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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

We have isolated a novel CSVTCG-specific tumor cell adhesion receptor and shown by immunohistochemical staining of human breast tumors that the receptor localizes to malignant ductal epithelium while no staining of epithelium in normal and benign tissues was observed. Preliminary studies suggest that the CSVTCG-specific receptor may function to promote the invasive behavior of breast epithelium and contribute to the development of malignancy. To test this hypothesis, we proposed to transfect full-length receptor cDNA in the sense or antisense orientation in order to either over-express or block receptor expression in breast carcinoma cell lines. After successful cloning of the receptor cDNA, clones were selected and evaluated for the expression level of the receptor. Stably transfected cell lines would then be evaluated for their in vitro cell invasive activities as well as their capacity to metastasize and form tumors in vitro and in athymic mice. These studies should provide information on the role of the CSVTCG-specific receptor in breast cancer progression and identify a new target for the development of anti-tumor therapeutics.

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Research Report

Tumor progression is a complex and multistep process characterized by adhesion of circulating tumor cells to the blood vessel wall, invasion of tumor cells through the vessel wall and growth of new tumor colonies. We have discovered that thrombospondin-1 (TSP-1), present in the environment around the tumor cells, helps the tumor cells to attach and migrate to other sites in the body. TSP-1 is composed of three identical disulfide-linked polypeptide chains. Each chain is composed of different domains and one of them the type I repeat domain has the cysteine-serine-valine-threonine-cysteine-glycine (CSVTCG) repeating sequence.

Our laboratory has shown that the CSVTCG sequence of TSP-1 functions as a tumor cell adhesion domain and CSVTCG peptides as well as anti-CSVTCG antibodies have anti-metastatic activity in a murine model of lung metastasis. We have also isolated a novel CSVTCG-specific TSP-1 receptor and have shown that anti-receptor antibodies blocked breast cancer adhesion and invasion as well as human breast cancer progression in athymic mice. These studies suggest that the CSVTCG-specific receptor functions in the promotion of the invasive behavior of breast cells and contributes to the development of malignancy. To test this hypothesis, MDA-MB-231 breast cancer cells were transfected with the full-length receptor cDNA in the sense and the antisense orientation in order to over-express and block the receptor expression.

As reported last year, cloning of the sense and antisense orientation cDNA was done in pTracer-SV40 and pTracer-CMV2 vectors as well as the pLXSN and MigRI retroviral vectors. It was found that cells expressing the GFP protein were unstable and lost their color soon after being selected. Then the transfection system was changed to a retroviral infection. The cells were sorted using the fluorescence-activated cell sorting (FACS) analysis. When the cells were permeabilized though, the fluorescence leaked out of the cells because the form of GFP was not a myristylated one. As a result of this we decided to clone our gene in a vector that has no GFP marker.

The pcDNA3.1/Zeo (+) and (-) vectors (Invitrogen,CA) were the vectors used for cloning the gene of interest. The (+) vector was designed to express the sense cDNA and the (-) one to express the antisense cDNA. The gene was cloned to the BamHI and EcoRI

sites. The CMV promoter of the vectors provides a high-level expression in a wide range of mammalian cells. Transfection was done by using Superfect reagent (Qiagen,CA). Superfect-DNA complex formation was performed in a serum free and antibiotic free medium. The cells were then selected with zeocin antibiotic. Several clones of the vector control, sense and antisense breast cancer cells were isolated and tested for the expression of receptor protein and mRNA.

Expression of the receptor in the transfected cells was assessed at the protein level by Westrern blot analysis. Cell lysates or stroma samples were blotted to a nitrocellulose membrane. As a positive control purified receptor protein was used. Nonspecific sites on the membranes were blocked with 5% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 hour at room temperature. The immunoblots were then incubated with the polyclonal anti-receptor Ab made from Covance. The bound antibody was detected using the enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL). The same membrane was incubated with β-actin to normalize for the loading of the samples. Figure 1 shows that the receptor expression level for sense clones is higher than the control or antisense clones.

The transfected breast cancer cells were also examined immunohistochemically for the presence of CSVTCG-specific receptor using the ABC immunoperoxidase staining procedure. The analysis was performed to show the expression of the receptor and determine its localization (figure 2), which is on the surface. The polyclonal anti-receptor Ab made from Covance was used in this study and as a negative control mouse serum was used. In addition, immunofluorescent labeling of the cells shown in figure 3 confirmed that the cells overexpress the receptor.

Northern blot analysis revealed that the receptor transfected cells expressed higher levels of receptor mRNA (figure 4). Total RNA is isolated from the cells by RNeasy Total RNA kit (Qiagen, CA) following the manufacturer's directions. 10 μg of total RNA is slot blotted onto a nylon membrane. The membrane is hybridized with an HRP-labeled receptor cDNA probe generated using the North2South kit (Pierce,IL). A chemiluminescent solution is used for development and then the blot is exposed to film.

