The Role of CD164 in Metastatic Cancer

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The spread of tumors, a process called metastasis, is a dreaded complication in the progression of many cancers. This complication is most severe when the tumors spread or 'home' to the bone, as they frequently do in breast or prostate cancers. Blood or hematopoietic cells also 'home' to bone during development and there is now compelling evidence that the growth factor/chemokine (a growth factor that attracts cells) stromal-derived factor-1 (SDF-1) plays a critical major role in the process. We predicted that SDF-1 and its receptor CXCR4, as well as genes activated by SDF-1, may play an essential role in targeting tumors to the bone marrow.

To investigate this hypothesis, we examined prostate cancer as a model for a tumor that frequently metastasizes to bone. Previously, we have demonstrated that prostate cancer cell lines express CXCR4, adhere to endothelium, and migrate through basement membranes in response to SDF-1 [1]. More recently, we determined that SDF-1 and CXCR4 are expressed by human prostate cancers *in vivo* [2]. To identify genes activated by SDF-1 in prostate cancers which might facilitate the establishment of bone metastases, we treated prostate cancers with SDF-1 and examined changes in gene expression at the mRNA level using gene arrays. For our investigations, we treated the metastatic human prostate cancer cell lines LNCaP and LNCaP C4-2B with SDF-1. Originally LNCaP cells were isolated from a lymph node of a patient with widespread bony and lymph node involvement. These cells were passaged in mice until a sub line was identified with increased bone homing capabilities (LNCaP C4-2B) [3]. The LNCaP cells and the LNCaP sub line C4-2B cells were originally obtained from UroCor, Inc (Oklahoma City, OK). Several prostate cancer cell lines were used in our studies.

In order to determine which genes in prostate cancers are altered by treatment with SDF-1, we utilized DNA Microarrays, a tool for the fast monitoring of a large number of genes at once. With this technology, we analyzed the expression of over 20,000 genes. In general, a microarray consists of gene sequences or fragments of genes called expressed sequence tags (ESTs). Very small amounts of hundreds or thousands of these ESTs are arranged on a single microarray substrate, often glass, usually by a robotic device. The genetic messenger (RNA or mRNA) which signals protein production interest is labeled, purified, and allowed to bind to the microarray. Later an imaging scanner reads the signal intensity (degree of hybridization) of a sample at each spot on the microarray. The analysis software formulates this information generated from the imaging scanner and uses the information to extract, manage and present the information in a usable fashion. In the past, we have only been able to conduct analyses on a few genes at once.

With the development of microarray technology, we can now examine thousands of genes at the same time. In order to perform the microarray analysis, RNA was collected by standard methods from SDF-1 stimulated LNCaP and LNCaP C4-2B cells and compared to RNA collected from cells that were not treated with SDF-1. For our analysis, we used 4 Affymetrix HG-U133A chips that contained signals for approximately one half of the human genome, obtained by the University of Michigan Dental School Microarray Facility. The raw data was transferred into the statistical software and then calculated expression values were performed using a Robust Multi-array Average (RMA).[4]). We chose this program to 'fit' a model to the data in order to calculate relative expression values [5]. After the expression values were calculated, the data was further analyzed using Significance Analysis of Microarrays (SAM), a program that calculates various statistical tests with adjustments for multiple comparisons using False Discovery Rate (FDR) [6]. The comparison of the SDF-1 (at a physiologic dose of 200 ng/ml) treated and untreated cells was straightforward and performed using a t-test that

