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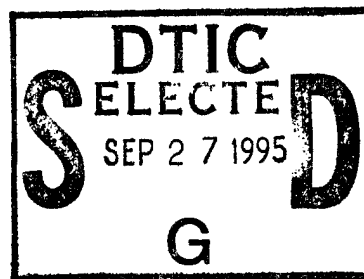
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28 JUL 1995

Date

## Molecular diagnosis for breast malignancy.

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## (5). INTRODUCTION

**Nature of the problem.** Recently, integral membrane proteases, maligrin and seprase, have been shown to localize predominantly to the invading front (invadopodia) of the breast cancer cells and to shed membrane vesicles. These molecules are promising indicators of the metastatic potential of breast cancer cells (malignancy antigens). Ideally, a measurable product of invasive cancer cells either localized at the invading front of the breast cancer tissues or shed in patient's blood would permit objective assessment of micrometastases formation. The purpose of this application is to perform a clinical evaluation of invasion-related membrane proteases as prognostic markers for node-negative breast cancer.

**Background of previous work.** During the past two years, we have been pursuing three observations that appear to be related directly to malignancy. The first observation was that malignantly transformed cells produced cell surface protrusions, invadopodia, and shed membrane vesicles that expressed active extracellular matrix-degrading proteinases including collagenases, a novel 170 kDa sulfhydryl membrane proteinase (seprase), and a putative serine membrane proteinase (maligrin) forming complexes at 180 kDa. The second observation was that, in established cell lines with differential invasiveness, maligrin and seprase are down-regulated in differentiated cells, up-regulated in benign hormone-dependent breast carcinoma, and over-expressed on the cell surface of invasive hormone-independent breast carcinoma. The third observation was that, in addition to the close relationship of collagenase, seprase, and maligrin to cell invasiveness as described above, maligrin complexes stood out as a super target cell surface molecule, which is a malignancy-associated immunogen. This is based upon unique structural features of the complexes and its striking association with malignantly transformed cells, particularly breast carcinoma derived cell lines.

While pursuing the study of membrane proteases that play a role in matrix degradation of the human melanoma cell line LOX, we observed that seprase, maligrin and 72 kDa MMP are present at high levels in shed membrane vesicles (Aoyama and Chen, 1990; Chen, 1992). Human malignant melanoma cells LOX (now we know that the breast carcinoma cell line MDA-MB-231 express 10 folds more maligrin-p29 complexes than LOX) produce a large amount of membrane vesicles that are highly enriched in maligrin and seprase. In the past year, we have completed several purification studies for maligrin and its associated protein complexes, which, when separated under reducing condition, dissociate into major p90 and p29 components.

**Purpose of the present work.** In this proposal, we will test whether overexpression of membrane proteases and their associated proteins is part of the invasive machinery of tumor cells. We will determine the distribution and concentration of protease and associated molecules in histological sections of human breast carcinomas as well as sera of node-negative breast cancer patients using mAbs and cDNA probes specific for these molecules. The information obtained will be compared with the previously established prognostic factors of breast carcinomas, such as tumor size, lymph node involvement, histology, hormone receptor status, and other factors, such as the rate of proliferation and neovascularization. This is performed in the contents of a larger study at the Lombardi Cancer Research Center in collaboration with the Armed Forces Institute of Pathology.

**Methods of approach.** As discussed above, p95 maligrin and its associated proteins are important cell surface antigens in breast cancer. An immediate goal of this proposal is to test the hypothesis that a newly discovered 29 kDa, maligrin-associated polypeptide may be an endogenous collagenase stimulating factor for host cells. The specific aims of this study are to:

- 1) perform experiments on isolation, cloning, sequence analysis, and expression of the 29 kDa molecule, and test the recombinant molecule in stimulating collagenases of host stromal cells.
- 2) produce mAbs directed against malignancy antigens that are useful in pathological studies described in Tasks 3 and 4 below,
- 3) examine immunohistochemically paraffin sections of a large set of breast cancer samples from the Armed Forces Institute of Pathology in order to predict possible node-negative breast cancer outcomes, and
- 4) develop a mAb sandwich ELISA to determine serum malignancy antigen levels in an experimental invasion and metastasis model, and, subsequently, those of breast cancer patients using the Serum Bank of the Lombardi Cancer Research Center.

## **(6). BODY**

### **Task 1: Characterization of breast cancer maligrin-associated protein p29.**

**1a. Clone a full length cDNA corresponding to the p29 from a human breast carcinoma cDNA expression library.** We have isolated and analyzed a full length cDNA of the p29 gene. The cDNA clones were sequenced and an open reading frame was identified as a 58-kDa human

tumor derived collagenase-stimulating factor, now called EMMPRIN. The p29 antigen was originated from bovine serum as identified by the sandwich ELISA (see below), and it bound to cancer cell surface invadopodia. Northern analysis of mRNA from six breast carcinoma cell lines showed that they all expressed high level of EMMPRIN message.

