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CO-EXPRESSION OF THE TOLERAGENIC GLYCOPROTEIN, CD200, WITH MARKERS FOR CANCER STEM CELLS

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Abstract

Tumor immunology fundamentals suggest immunological surveillance has the ability to recognize malignant cells and kill them before a tumor develops. However, cancer cells employ evasion mechanisms whereby the immune system may be actively suppressed or even tolerized to the tumor. Recently cancer stem cells were linked to tumor initiation and formation. However, no reports have addressed whether these cells participate in a tumor's ability to evade immune surveillance. Recently the glycoprotein CD200, expressed within the innate immune system and other tissues and cells, was shown to be involved in tolerance. Here we describe CD200 co-expression with stem cell markers found on prostate, breast, brain, and colon cancers. This is the first report describing an immunomodulatory molecule on epithelial cancer stem cells. This important finding suggests a mechanism by which a tumor might evades immune system detection.

Keywords

tumor immunity; cell surface molecules; tolerance; CD200; cancer stem cells

INTRODUCTION

Representing a small subpopulation of the tumor mass, cancer stem cells (CSCs) are believed to be the initiators of tumorigenesis. Furthermore, they seem to be resistant to conventional chemotherapy and serve as a reservoir of self-renewing cells potentiating tumor relapse. [1]. The cancer stem cell hypothesis has been evolving for the past 150 years, and strong evidence for their existence was recently reported [2]. CSCs were first described in acute myeloid leukemia (AML), where a small, distinct population of cells was capable of generating leukemia in mice [3;4]. The demonstration that a CSC population initiates leukemia has galvanized scientific research to identify stem cells in other cancers. To date, human CSCs were identified in a variety of other tissues including breast, brain, prostate, lung, colon and pancreatic tumors and their corresponding cell lines [5–10]. For the most part, CSCs have been phenotypically identified by specific clusters of differentiation (CD) markers previously found on hematopoietic cells.

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A hallmark of tumor progression is the ability to avoid discovery by the immune system. Molecular signaling in the tumor environment helps create an immunosuppressive network whereby the cancer escapes detection [11]. CSCs may play an important role in the tumor's ability to evade immune detection. However, it is unknown whether CSCs are even recognized by the immune system, since the majority of experimental tumor immunology is based on whole populations of cells. Tumor tolerance is a mechanism by which a tumor can escape recognition by the immune system thus allowing transformed cells to grow out. The glycoprotein, CD200 (OX-2) has been studied as a therapeutic target for its role in immunoregulation and tolerance [12]. CD200 is a highly conserved member of the Ig superfamily and is commonly expressed in cells of myeloid lineage such as dendritic and neuronal cells, as well as other cell types. CD200 is also a marker of human hair follicle bulge stem cells [13]. Thus, the identification of CD200 as both a potential stem cell marker and as an immune suppressor suggests that CSCs might evade the immune system through a CD200 dependent mechanism.

To determine if CD200 expression is a common characteristic of CSCs, we evaluated the prevalence of CD200 on prostate, breast, glioblastomas, and colon cell lines, and on normal human mesenchymal stem cells. The expression of CD200 on epithelial cancer stem cells may be important in understanding the mechanism used by tumors to evade the immune system.

MATERIALS AND METHODS

Cell Culture

All cells were cultured as previously described [14–17]. VCaP cells were a generous gift from Ken Pienta, University of Michigan.

Fluorescence-Activated Cell Sorting (FACS) and Analysis

LNCaP cells were washed in FACS buffer (PBS plus 1–2% BSA and 5 mM EDTA) and incubated at 4°C for 15 minutes with 10⁶ cells of anti-CD24 conjugated FITC (Invitrogen, Carlsbad, CA) and anti-CD44 conjugated to PE (Invitrogen, Carlsbad, CA). Cells were washed in FACS buffer, re-suspended at 20×10⁶ cells/ml, and separated on either an Aria (BD Biosciences, San Jose, CA) or a MoFlo High Performance cell sorter (Dako Cytomation, Carpinteria, CA). Surface expression was determined with the following antibodies: anti-CD200-FITC- or PE-conjugated anti-human antibody (Serotec, Raleigh, NC), anti-CD44-APC (BD Biosciences PharMingen, Franklin Lakes, NJ), anti-CD24-FITC (Invitrogen, Carlsbad, CA), and anti-CD133-PE (Miltenyi Biotec, Auburn, CA). Cells were trypsinized, counted, resuspended at 10⁶ cells per tube in FACS buffer and stained for 15 minutes at 4°C. Cells were washed with buffer and analyzed by FACS.

RNA Extraction, Amplification and Microarray Analysis

Isolation and amplification of total RNA was done as previously described [18]. Hybridization, preparation and analysis of microarrays were done as previously described [18]. The Cy5/Cy3 ratios for each gene were median centered and clustered using the Cluster program and visualized using the TreeView program, offered by Michael B. Eisen as freeware (<http://rana.lbl.gov/EisenSoftware.htm>).

