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Stage-specific embryonic antigen: determining expression in canine glioblastoma, melanoma, and mammary cancer cells

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The expression of stage-specific embryonic antigens (SSEAs) was determined in several types of canine cancer cells. Flow cytometry showed SSEA-1 expression in glioblastoma, melanoma, and mammary cancer cells, although none expressed SSEA-3 or SSEA-4. Expression of SSEA-1 was not detected in lymphoma, osteosarcoma, or hemangiosarcoma cell lines. Relatively stable SSEA-1 expression was observed between 24 and 72 h of culture. After 8 days in culture, sorted SSEA-1$^-$ and SSEA-1$^+$ cells re-established SSEA-1 expression to levels comparable to those observed in unsorted cells. Our results document, for the first time, the expression of SSEA-1 in several canine cancer cell lines.

Keywords: canine cancer, flow cytometry, magnetic-activated cell sorting, stage-specific embryonic antigens

Stage-specific embryonic antigens (SSEAs) are developmentally-regulated carbohydrate antigens that have been shown to be tumor-specific or tumor-associated antigens [4]. It has been shown that SSEAs are involved in key pathophysiological processes during tumor progression [2]. For example, SSEA-1-positive (SSEA-1$^+$) human glioblastoma cells were shown to have higher tumorigenic potential than SSEA-1-negative (SSEA-1$^-$) cells, and they were capable of forming secondary tumors following *in vivo* passage, suggesting that SSEA-1 might serve as a marker for tumor-initiating cells in glioblastoma [12]. In addition, SSEA-3$^+$ colorectal cancer cells were shown to possess increased tumorigenic potential and higher proliferative ability, although they had lower *in vitro* sphere formation capacity than SSEA-3$^-$ cells. Furthermore, immunofluorescence-based analysis of colorectal cancer specimens indicated that SSEA-3 expression, which was limited to stromal cells in the normal mucosa, was widely distributed in poorly differentiated adenocarcinomas, suggesting that SSEA-3$^+$ cells may function as tumor transient amplifying cells or delayed-contributing tumor-initiating cells in this tumor [14]. In oral squamous cell carcinoma, CD44$^+$SSEA-4$^+$ cells had cancer stem-like characteristics including preferential expression of stemness genes, self-renewal, resistance to anticancer agents, and greater tumorigenic potential than cells lacking either marker [8].

The biology and natural behavior of human and canine tumors share many similarities [3]. The expression of SSEAs has been previously examined in embryonic and adult canine mesenchymal stem cells [15], and the distribution and function of SSEA-1 have been examined in metastatic canine mammary tumor cells [6]. However, the expression of SSEAs has not been examined systematically in canine cancer cells. In this report, we describe the expression patterns of SSEA-1, SSEA-3, and SSEA-4 in canine glioblastoma, melanoma, sarcoma, lymphoma, and mammary carcinoma cell lines.

The canine glioblastoma cell lines Candy and Mac were generously provided by John Ohlfest and Elizabeth Pluhar (University of Minnesota, USA). The melanoma cell lines TLM1, CMGD2, and CMGD5, osteosarcoma cell lines OSCA32 and OSCA40, and hemangiosarcoma cell lines DD-1 and EFB were isolated and maintained in our laboratory. The COSB hemangiosarcoma cell line was derived by *in vivo* passage from the SB-HSA cell line originally established by Stuart Helfand (Oregon State University, USA) and Erin Dickerson (University of Minnesota). The lymphoma cell line CLBL1 was the kind gift of Barbara Rütgen (University of Vienna, Austria), and canine mammary tumor cell lines CMT9, CMT12, CMT25, CMT27, CMT28, and CMT83 were generously provided by

Curtis Bird (Auburn University, USA). Human MCF7 cells (ATCC, USA) were used as a positive control for antibody performance [5] and SSEA expression. Melanoma, osteosarcoma, and mammary cancer cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco-BRL, USA) supplemented with 10% fetal calf serum (FCS; Atlas Biologicals, USA), 2 mM L-glutamine (Mediatech, USA), and 100 μg/mL primocin (InvivoGen, USA). Glioblastoma cell lines were cultured in DMEM/Hams F12 medium supplemented with 10% FCS, L-glutamine, primocin, 1× NEAA, N2, and B27 (Gibco-BRL). Hemangiosarcoma cells were cultured in Hams F12 medium supplemented with 30 μg/mL endothelial cell growth supplement (Fisher Scientific, USA), 100 μg/mL heparin (Sigma-Aldrich, USA), 10 mM 4-(2-hydroxyethyl)-1-piperazineethaneusulfonic acid (Mediatech) and primocin. CLBL1 cells were cultured in Iscove's Modified Dulbecco's Medium (Gibco-BRL) supplemented with 20% FCS, L-glutamine, and primocin. All cells were grown at 37°C in a relative humidity of 100% and an atmosphere of 5% CO_2.

