α and β overexpression on the progression of breast cancer cells from a less invasive to a more invasive state. These studies used a moderately invasive human breast cancer cell line, MDA-MB-231, which does not express either meprin α or β under normal culture conditions. Stable transfection and expression of meprin cDNAs in MDA-MB-231 cells creates in a more invasive phenotype as measured in vitro. Meprin α expression alone significantly increased invasiveness over that seen in nontransfected or vector-only transfected cells. In addition, co-expression of meprin α and β increased invasiveness above that in meprin α only transfectants. These results indicate that meprin α and β both can contribute to the invasive phenotype of breast cancer cells.

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Introduction

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Body of the Report

The objective of this project was to determine if the metalloproteases meprin α and β contribute to the progression of human breast cancers from a less invasive to a more invasive phenotype. Full-length cDNA clones encoding the meprin α and β subunits in the constitutive mammalian expression vector pcDNA 3.1(+)(Invitrogen) were made. Their expression was tested by independently transfecting the cDNAs into HEK 293 cells. By Western blotting, the media fraction was examined for expression of the α subunit and the cell membrane fraction tested for β subunit expression. Both meprin cDNAs exhibited correct localization and expression in 293 cells.

The MDA-MB-231 human breast cancer cell line, obtained from Dr. Dan Welch, Jake Gittlen Cancer Center, Penn State College of Medicine, is moderately metastatic in nude mice models and can be easily transfected using lipid-based transfection reagents. MDA-MB-231 cells were screened for endogenous expression of the meprin α and β mRNAs by RT-PCR and for meprin protein by Western blot. Western blots of media and cell membrane fractions were probed with anti-meprin α and meprin β antibodies, and no detectable meprin protein was found in either the MDA-MB-231 cell or media fractions. Likewise, RT-PCR did not detect either of the meprin mRNAs in MDA-MB-231 cells. Thus, the lack of endogenous meprin expression in these cells make them good recipients

for stable meprin expression and subsequent analysis of the effects of meprin expression on invasiveness.

The meprin α and β cDNAs which gave consistent expression in HEK 293 cells were used to transfect MDA-MB-231 cells. Using Lipofectamine 2000 (Invitrogen), MDA-MB-231 cells were independently transfected with the meprin α and β cDNAs as well as with the vector plasmid only (pcDNA 3.1+) as a negative control. MDA-MB-231 clones expressing the highest levels of meprin protein, based on Westerns, were selected for further study.

MDA-MB-231 clones expressing high levels of meprin α or meprin β were fractionated into soluble and membrane bound-proteins, and the media containing secreted protein was also collected and concentrated. Membrane-bound protein was released by treatment with 1% octylglucoside, and the presence of meprin in the soluble, membrane, and media protein fractions were detected by Westerns. As in the mouse kidney and intestine and in HEK 293 cells, the meprin α subunit protein is secreted from the cell if meprin β is not present, while the meprin β subunit protein stays anchored at the cell surface through a short transmembrane domain. Most of the meprin β subunit protein is extracellular, and through covalent and noncovalent α/β interactions, the meprin α protein can associate with meprin β subunit protein and be maintained at the cell membrane. Fractionation experiments showed that the transfected MDA-MB-231 cells secrete the meprin α subunit protein into the media and retain the meprin β subunit protein at the cell membrane. Because no meprin β protein is present on the meprin α transfected MDA-MB-231 cells, all the meprin α protein was secreted instead of being associated with the cell membrane. Vector transfected control cells showed no evidence of meprin protein on the cell membrane or in the media.

To test the stability of the meprin proteins expressed in MDA-MB-231 cells, isolated cell fractions were treated with a mild trypsin solution (25 ng/μl) for 30 minutes at room temperature and used in Western blots. Previous work in our lab has demonstrated that misfolded meprin proteins, such as mutant proteins with truncations or deleted domains, are susceptible to degradation by trypsin. However, the secreted and membrane-bound meprins from MDA-MB-231 cells were not degraded by the mild trypsin treatment. This implies that the meprin proteins produced by the MDA-MB-231 cells are folded properly and are stable.

Another characteristic of most extracellular proteases is a high degree of protein glycosylation. However, cancer cells can alter protein glycosylation patterns. The deglycosylating enzymes EndoH, which removes high mannose type glycosylation, and PNGaseF, which removes all N-linked sugars, were used to analyze the type and degree of glycosylation on the meprin proteins. After overnight treatment with the deglycosylating enzymes, meprins were subjected to Western blotting. The meprin proteins expressed in MDA-MB-231 cells showed a pattern of deglycosylation identical to that of meprins expressed in HEK 293 cells. No high mannose-type sugars were present on the protein, indicating that the meprin proteins were complex glycosylated, and the size of the untreated and deglycosylated proteins was identical, indicating that the degree of meprin glycosylation was similar.

Once it was established that MDA-MB-231 cells will correctly express stable meprin α and β proteins, experiments addressing whether meprins can affect the invasive phenotype of MDA-MB-231 cells were done. For in vitro invasion studies, Matrigel-coated invasion chambers (Becton Dickinson) and control chambers with no Matrigel were used. Log phase cells were detached from plates by scraping, and $5x10^4$ cells in serum-free media were added to the upper chamber. Media with 5% serum as a chemoattractant was added to the lower chamber. After 24 hours, the cells on the lower surface of the Matrigel invasion chambers and filter only control chambers were stained and counted. Between 10 and 20% of untransfected or vector-transfected MDA-MB-231 cells were invasive, and the meprin β only transfected cells showed a similar level of invasiveness. However two, independently transfected clones of meprin α in MDA-MB-231 both showed increased invasiveness through Matrigel. Although not significantly different from each other, the meprin α transfected clone were about twice as invasive as vector-only transfectants or untransfected cells. This indicates that meprin α, but not meprin β, affects the ability of breast cancer cells to invade through basement membrane in vitro.

