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13. ABSTRACT ***(Maximum 200 Words)***
Matrix Metalloproteinases (MMP) are a family of enzymes that collectively degrade components of the extracellular matrix (ECM). A family of proteins called TIMP (Tissue Inhibitor of Metalloproteinase) are considered the primary protein inhibitors that modulate MMP activity. However, other polypeptides have been shown to inhibit MMP in vitro. MMP have been implicated in tumor growth and metastasis in general and breast tumors in specific. In the breast, MMP may also be involved in tumor fibrosis because the synthesis and degradation of ECM is no longer in balance. Understanding factors that modulate MMP activity is important to understanding breast tumor biology. Previously, we identified a non-TIMP inhibitor that was the C-terminal region of Procollagen C-terminal Proteinase Enhancer. This novel inhibitor was designated CT-PCPE. Intact PCPE has no metalloproteinase inhibitor activity. Activity is revealed by proteolytic processing of the parent PCPE. We have observed CT-PCPE and other small non-TIMP inhibitors in the medium conditioned by an aggressive breast tumor cell line. To investigate the role of CT-PCPE in breast tumors, PCPE and CT-PCPE have been expressed for the purpose of characterization of inhibitor activity and CT-PCPE production. Additionally, another putative non-TIMP inhibitor has been identified in breast tumor cell conditioned medium.

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Introduction

It is widely accepted that for tumors to grow and metastasize components of the extracellular matrix must be degraded and remodeled. Many proteinases have been implicated in this process including Matrix Metalloproteinases (MMP) (Woessner, 1991). A family of proteins called TIMP (Tissue Inhibitor of Metalloproteinase) are considered the primary protein inhibitors that modulate MMP activity. In contrast to tumor growth and metastasis, breast tumor fibrosis requires excess deposition of connective tissue macromolecules, such as collagen, and the suppression of connective tissue-degrading proteinases. Molecular mechanisms responsible for breast tumor fibrosis and the significance of that fibrosis in breast cancer are poorly understood. Collagen deposition, normal or abnormal, involves a series of extracellular molecular events. Nascent collagen must undergo N-terminal and C-terminal processing mediated by a specific procollagen N-terminal proteinase and a **p**rocollagen **C**-terminal **p**roteinase (PCP) (Prockop, et al., 1998). **P**rocollagen **C**-terminal **P**roteinase **E**nhancer (PCPE) enhances the activity of PCP. However, the enhancer activity resides in the N-terminal domain of the PCPE molecule (Takahara, K. et al., 1994). We have identified a metalloproteinase inhibitory activity associated with the C-terminal domain of PCPE (CT-PCPE) (Mott, et al., 2000). This activity was originally associated with fatal brain tumors; however, we have also observed this inhibitor in a highly invasive breast tumor cell line. This novel activity is revealed only when the parent PCPE molecule is proteolytically processed in the extracellular milieu.

The objectives of the proposed studies are to identify structural requirements for CT-PCPE to function as a metalloproteinase inhibitor; to determine mechanisms that generate CT-PCPE from PCPE; and to determine if increased expression of PCPE and/or CT-PCPE correlates with the invasive nature of breast tumor cells.

Body

Task 1 Identifiction of Functional Domain of CT-PCPE: Previous studies investigating metalloproteinase inhibitors in brain tumor conditioned medium revealed that in addition to the presence of TIMP a fourth activity was observed (Apodaca, et al., 1990). This activity was purified and identified by amino acid sequence analysis. Results indicated the polypeptide responsible for the inhibitor activity was the C-terminal domain of a protein previously identified as PCPE. Partial characterization of the inhibitory activity of CT-PCPE against MMP has been examined (Mott et al, 2000). In addition CT-PCPE was observed in the conditioned medium of an aggressive breast tumor cell line MDA MB-231. CT-PCPE was identified in the breast tumor conditioned medium by inhibitory activity on reverse zymography and by western blot analysis with anti-PCPE antibodies. However, the inhibitors observed in the beast tumor conditioned medium differed slightly from the brain tumor inhibitor. Several inhibitor bands were observed at smaller molecular weights than CT-PCPE, and these bands did not react with the anti-PCPE. Two possible explanations for these observations were that the smaller bands were other fragments of PCPE that did not react with the antibody, or these polypeptide inhibitors were not derived from PCPE.