For flow cytometric analysis, cells were incubated with anti-dog IgG antibody (Jackson ImmunoResearch Laboratories, USA) to prevent non-specific binding of antibodies to Fc receptors and stained with phycoerythrin (PE)-conjugated antibodies against human SSEA-1, SSEA-3, and SSEA-4 (Millipore, USA). All three antibodies are reported to recognize their respective canine targets. Subsequently, 0.5 μg/mL 7-amino-actinomycin D (7-AAD; eBioscience, USA) was added to each FACS tube prior to analysis. Cells were gated based on their light scatter properties and dead cells were excluded by using 7-AAD staining. Flow cytometry was performed on a BD Accuri C6 flow cytometer (BD, USA) and 10,000 live cells for each condition were used for analysis. For magnetic cell sorting, cells were dissociated into single-cell suspensions, labeled with anti-SSEA-PE antibody, and incubated with anti-PE microbeads (STEMCELL Technologies, Canada). Cells were then separated using the StemSepsystem (STEMCELL Technologies). Two separation columns were used consecutively to ensure high purity of sorted populations. Cell purity was assessed by using flow cytometry, and sorted SSEA$^+$ and SSEA$^-$ cells were then cultured for 8 days to determine SSEA expression.

We evaluated expression of SSEA-1, SSEA-3, and SSEA-4 in two or more canine glioblastoma, melanoma, osteosarcoma, hemangiosarcoma, and mammary cancer cell lines, and in a single canine lymphoma cell line. SSEA-1 was detectable in a small percentage of cells from one of two glioblastoma cell lines, two of three melanoma cell lines, and two of six mammary cancer cell lines (Table 1; panel A in Fig. 1), but it was not expressed in any of the osteosarcoma, hemangiosarcoma, or lymphoma cell lines tested. None of the canine cancer cell lines expressed SSEA-3 or SSEA-4 (data not shown). To investigate the patterns of SSEA-1 expression during cell growth, we determined the expression of SSEA-1 in CMT83 mammary

cancer cells after 24, 48, and 72 h in culture. Flow cytometry revealed relatively stable SSEA-1 expression in the CMT83 cells over time (5%−10% of total cancer cells), although the percent of SSEA-1$^+$ cells increased slightly as the cells reached 80% to 90% confluence after 3 days in culture (panel B in Fig. 1). To evaluate whether SSEA-1 expression was restricted to a unique subset of cells within the CMT83 cell line, we used immunomagnetic separation to obtain SSEA-1$^+$-enriched and SSEA-1$^+$-depleted CMT83 cells. This separation yielded approximately 17.0% SSEA-1$^+$ cells in the enriched population. SSEA-1$^+$ cells in the depleted population were below the limit of detection (0%), which we considered as the SSEA-1$^-$ population (Fig. 2). The sorted SSEA-1$^+$-enriched and the SSEA-1$^-$ cells were then cultured for 8 days and subjected to re-analysis of SSEA-1 expression. The results show that the SSEA-1$^-$ cells recovered SSEA-1 expression to levels approximating those observed prior to immunomagnetic depletion (panel B in Fig. 1 and Fig. 2), and the percentage of SSEA-1$^+$ cells was similarly reduced to levels seen prior to enrichment. These results suggest that SSEA-1 expression is tightly regulated to achieve a steady state in canine cancer cells.