Preliminary data show that the cells transfected with receptor cDNA in the sense direction are more adherent to TSP-1 compared to the vector control transfected cells

(figure 5). In 96 well plate, duplicate wells were covered with TSP-1 (40μg/ml), fibronectin (40μg/ml) and 1% BSA. The wells were dried out overnight and then blocked with BSA. 100μl of a suspension containing 50,000 cells were plated in the protein covered wells and incubated at 37°C for 1 hour. The non-adherent cells were removed and the wells were washed with a Hepes buffer. The total cell associated stain was determined by dissolving the attached cells directly in the wells with 100 μl of 10% acetic acid solution. The absorbance of each well was measured at 595 nm with a microtiter plate reader (Biotek, VT). Cells adhering to BSA were considered background while cells adhering to fibronectin were the positive control. As shown on figure 5 the receptor transfectants adhered to TSP-1 more rapidly and 25% increase of adhesion was observed as compared to the vector control transfectants.

Moreover, the proliferetion of the cells was measured by using a colorometric assay that provides the number of viable, proliferating cells. The MTS assay was performed according to the procedure provided by Promega (Madison,WI). The principle of the assay is that MTS (Owen's reagent) is bioreduced by cells into a formazan that is soluble in tissue culture medium. Dehydrogenase enzymes found in metabolically active cells generate the formazan. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture. In a 96 well microtiter plate, 10,000 cells were allowed to adhere and 2 hours after the MTS solution was added the absorbance at 490 nm was recorded. Enough replicate wells were plated so that cells could counted on days 0, 1, 2 and that the average value of triplicate cultures could be determined per time point. The sense transfected cells showed a 14% increase in proliferation rate compared with the control or antisense transfected cells, as it is shown in figure 6.

The significance of these findings with respect to protein expression is currently being investigated. The stably transfected clones are being evaluated for their in vitro cell invasive activities as well as their capacity to metastasize and form tumors in vitro and in athymic mice. This vector will also be used for transfection of other breast cell lines and their in vitro as well as their in vivo functions in cancer progression will be described.

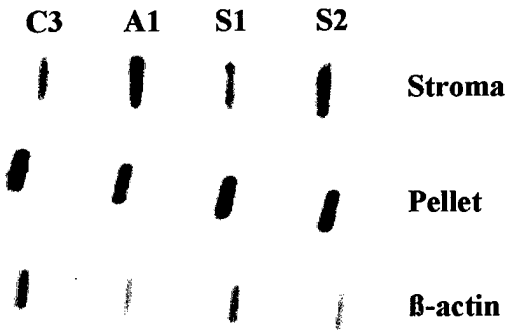

Fig. 1. Western blot analysis of C3 -vector control, A1-antisense and S1, S2-sense receptor transfected clones of MDA-MB-231 cells

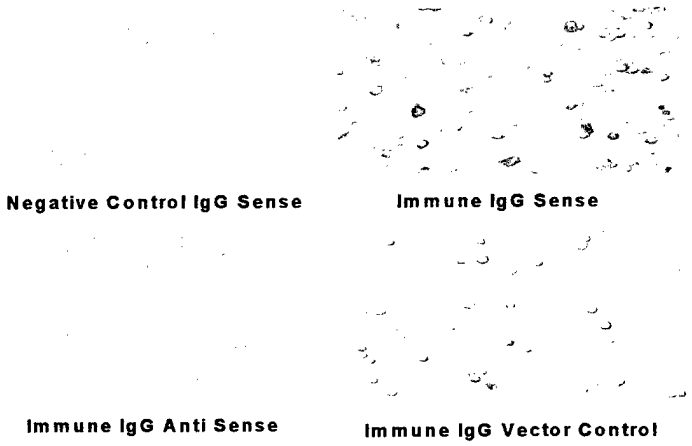

Fig.2. Immunohistochemical staining of the CSVTCG-specific receptor in the transfected MDA-MB-231 cells.

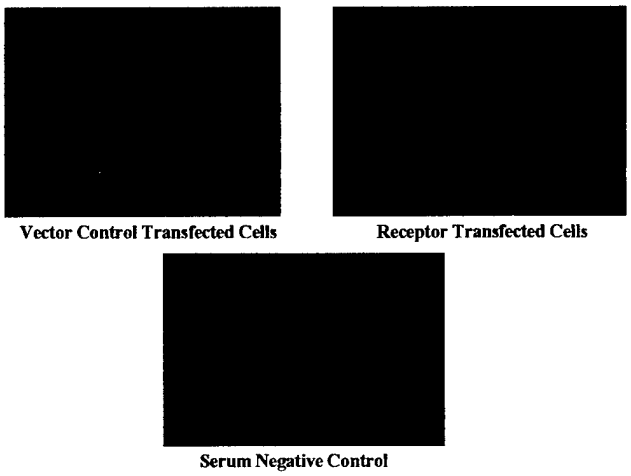

Fig 3. Immunofluorescent staining of the receptor in the MDA-MB-231 transfected cells.