To investigate these other inhibitors, a pool of MDA MB 231 conditioned medium enriched in inhibitor activity was made by fractionating the medium over the following columns: gelatin Sepharose, Heparin Sepharose, Lentil Lectin and SP-Sepharose. Proteins were electrophoresed and transferred to to a polyvinylidene fluoride membrane for direct amino acid sequence analysis. Results indicated that one of the unidentified small molecular weight

inhibitors was a fragment of a protein previously identified as Arginine Rich Protein (ARP). Very little is known about the function of ARP. Mutations within this gene have been associated with a variety of cancers, including breast cancer (Shridhar, V, et al., 1996, 1997 and Shridhar, R., 1996). However, other reports suggest that these "mutations" may be normal polymorphisms (Evron, E., et al., 1997). One of the most intriguing observations made from the comparison of the amino acid sequence of CT-PCPE, TIMP inhibitory domain, and the ARP fragment is that they contain six to eight cysteine residues. The metalloproteinase inhibitory activity of TIMP is dependent on the integrity of the disulfide bonds and reducing agents such as β-mercaptoethanol abolish this activity (Gomez et al., 1997). Thus, this fragment of ARP may act as a metalloproteinase inhibitor in the same manner as proposed for CT-PCPE. Both ARP and CT-PCPE may fold into a structure that can mimic TIMP and this folded structure is dependent on correct disulfide bond pairing.

We have proposed that CT-PCPE mimics TIMP due to the folded structure of CT-PCPE. In order to investigate CT-PCPE folding and to determine the smallest functional unit of CT-PCPE, we have begun collaborating with Dr. Richard Williamson of the University of Kent in Canterbury, U.K. Dr. Williamson's laboratory has experience with refolding expressed TIMP molecules and determining their structure with NMR (Williamson et al., 1994). Within the TIMP family of four proteins, the amino acid sequence identity is low. However, the tertiary structural homology is thought to be high due to the conserved placement of twelve cysteine residues. The metalloproteinase inhibitory domain of TIMP resides in the N-terminus of the molecule where six of the twelve cysteines are present (Gomez et al., 1997). CT-PCPE also contains six cysteines and reduction and alkylation abolishes metalloproteinase inhibitor activity (Mott, et al., 2000). Computer modeling suggests that the tertiary structure of CT-PCPE may be homologous to the amino domain of TIMP (Banyai and Patthy, 1999). This could account for the metalloproteinase inhibitory activity of CT-PCPE. Therefore, in collaboration with Dr. Williamson's group, CT-PCPE was expressed in bacteria. The region of CT-PCPE originally expressed began with cysteine 318. This is the region of CT-PCPE suggested by Banyai and Patthy (1999) to show the greatest structural homology to TIMP. Additionally, the N-terminal cysteine residue of TIMP is critical for TIMP inhibition of metalloproteinases.

Base pairs coding for amino acids 318-449 of PCPE have been cloned by PCR into pPCR-Script (Stratagene). Clones were sent to Dr. Williamson's laboratory where CT-PCPE was expressed in bacteria and refolded. The refolded CT-PCPE was examined for disulfide bond pairing. As predicted the CT-PCPE formed disulfide bond pairs that were positioned identically to those of TIMP. However, the bacterially expressed CT-PCPE has shown no inhibitory activity against MMPs *in vitro*. One possible explanation for this result is that although the disulfide bond pairing is correct and identical to TIMP, other portions of CT-PCPE may be folded incorrectly. Another possibility is that unlike TIMP, a cysteine residue can not be the first N-terminal residue for CT-PCPE to manifest metalloproteinase inhibitor activity. It is possible that other amino acid residues must be present at the N-terminus of CT-PCPE for it to function. This is supported by the fact that in conditioned medium we have not observed a functional fragment of CT-PCPE as small as the molecule expressed in bacteria. This suggests that the CT-PCPE fragment beginning with cysteine 318 is too small. Larger CT-PCPE fragments will expressed in bacteria in order to narrow the functional size of CT-PCPE required for metalloproteinase inhibitory activity.

Task 2. To determine the biologically relevant proteinases from breast tumor cells that process intact PCPE to the CT-PCPE metalloproteinase inhibitor. Based on the amino termini of the CT-PCPE identified in brain tumor conditioned medium, the amino termini of five of the six