It has been shown that embryonic development and cellular activation in vertebrates are typically accompanied by changes in cellular glycosylation profiles [9]. It is therefore not surprising that glycosylation alteration is a universal characteristic of malignant transformation and tumor progression [5,11]. SSEAs were originally identified by monoclonal antibodies recognizing defined carbohydrate epitopes associated with lacto- and globo-series glycolipids [4], and they are often used as markers for stem cell differentiation [10,13]. Recently, aberrant expression of SSEAs has been detected in several human cancers and found to be actively involved in tumor progression [8,14]. However, the expression and biological

Table 1. Expression of stage-specific embryonic antigen (SSEA)-1 in canine cancer cell lines

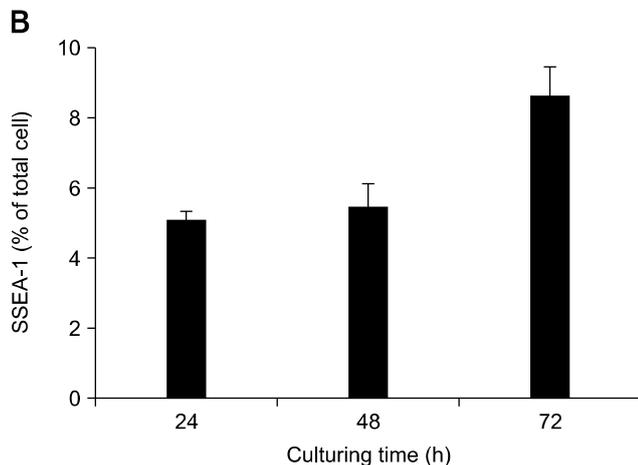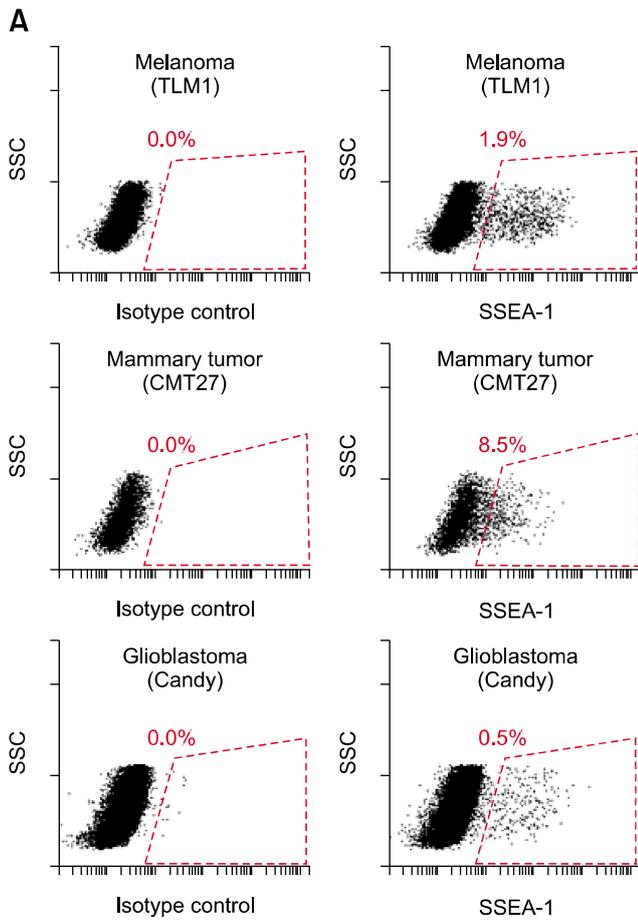| Tissue derivation	Cell lines	SSEA-1$^+$ (%)	Number of SSEA-1$^+$ cell lines over the total number of cell lines tested (%)
Glioblastoma	Candy	∼0.6	1/2 (50%)
	Mac	0	
Melanoma	TLM1	∼2	2/3 (66.7%)
	CMGD2	∼0.4	
	CMGD5	∼2.6	
Mammary cancer	CMT9	0	2/6 (33.3%)
	CMT12	0	
	CMT25	0	
	CMT27	∼4	
	CMT28	0	
	CMT83	∼7	