RESULTS AND DISCUSSION

Genomic expression of CD200 is increased in putative prostate cancer stem cells

Previous work in our lab and others characterized a putative CSC subpopulation in prostate cancer cell lines [19]. A marker characterizing a prostate CSC is CD44. The LNCaP prostate cell line is a useful model to assess CSC properties in an *in vitro* setting. Microarray analysis

of the LNCaP stem cells showed an increased expression of CD200, a protein involved in immunosuppression and immune tolerance [12]. CD200 expression was significantly higher (14-fold) in the putative stem cell population compared to the non-stem or total cell population (Fig. 1). Moreover, expression of other well described immunomodulatory molecules were not increased in CSCs [20;21] (Fig. 1). This is the first evidence of expression of a toleragenic molecule on CSCs. To determine the prevalence of CD200 expression, we next evaluated its expression on other cancer stem cells.

CD200 co-expresses with markers of cancer stem cells

Most human CSCs were identified by expression of phenotypic markers originally described on hematopoietic stem cells. Recent evidence suggests that CD44 is a marker for prostate cancer stem cells derived from cell lines [19]. We therefore evaluated whether CD200 and CD44 co-expressed in the prostate cancer cell lines, DU145, VCaP and PC-3. CD200 is expressed on a small, but significant population of prostate cells (Fig. 2). All live events were gated on forward and side scatter. DU145 and PC-3 had a significant proportion of CD200 expressing cells (64–99%) within the CD44⁺ population (Table 1). Interestingly, PC-3 cells, a highly invasive and metastatic cell line, had a population of CD200⁺ cells exclusively expressing CD44 (Fig. 2).

Extracellular markers for breast CSCs are CD44⁺CD24⁻ [5]. The breast cell line, MDA-MB231 contain a high percentage of CD44⁺CD24⁻ expressing cells whereas MCF-7 cells were lower in CD44⁺CD24⁻ expression (Fig. 3). Interestingly, the vast majority of CD200⁺ cells in MDA-MB231 were both CD44⁺ and CD24⁻ (Fig. 3). The majority of CD200 expressing cells (88%) were found on the putative stem cell population (Table 1). Only 12% of the cells that expressed CD200 were found on the cells depleted of CD44⁺CD24⁻. Furthermore, this cell line is highly tumorigenic and invasive [22]. The less tumorigenic cell line, MCF-7, had a much smaller proportion of CD200⁺ cells that were CD44⁺CD24⁻ (Fig. 3). However, there still was a distinct subpopulation of cells (5.2%) that were CD200⁺CD44⁺CD24⁻ (Table 1).

The CSC marker for glioblastoma is CD133 [10]. The glioblastoma cell lines SF295, SNB75 and U251 have a distinct subpopulation of CD200 expressing cells that are also CD133⁺ (Fig. 4a). There was a 47–73% range of CD200 cells that were also CD133⁺ (Table 1). Recent evidence implicates CD133 as a colon CSC marker.[8]. The colon cell lines HT-29, HT-116, and SW48 also have a discrete population of CD200⁺ cells (Fig. 4b). Of the CD200 expressing cells, 83–97% were also CD133⁺ (Table 1).

The characterization of CSCs is constantly developing; therefore CD200 expression may be a further delineation of CSCs. These cells may be all that is required to maintain tumor growth, self-renew, metastasis and evade the immune system. Another possibility is that there is functional heterogeneity in the CSCs whereby CD200 expressing cells maintain cancer stem cell survival and tumor tolerance. The CD200 expressing CSCs could act to tolerize the growing tumor from the host immune system. There may be other subpopulation of CSCs that serve other functions such as driving tumor growth and pluripotency maintenance. However, additional work is needed in order to address these questions.

CD200 expression on normal stem cells

Lastly, CD200 expression was checked on normal human stem cells. We used primary human bone marrow mesenchymal stem cells (hMSC, Lonza Corp., NJ) as a source of adult stem cells. These pluripotent cells can differentiate into a variety of cell types. A marker for hMSCs is CD44. There was a distinct subpopulation of hMSCs that also express CD200, further supporting the idea that CD200 is found on a subpopulation of stem cells (Fig. 5). Our finding

that CD200 is found on normal human stem cells suggests the possibility that these cells use the CD200 system to prevent the immune system from attacking stem cells.

There is an impressive body of evidence implicating CD200-CD200R interactions on immune tolerance, transplantation, autoimmunity and therapy [23–25]. Notably, CD200-deficient mice develop a number of autoimmune disorders including experimental allergic encephalomyelitis, collagen-induced arthritis, and autoimmune alopecia [23;24]. In a recent study, anti-CD200 antibodies almost completely attenuated tolerance and allowed immune rejection of CD200⁺ B-cell lymphoma cells in a mouse xenograft model that incorporated human immune cells [26]. They further demonstrated that CD200 overexpressing cells were better able to survive lymphocyte eradication compared to vector controls. This study, and our finding that CD200 is found on CSCs, suggests an intriguing model whereby a tumor is able to evade the immune system via a CD200 expressing CSC. Additionally, since CSCs are thought to be responsible for relapse, targeted therapy of these cells may be a viable option in the prevention of cancer recurrence and vaccine designs against CSCs, including protocols blocking CD200 function.