fragments have the signature of serine proteinase cleavage. Moreover, PCPE expressed in a baculovirus system can be processed *in vitro* with plasmin to generate smaller fragments of approximately the same size as CT-PCPE. Some of these small fragments have inhibitory activity as determined by reverse zymography (Mott, et al., 2000). Taken together, this information suggests that serine proteinases are most likely the enzymes that process PCPE. Therefore, medium conditioned by MDA MB-231 cells was passed over a lysine Sepharose column. Bound material was eluted with 0.2 M ε-aminocaproic acid. The recovered protein was analyzed by gelatin and casein zymography, and a proteinase of apparent molecular weight of 45,000 was observed on both types of zymography. This proteinase was also present in the medium conditioned by brain tumor cells that process PCPE to CT-PCPE. In addition, the transformed epithelial cell line HEK-293 also produces the inhibitory fragment CT-PCPE and this proteinase activity can be isolated from HEK-293 conditioned medium. However, this proteinase was not present in medium conditioned by Hs 27 cells. These cells are normal fibroblasts and CT-PCPE has not been observed in Hs 27 conditioned medium. These data suggest that the 45,000 molecular weight proteinase is an excellent candidate for a PCPE processing enzyme. Further investigation of this proteinase has suggested that the activity observed at 45,000 is a processed form of a parent molecule of much larger molecular weight. Many proteinases are processed during enzyme activation and this may be the case with this particular proteinase. This proteinase is very active in zymography, but not enough protein has been collected to attempt amino acid sequence analysis. Conditioned medium is currently being collected from MDA MB-231 and HEK 293 in order to attempt amino acid sequences of this enzyme. In order to test whether this enzyme is capable of processing PCPE to CT-PCPE, purified enzyme was mixed with purified PCPE expressed in a mammalian cell line. After incubation, the mixture was analyzed by reverse zymography and SDS-PAGE. Results showed that PCPE is a substrate for the putative processing enzyme. However, whether this proteinase can process PCPE to release the metalloproteinase inhibitor CT-PCPE has yet to be determined. Experiments are currently in progress to identify and characterize the degradation products by reverse zymography and western blot.

Task 3: <u>To determine if increased expression of PCPE and/or CT-PCPE correlates with prognosis and the invasive nature of breast tumor cells.</u> MDA MB-231 cells are a highly invasive cell line which shows signs of epithelial to mesenchymal transition (Sommers et al., 1994 and Gilles and Thompson 1996). The production of CT-PCPE may be specific to cells that are at this late stage of transformation. To test this hypothesis, we are investigating whether different breast tumor cell lines express PCPE and process it to CT-PCPE. Previously we examined MCF-7 conditioned medium and have not observed the inhibitory activity associated with CT-PCPE. The cell lines currently under examination include MCF 10A, BT-483, MDA MB-468 and MDA MB-435. Examination of the conditioned medium from MCF 10A by reverse zymography showed no metalloproteinase inhibitor activity except for TIMP-1. Analysis of MDA MB-468 by reverse zymography indicated that TIMP-1 and –2 were present and possibly a small amount of inhibitor activity that is consistent with CT-PCPE. The analysis of MDA MB-435 and BT-483 is currently under way.

Key Research Accomplishments

1. Expression of the smallest possible fragment of CT-PCPE in bacteria indicated that the disulfide bond pairing in CT-PCPE is identical to that of TIMP.

2. The identification of another putative non-TIMP metalloproteinase inhibitor, Arginine Rich Protein (ARP), from MDA MB-231 conditioned medium. Like CT-PCPE, ARP may act as metalloproteinase inhibitor due to its folded structure held in place by specific disulfide bond pairs similar to TIMP.
3. Expression of full-length PCPE in a mammalian cell line for use in the characterization of a serine proteinase isolated from conditioned medium that may process PCPE to CT-PCPE.

Reportable Outcomes

Mott, J.D. and Banda, M.J. (2001) "Non-TIMP Metalloproteinase Inhibitors Produced by the Breast Tumor Cell Line MDA MB-231" Abstract Number 3987. Annual Meeting of American Society of Cell Biology.

Conclusions

The non-TIMP metalloproteinase inhibitor CT-PCPE has not been observed in the normal human fibroblast cell line Hs 27, nor has this activity been observed in the aggressive fibrosarcoma cell line HT-1080. The small molecular weight inhibitor CT-PCPE has been observed in brain tumor cell lines, breast tumor cell lines and the transformed kidney epithelial cell line HEK-293. Take together, these observations suggest that CT-PCPE production may only occur in transformed epithelial cells. The biology of MMP and their classical inhibitors, TIMP, has typically been studied in fibroblasts, other stromal cells, or vascular endothelial cells. However, one of the most striking observations from our research is that the non-TIMP metalloproteinase inhibitor we have designated CT-PCPE appears to be present exclusively in transformed epithelial cells. Thus, the production of CT-PCPE from PCPE may be a marker for transformed and potentially invasive epithelial cells. We will be continuing to examine a number of breast tumor epithelial cell lines both invasive and non-invasive to determine if CT-PCPE is a marker for the invasive nature of breast epithelial cells. Information gathered from this data may be useful in designing assays to screen for CT-PCPE. This information may be helpful in deciding the course of treatment for specific breast tumors.