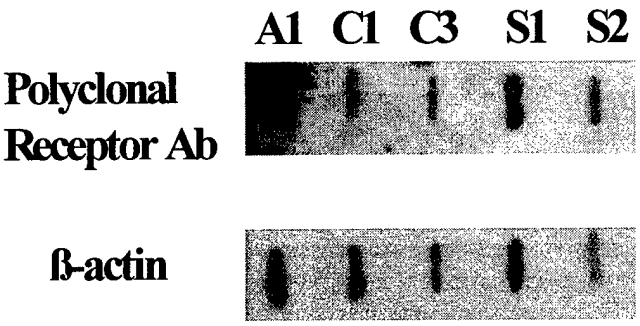

Fig 4. RNA expression level among the different transfected cell lines. A1is antisense, C3 is vector control and S1, S2 are sense clones.

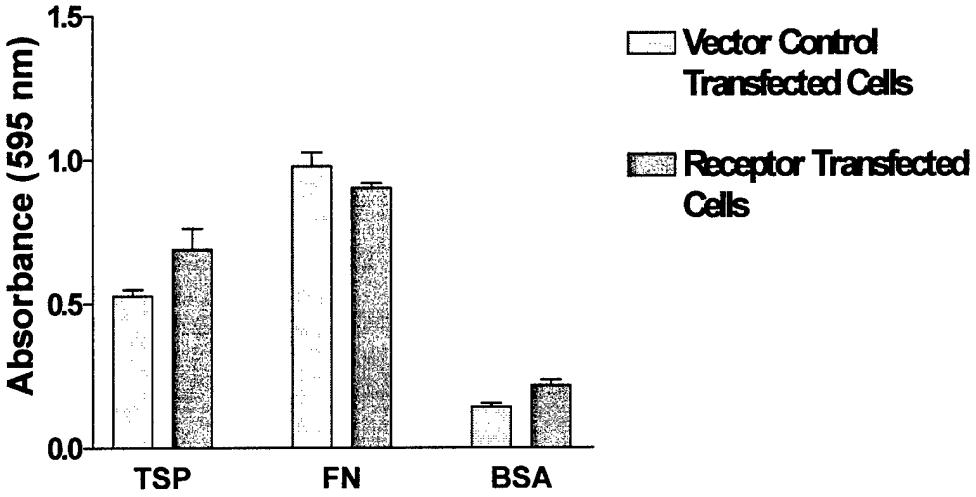

Fig. 5. Adhesion assay of vector control transfected and CSVTCG-specific receptor transfected MDA-MB-231 cells.

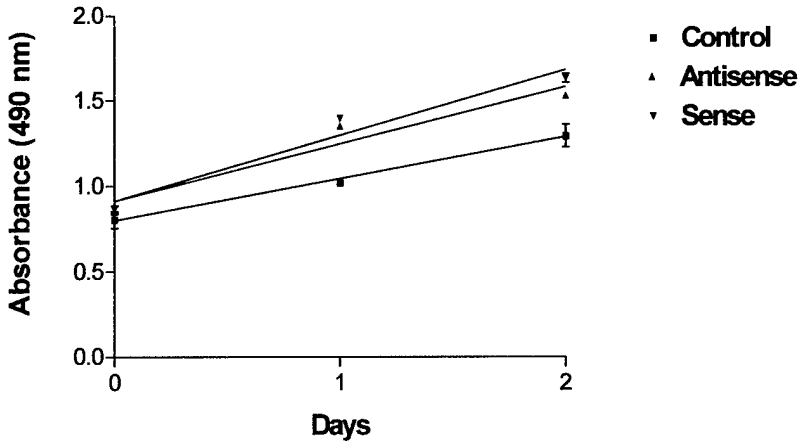

Fig. 6. Proliferation assay of the transfected MDA-MB-231 cells.

Key Accomplishments

- Construction of pcDNA3.1/Zeo (+) vector + CSVTCG specific receptor cDNA
- Transfection of MDA-MB-231 cells with the above DNA using the Superfect reagent and generation of stable transfected cells
- Construction of pcDNA3.1/Zeo (-) vector + CSVTCG specific receptor cDNA
- Transfection of MDA-MB-231 cells with the antisense receptor cDNA using the Superfect reagent and generation of stable transfected cells
- Generation of clones of vector control, sense and antisense receptor expressing cells by antibiotic selection
- Evaluation of the expression of receptor at the protein and mRNA levels
- Evaluation of functional characteristics of the cells