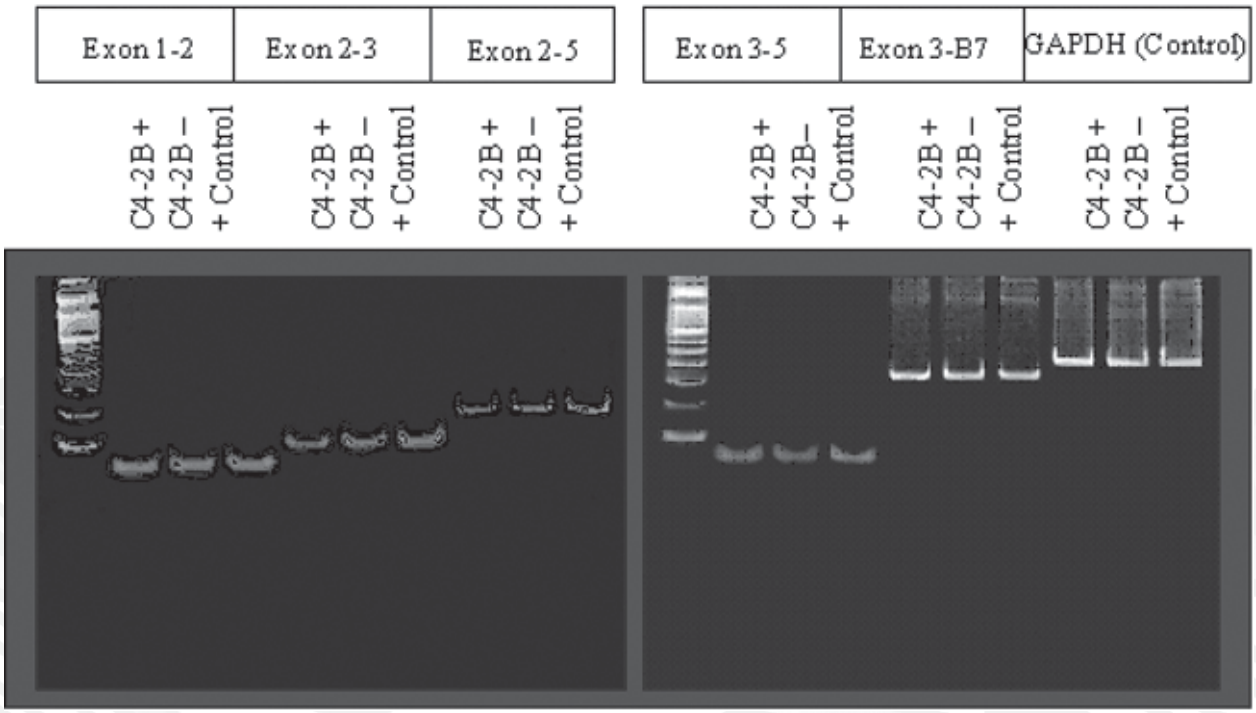

Figure 1: Gel Electrophoresis of CD164 Exon 1-6 This non-quantitative polyacrylamide gel shows the DNA sequences depicted over the designated exons (coding regions of DNA). Three types of cDNA are shown for each set of primers (probes for detecting the presence of the DNA) presented. Data is for LNCaP C4-2B cells treated with SDF-1(+), or cells that were not treated (-). The human hematopoietic cell line KG1a was utilized as a positive control. A molecular weight ladder corresponding to differences in 100 base pairs is shown to the left of each figure.

compares the mean of the expression values for each gene (at each time).

Initially our analysis revealed that there were ~300 genes in which expression increased in response to SDF-1. From these, the gene CD164 was identified as a SDF-1-responsive gene that is potentially involved in the homing process of prostate cancers to the marrow. CD164 is a protein of ~160 kDa initially identified on very primitive blood cell precursors. CD164 is known to function as an adhesion receptor during blood development, facilitating the adhesion of these early cells to their support cells in the bone marrow (or stromal cells) [7,8].

To verify the results from the microarray, we examined several prostate cancer cells for the expression of CD164 by Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR). This technology was first developed in the late 1980's and allows one to rapidly assess the presence or absence of a gene in a given sample of messenger RNA. We chose reagents for these analyses that were designed to cross intron/exon boundaries to ensure that we were not obtaining false positive signals. The first step in this technology was to isolate mRNA from the cells of interest and to then convert the mRNA into a complementary copy of DNA. Polymerase Chain Reaction or PCR was then used to make many copies of the gene CD164, and we incorporated many controls into our experiment to control for false signals. The PCR product was then analyzed in a DNA or polyacrylamide gel, where the amount of DNA base pairs can be quantified. As illustrated in Figure 1, gel electrophoresis shows that CD164 is expressed by the bone homing prostate cancer cell line LNCaP C4-2B, and the major mRNA produced by the tumor cells is the full length version of the mRNA (unspliced species). We cloned and sequenced the RT-PCR product to confirm this result. (Figure 2). The RT-PCR result provided further validation of the microarray results.