In addition to evaluating the correlation of CT-PCPE with the aggressive nature of breast tumor cells, we are continuing to establish the importance of CT-PCPE as a metalloproteinase inhibitor. In an effort to understand mechanisms by which CT-PCPE inhibits MMP, the structure of CT-PCPE is being determined. We have made progress in determining the structure of CT-PCPE through our collaboration with Dr. Richard Williamson. The disulfide bond structure of CT-PCPE has been determined and we have data to show that the disulfide bond pairing is identical to TIMP. Understanding mechanisms that modulate MMP activity is important for the design of compounds that can specifically inhibit MMP. Modulation of MMP activity can important implications for controlling breast tumor cell invasion and metastasis.

References

Apodaca, G., Rutka, J.T., Bouhana, K., Berens, M.E., Giblin, J.R., Rosenblum, M.L., McKerrow, J.H. and Banda, M.J. (1990) Expression of metalloproteinase inhibitors by fetal astrocytes and glioma cells. Cancer Res. 50:2322-2329.

Banyai, L., and Patthy, L. (1999) The NTR module: Domains of netrins, secreted frizzled related proteins and type I procollagen C-proteinase enhancer protein are homologous with tissue inhibitors of metalloproteases. Protein Sci. 8:1636-1642.

Evron, E., Cairns, p., Halachmi, N., Ahrendt, S.A., Reed, A.l. and Sidransky, D. (1997) Normal polymorphism in the incomplete trinucleotide repeat of the arginine-rich protein gene. Cancer Res. 57:2888-2889

Gilles, C., and Thompson, E.W. (1996) The epithelial to mesenchymal transition and metastatic progression in carcinoma. Breast J. 2:83-96

Gomez, D.E., Alonso, D.F., Yoshiji, H. Thorgeirsson, U.P. (1997) Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. Eur. J. Cell Biol. 74:111-122.

Mott, J.D., Thomas, C.L., Rosenbach, M.T., Takahara, K., Greenspan, D.S. and Banda, M.J. (2000) Post-translational proteolytic processing of procollagen C-terminal proteinase enhancer releases a metalloproteinase inhibitor. J. Biol. Chem. 275:1384-1390.

Prockop, D.J., Sieron, A.L., and Li, S. (1998) Procollagen N-proteinase and procollagen C-proteinase. Two unusual metalloproteinases that are essential for procollagen processing probably have important roles in development and cell signaling. Matrix Biol. 16:399-408.

Shridhar, R., Shridhar, V. Rivard, S., Siegfried, J.M., Pietraszkiewicz., H., Ensley, J.,Paul., R., Grignon, D., Sakr, W., Miller, O.J. and Smith, D.I. (1996) Mutations in the arginine-rich protein gene, in lung, breast and prostate cancers, and in squamous cell carcinoma of the head and neck. Cancer Res. 56:5576-5578.

Shridhar, V., Rivard, S., Shridhar, R., Mullins, C., bostick, L., Sakr, W., Grignon, D., Miller, L.J., and Smith, D.I. (1996) A gene from human chromosomal band 3p21.1 encodes a highly conserved arginine-rich protein and is mutated in renal cell carcinomas. Oncogene 12:1931-1939.

Shridhar, V., Rivard, S., Wang, X., Shridhar, R., Paisley, C., Mullline, C., Beirnat, L., Dugan, M., Sarkar, F., Miller, O.J., Vaitkevicius, V.K., and Smith, D.I. (1997) Mutations in the arginine-rich protein gene(ARP) in pancreatic cnacer. Oncogene 14:2213-2216.

Sommer, C.L., Byers, S.W., Thompson, E.W., Torri, J.A. and Gelmann, E.P. (1994) Differentiation state and invasiveness of human breast cancer cell lines. Breast Cancer Res. and Treat. 31:325-335.

Takahara, K., Kessler, E., Biniaminov, L., Brusel, M., Eddy, R.L., Jani-Sait, S., Shows, T.B., and Greenspan, D.S. (1994) Type I procollagen COOH-terminal proteinase enhnacer protein: Identification, primary structure and chromosomal location of the cognate human gene (PCOLCE). J. Biol. Chem. 269:26280-26285.

Williamson, R.A., Martorell, G., Carr, M.D., Murphy, G., Docherty, A.J.P., Freedman, R.B., and Feeney, J. (1994) Solution structure of the active domain of tissue inhibitor of metalloprtoeinases-2. A new member of the OB fold protein family. Biochem. 33:11745-11759

Woessner, J.F. Jr (1991) Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J. 5:2145-2154.