To test if the coexpression of meprin α and β in the same cell would affect invasiveness, a meprin α expressing MDA-MB-231 clone was transfected with a meprin β cDNA. In the double transfectant, both meprin α and meprin β protein were detected in the membrane fraction, presumably as α/β cell surface heterooligomers. Meprin α was also detected in the media, indicating some of the meprin α was secreted as a homooligomer. Coexpression of meprin α and β in MDA-MB-231 cells resulted in an even more invasive phenotype than expression of meprin α alone. Meprin α/β double transfectants were three times more invasive than untransfected cells. Since meprin β alone did not significantly

affect invasiveness, it is likely that the localization of meprin α to the cell surface in α/β heterooligomers increases the ability of the breast cancer cells to invade through Matrigel. This result is similar to the serine protease urokinase-type plasminogen activator/receptor (uPA/uPAR) system, where uPA increases cancer cell invasiveness when bound to the cell surface uPAR (Holst-Hansen et al., 1996, Duggan et al., 1995).

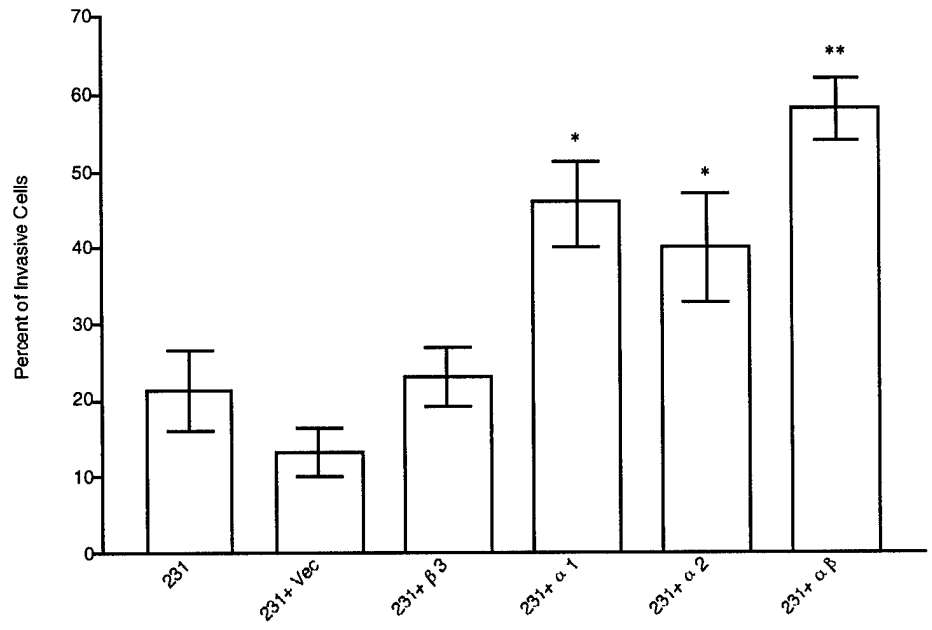

Figure 1. Invasion of untransfected, vector transfected, or meprin transfected MDA-MB-231 cells through Matrigel. Bars represent the standard error of the mean of at least 10 independent assays.

Because of the time required to construct the meprin α/β double transfectant, and a temporary shortage of space in the College of Medicine animal facility, Objective 4 has not been completed. However, the experiments on the invasiveness of MDA-MB-231 tranfectants in vivo are continuing as space becomes available.

Key Research Accomplishments

- The transfection and selection of MDA-MB-231 cells that stably express cDNAs for meprin α, meprin β or both genes simultaneously.
- The characterization of the stable meprin transfectants for expression levels, localization of the protein, correct post-translational modifications, and stability.

- The determination that meprin α expression, particularly at the cell surface, increases the invasiveness of breast cancer cells in vitro.

Reportable Outcomes

1. Based on the results of these experiments, I have submitted a proposal for further funding of this project to the Penn State Cancer Institute Research Grant Program. It is entitled "The protease meprin in metastatsis of breast and colon cancer". The status of the proposal is pending.

2. A manuscript detailing the results of the invasion assays is in preparation, and I expect to submit it for publication this summer.

3. Published Manuscripts:

Bertenshaw, GP, BE Turk, SJ Hubbard, **GL Matters**, JE Bylander, JM Crisman, and JS Bond (2001) *J. Biol. Chem.* 276: 13248-13255.

Jiang, W, JM Kumar, **GL Matters**, and JS Bond (2000) *Gene* 248: 77-87.

Matters, GL, and JS Bond (1999) *Mol. Carcingen.* 25: 169-178.

Matters, GL, and JS Bond (1999) *APMIS* 107: 19-27

4. Conference Presentations:

June 2000 - Era of Hope, Atlanta, Georgia

July 2000 - Gordon Research Conference, Colby-Sawyer College, New London, NH, "Proteolytic Enzymes and their Inhibitors".

October 2001 - International Proteolysis Society, Munich, Germany

Conclusions

The key finding of this project is that meprin α increases the ability of human breast cancer cells to invade through extracellular matrix. The localization of meprin α to the cell surface appears to be critical for producing the most invasive phenotype.

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Duggan, C., T Maguire, E. McDermott, N. O'Higgins, JJ Fenelly, and MJ Duffy (1995) *Int. J. Cancer* 61: 597-600.

Holst-Hansen, C., B. Johannessen, G. Hoyer-Hansen, J. Romer, V. Ellis, and N. Brunner (1996) *Clin. Exp. Metastasis* 14: 297-307.