In order to determine whether prostate cancer cells actually make the protein coded for by the mRNA

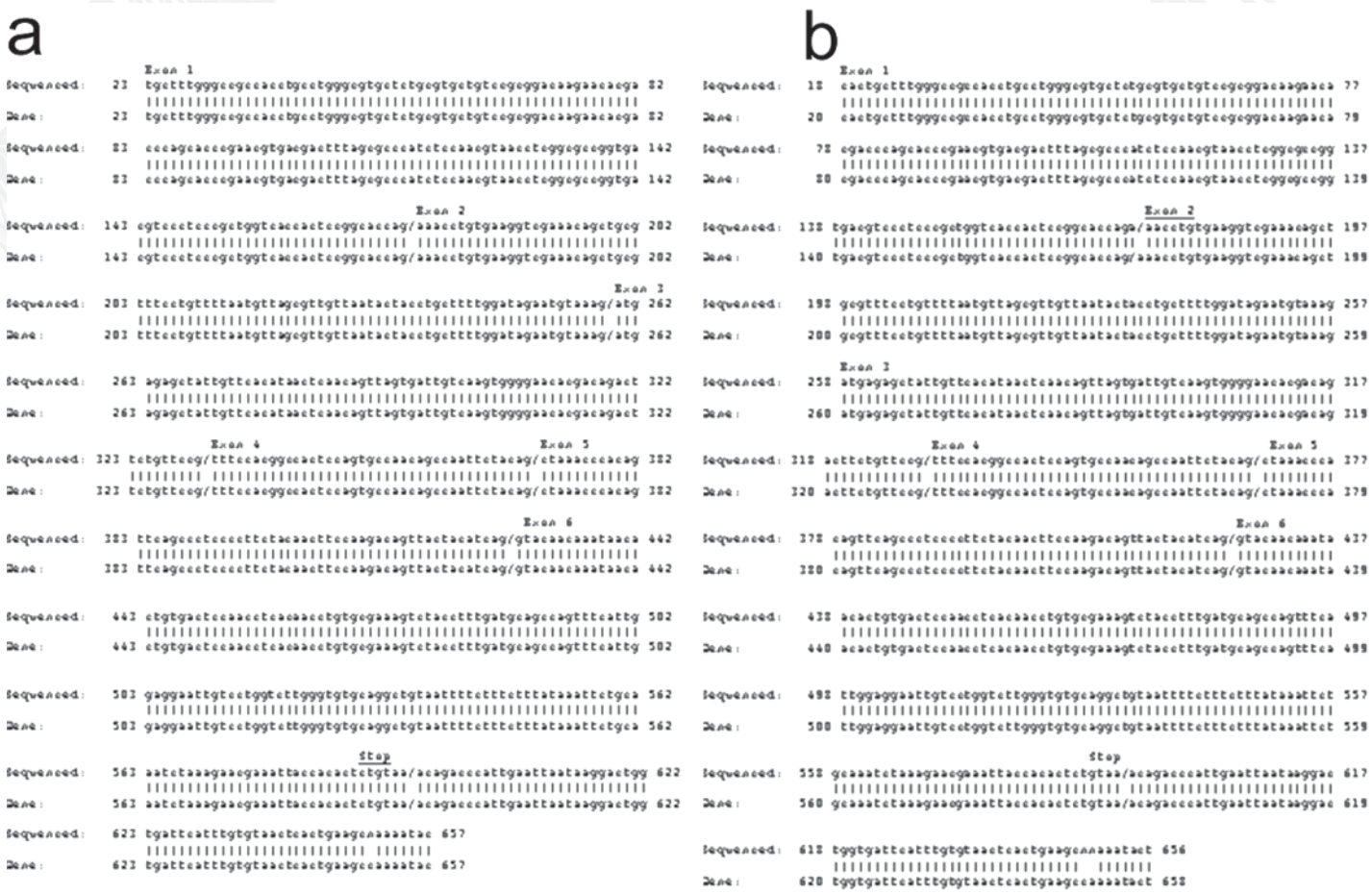

Figure 2: DNA Sequence Results DNA sequencing was performed by extracting the DNA from the gels in figure 1. Sequencing results along with the matched sequence of the gene CD164 cDNA (AF299341) in Gene Bank are shown, demonstrating Intron-Exon boundaries. (A.) The cDNA sequenced was C4-2B SDF-1 + between primers that are targeted for the full length transcript (F164 to B7). (B.) The cDNA sequenced was C4-2B SDF-1 – between the primers that are targeted for the full length transcript (F164 to B7).