**Task 2. Production of monoclonal antibodies directed against malignancy antigens which are useful for clinical studies.**

**2 a. Production of mAbs.** Hybridoma protocols have been established in our laboratory that allow for production of mAbs to specific domains of maligrin, seprase and DPP IV. We have already had over 150 promising mAbs directed maligrin, and our primary goal is to screen this panel of mAbs for antibodies that are useful for clinical studies. Second, we have generated a new panel of mAbs (panel A, i.e., mAb A27) for histochemical staining of breast cancer in paraffin sections.

**2a. Preparation of breast cancer cells, MDA-MB-231, in paraffin sections for screening of clinically useful mAbs.** To obtain mAbs that could be used as a measure of malignancy antigen in archival formalin-fixed, paraffin-embedded tissues, we have established an immunohistochemistry technique with an appropriate "antigen retrieval" treatment for formalin-fixed, paraffin-embedded breast carcinomas according to a previously described method (Siitonen et al., 1993). We found that mAb V4 and rat polyclonal antibody directed against seprase stained specifically a subset of lymphocytes and epithelia in tonsil, prostate and skin. In addition, anti-seprase mAbs D8, D28, and D43 stain a subpopulation of cancer cells in melanoma biopsies.

**Tasks 3 & 4. Tissue and serum prognostic markers for node-negative breast cancer.**

**3&4a. Monoclonal antibody-based ELISA.** We have established mAb-based "sandwich" ELISA for the quantitative detection of membrane proteases in culture media conditioned by breast cancer and melanoma cells. The assay was able to measure the protease molecules in cell culture supernatants derived from MDA-MB-231 and LOX cells. We found that the assay had a sensitivity of about 200 ng/l for DPP IV and seprase, 100 ng/l for p29, and 10 ng/l for p29-p90 maligrin complex (using mAbs S2 and S9). The precision is better than 10%. The result is comparable with that for detecting human tumor necrosis factor-binding protein (Adolf and Apfler, 1991) and for CD-10 (Howell et al., 1991).

## (7). CONCLUSIONS

(i). We have isolated and analyzed a full length cDNA encoding EMMPRIN. Northern analysis of mRNA from six breast carcinoma cell lines showed that they all expressed high level of EMMPRIN message. This result is a surprise to our original hypothesis that the protein may be an endogenous inhibitor of maligrin. However, it now open up an exciting possibility that EMMPRIN on breast carcinoma invadopodia, when it contact directly the host cells, serves as a collagenase-stimulating factor. Future isolation and expression of EMMPRIN will be sought to test the recombinant molecule in stimulating production of collagenases by tumor-stromal cells.

(ii). We have generated a new panel of mAbs (panel A, i.e., mAb A27) for histochemical staining of breast cancer in paraffin sections. To obtain mAbs that could be used as a measure of malignancy antigen in archival formalin-fixed, paraffin-embedded tissues, and that their staining results have long-term prognostic significance in axillary node-negative breast cancer, we have established an immunohistochemistry technique with an appropriate "antigen retrieval" treatment for formalin-fixed, paraffin-embedded breast carcinomas as these sections are generally not reactive to mAbs (Siitonen et al., 1993). MDA-MB-231 cells overexpress membrane proteases and MCF-7 cells underexpress them. These two cell lines have been chosen as an experimental pair to prepare conventional archival sections. With this technique, we found that mAb V4 and rat polyclonal antibody directed against seprase stained specifically a subset of lymphocytes and epithelia in tonsil, prostate and skin. In addition, anti-seprase mAbs D8, D28, and D43 stain a subpopulation of cancer cells in melanoma biopsies.

(iii). We have established mAb-based "sandwich" ELISA for the quantitative detection of membrane proteases in culture media conditioned by breast cancer and melanoma cells. Over 3 mAbs directed against p95 maligrin, p29, seprase, and DPP IV, receptively, are available. These antibodies recognize different epitopes on individual molecules. Two of the antibodies were used to develop an ELISA with horseradish peroxidase as the marker enzyme. The assay was able to measure the protease molecules in cell culture supernatants derived from MDA-MB-231 and LOX cells. Culture supernatants of 6 human breast cancer cell lines with different invasiveness have been assayed for the amount of protease antigens. The assay will be a useful tool to detect malignancy antigens in sera of mice and cancer patients.

**(8) REFERENCES**

- Aoyama, A. and Chen, W.-T. (1990). A 170-kDa membrane-bound protease is associated with the expression of invasiveness by human malignant melanoma cells. *Proc. Natl. Acad. Sci. U. S. A.* 87, 8296-8300.
- Chen, W.-T. (1992). Membrane proteases: roles in tissue remodeling and tumour invasion. *Curr. Opin. Cell Biol.* 4, 802-809.
- Howell, S., Murray, H., Scott, C.S., Turner, A.J., and Kenny, A.J. (1991). A highly sensitive e.l.i.s.a. for endopeptidase-24.11, the common acute-lymphoblastic-leukaemia antigen (CALLA, CD-10), applicable to material of porcine and human origin. *Biochem. J.* 278, 417-421.
- Siitonen, Sanna M., Olli-Pekka Kallioniemi, and Jorma J. Isola. (1993) Proliferating cell nuclear antigen immunohistochemistry using monoclonal antibody 19A2 and a new antigen retrieval technique has prognostic impact in archival paraffin-embedded node-negative breast cancer. *American J. Path.*, 142: 1081-89.