This study demonstrates that CD200 is expressed on CSCs. In each case, there was a subpopulation of cells that expressed CD200 and their corresponding cancer stem cell marker, implicating CD200 as a potential marker of CSCs. In ten of the twelve cell lines tested, the majority of cells expressing CD200 were found in the CSC population. In addition, the role of CD200 in immune tolerance could suggest CD200 marks a subclass of cells involved in the ability of cancer cells to evade the immune system. The finding that CD200 is found on non-stem cells could suggest a transient population in which CD200 is expressed during the differentiation of CSCs towards the progeny cells. Alternatively, any cell, including the CSCs may have an altered immunological response due to CD200; however, it is of interest that its expression on LNCaP is strikingly fourteen fold higher in the CSC. Additional studies must be done to address these issues.

This is the first study to recognize a toleragenic marker on human normal and cancer stem cells. In addition, it is also the first study to identify CD200 on epithelial-derived tumors. The co-expression of CD200 on a cancer stem cell subpopulation provides a potential mechanism whereby cancer stem cells are able to avoid detection by the host immune system and remain as residual disease. Lastly, our results of CD200 co-expression on human CSCs remain provocative to the issue of tolerance, tumor immunity, and therapy. The identification of CD200 on CSCs may offer opportunities in the designs of therapies that target CSCs in the prevention of relapse.

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Figure 1.

Microarray analysis of proteins known to be involved in tolerance in a putative prostate cancer stem cell. CD44⁺CD24⁻ and CD44⁺CD24⁻-depleted cells were isolated from the prostate cell line, LNCaP, and hybridized along with Universal Reference RNA on an oligonucleotide array. Relative expression is shown with red representing a relative over-expression, green representing a relative under-expression, black representing no change, and gray representing an absence of data.

Figure 2.

CD200 expression in prostate and breast cancer cell lines. A. Prostate cell lines DU145, PC-3 and VCaP were FACS analyzed for expression of CD200 and CD44. Cells were labeled with PE-coupled antibodies to CD200 and FITC-labeled antibodies to CD44. Percentage of gated population for each subgroup, CD44⁺CD200⁻, CD44⁺CD200⁺ and CD44⁻CD200⁺, is noted in their corresponding quadrants (upper-left, upper-right and lower right, respectively). B. Breast cancer cell lines were FACS analyzed for expression of CD200, CD44 and CD24. Cells were labeled with PE-coupled antibodies to CD200, FITC-labeled antibodies to CD24 and APC-coupled antibodies to CD44. For each cell line, top panels indicate CD200⁺ (y-axis) expression in the CD44⁺CD24⁻ population. Bottom panels indicate CD200⁺ (y-axis) expression in the cells depleted of CD44⁺CD24⁻. Depleted indicates the population of cells not contained in the CD44⁺CD24⁻ population. Percentages indicate the number of CD200⁺ cells of a given population divided by the total number of CD200⁺ cells. Percentages in parenthesis indicate the number of CD200⁺ of a given population divided by the total number of cells analyzed. Live events were gated based on forward and side-scatter.

Figure 3.

CD200 expression in glioblastoma and colon cancer cell line. A) Glioblastoma and B) colon cell lines were analyzed for expression of CD200 and CD133. Cells were labeled with FITC-coupled antibodies to CD200 and PE-labeled antibodies to CD133. Percentage of gated population for each subgroup, CD133⁺CD200⁻, CD133⁺CD200⁺ and CD133⁻CD200⁺, is noted in their corresponding quadrants (upper-left, upper-right and lower right, respectively). Live events were gated based on forward and side-scatter.

Figure 4.

CD200 expression in human mesenchymal stem cells. Primary hMSCs were analyzed just as the prostate cancer cell lines (see Figure 2). Cells were passaged a maximum of five times before being analyzed. Live events were gated based on forward and side-scatter.

TABLE 1

Co-expression of CD200 with cancer stem cell markers Left panel indicates type and name of cell lines and parenthesis specifies cancer stem cell marker. Right panel is the number of stem cells expressing CD200 as a percentage of the total number of CD200-expressing cells

Cancer cell line (stem cell marker)	%CD200 stem/total CD200
PROSTATE (CD44⁺)	76.2
DU145	63.9
VCAP	99.2
PC-3	
BREAST (CD44⁺CD24⁻)	88.6
MDA-MB231	5.2
MCF-7	
GLIOBLASTOMAS (CD133⁺)	68.1
SF295	47.7
SNB75	73.6
U251	
COLON CARCINOMAS (CD133⁺)	83.3
HT-29	95.4
HT-116	97.6
SW48	
MESENCHYMAL STEM CELLS (CD44⁺)	99.9