that we detected by microarray and RT-PCR, we turned to Fluorescence Activated Cytometry (FACs). This technology allows detection of proteins on the surface of cells using colored antibodies and lasers. Here we examined the relative protein expression levels of CD164 on LNCaP C4-2B cells, PC3 cells (originally isolated from a vertebral metastasis of a human prostate cancer patient) or MCF-7 (a breast cancer cell line) with a commercially available antibody directed against CD164. For these investigations, cells were incubated over a three-hour period in the presence or absence of SDF-1 and subsequently stained with the antibody to CD164 (or an antibody control). Thereafter, members of the University of Michigan Cancer Center Cytometry Core Facilities examined the cells for the expression of CD164. The data demonstrates that by three hours both the LNCaP C4-2B and PC3 prostate cancer cells increased their expression of CD164 in the presence of SDF-1 (Figure 3). Surprisingly, the breast cancer cell line, which also homes to bone, did not alter the expression of CD164, suggesting that there may be alternative mechanisms involved in marrow homing by different tumor types (Figure 3).

Finally, to further verify that prostate cancer cells express CD164, we measured the expression of CD164 in human prostate cancer cells and human lymph node. An antibody to CD164 that was hooked to an enzyme was used to stain biopsies of patient samples. The expression of CD164 was detected by

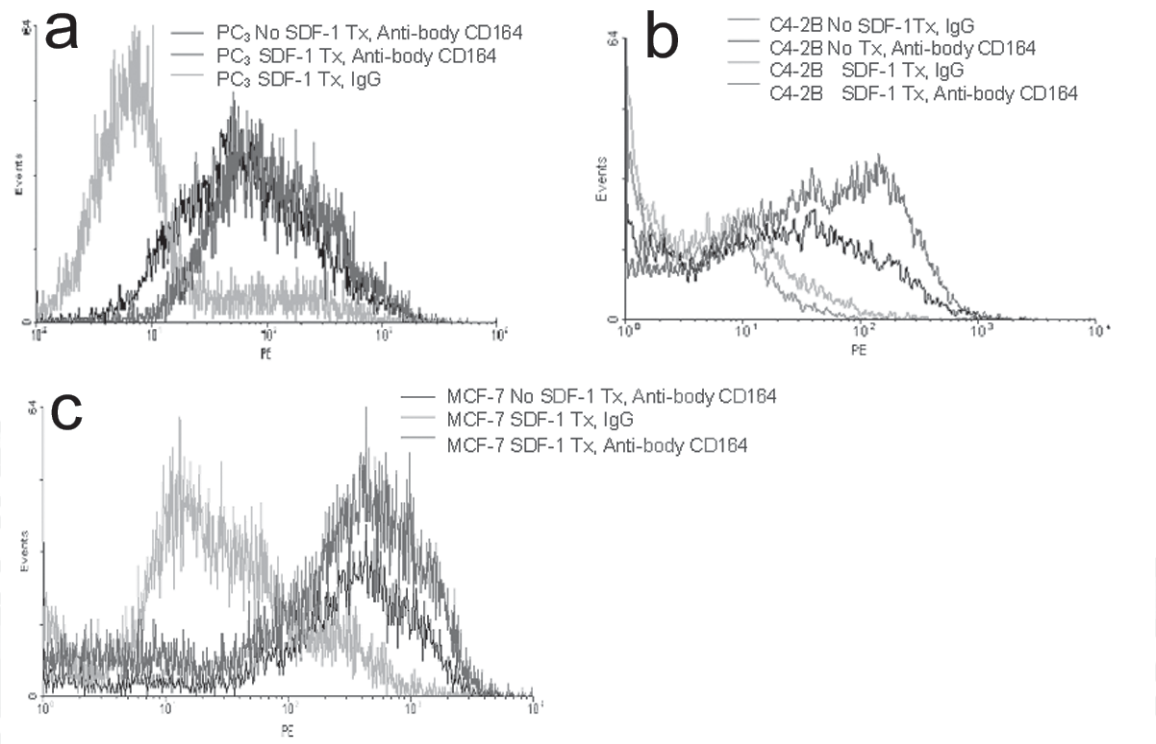

Figure 3: Flow Cytometry Analysis for SDF-1 Stimulated PC3 or LNCaP C4-2B Cells A.) Flow cytometry results of PC_3 cells treated with SDF-1. The slight shift of the antibody-stained cells of SDF-1 treatment for 3 hours shows that more PC_3 cells were stained during analysis. B.) Flow cytometry results of LNCaP C4-2B cells treated with SDF-1. The shift of the antibody-stained cells with SDF-1 treatment for 2 hours shows that the expression of CD164 is increased as the levels of SDF-1 are heightened when compared to the antibody-stained cells with no SDF-1 treatment. C.) Flow Cytometry results of MCF-7 cells treated with SDF-1. Both graphs show an IgG control.

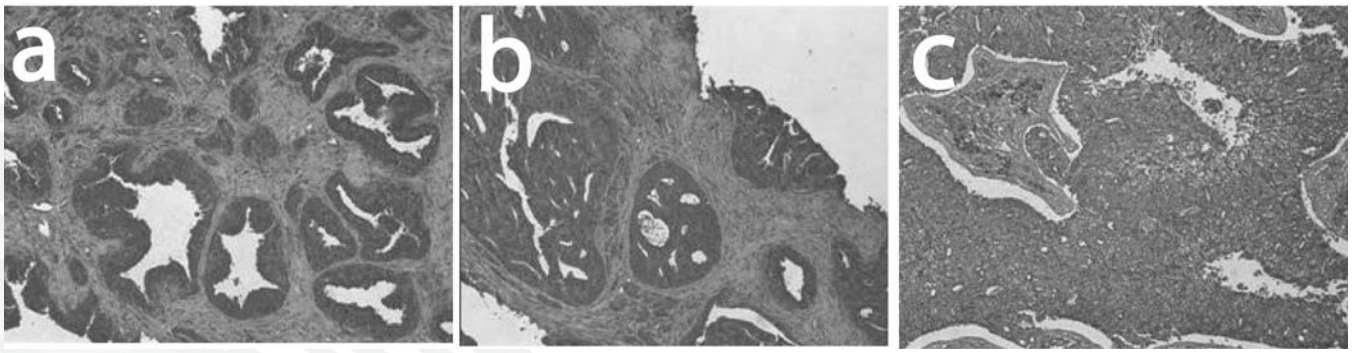

Figure 4: Immunohistochemistry of Prostate Cancer and Lymph Node
Immunohistochemistry was performed on patient samples of both prostate cancer and lymph node cells. The samples were stained with an antibody to CD164. (A, B) Human prostate cancer tissue biopsy demonstrated the intense brown staining of CD164, indicating the presence of CD164 in human prostate cancer. (20X) (C.) Positive control staining of a human lymph node at 20x magnification.

the ability of the enzyme to break down a substrate that turns a color – the result in this case is the presence of a brown stain on the tissue samples (Figure 4). The data demonstrates that CD164 is expressed by prostate cancer cells in patients and is not merely related to a culture artifact.

In summary, we have identified CD164 as a SDF-1 responsive gene in prostate cancers that is a reasonable candidate for a protein possibly involved in homing to the bone marrow. Thus far we have determined that CD164 mRNA is expressed by prostate cancer cell lines in relation to SDF-1 treatment. In addition, we have determined the protein expression of CD164 through flow cytometry or FACs analysis of metastatic cancer cell lines. At this point we have also found that the gene CD164 is responsive to heightened levels of SDF-1 and believe that CD164 is a gene responsible for adhesion molecules involved in prostate cancer homing to the bone marrow. Further studies are underway to determine the relative expression of CD164 in a large number of human samples relative to tumor aggressiveness. These preliminary studies may ultimately lead better targeted therapy to prevent prostate cancer metastasis.

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