Fig. 1. Stage-specific embryonic antigen (SSEA)-1 expression in canine cancer cells. (A) Flow cytometric analysis of SSEA-1 expression in canine melanoma TLM1, mammary cancer CMT27, and glioblastoma Candy cancer cell lines. Cells were incubated with anti-SSEA-1-PE antibody. A minimum of 10,000 events were collected per sample. Data shown are representative of several independent experiments. (B) SSEA-1 expression in canine mammary cancer CMT83 cells at various culture times. Cells were incubated with anti-SSEA-1-PE antibody. Data are expressed as mean ± SD.
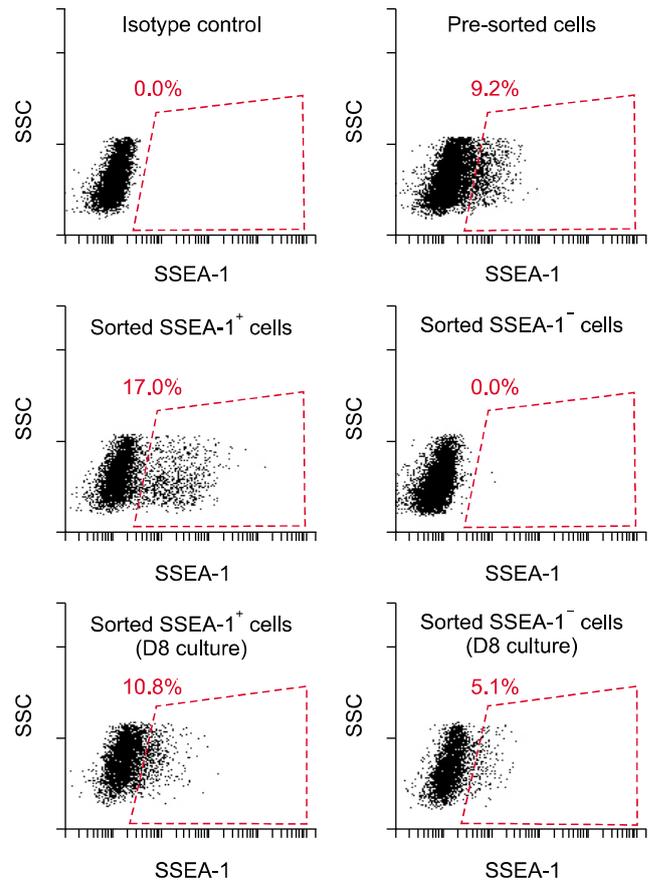
Fig. 2. Flow cytometric analysis of stage-specific embryonic antigen (SSEA)-1 expression in mammary cancer CMT83 cell line. The sorted SSEA-1 positive (+) and negative (−) cells were cultured separately and harvested for re-analysis of SSEA-1 expression after 8 days of culture. Values are taken from one of three independent experiments.

features of SSEAs are not fully characterized.

In this study, we identified SSEA-1, SSEA-3, and SSEA-4 in canine glioblastoma, melanoma, osteosarcoma, hemangiosarcoma, lymphoma, and mammary cancer cell lines, and found SSEA-1 was expressed in some, but not all of the glioblastoma, melanoma, and mammary cancer cell lines. SSEA-1 was not expressed in any of the lymphoma, osteosarcoma and hemangiosarcoma cell lines tested, and none of the cell lines expressed SSEA-3 or SSEA-4. To our knowledge, this is the first report documenting SSEA-1 expression in cultured canine cancer cells, and the findings suggest that expression of the SSEA-1 carbohydrate antigen, modified with a fucosylated structure, is similar in canine and human cancers. Our results also show that SSEA-1 expression was relatively stable over time, but at the same time, it was a dynamic process: Not only were SSEA-1- cells able to upregulate this antigen, but also a proportion of SSEA+ cells seemed to downregulate its expression after enrichment to re-establish a stable steady state. This

suggests that sorted SSEA1$^-$ cells retain active β1,4-galactosyltransferases 1 and α1,3-fucosyltransferase, which are required to generate SSEA-1 [4].

High expression of SSEA-1 carbohydrate antigen is associated with proliferation, migration, and resistance to chemotherapeutic agents in human cancer cells [1]. In addition, SSEA-1 antigen is involved in selectin-mediated adhesion of cancer cells to vascular endothelium, which seems to correlate with hematogenous cancer metastases, and it has been proposed as a marker for cancer stem cells or for transit-amplifying cells [8,12,14]. Given the preferential expression of SSEA-1 in normal stem cells of humans, dogs, and mice [4,10,17], it could be surmised that this trait is shared between normal stem cells and cancer stem cells. However, recent data suggest that proliferation, self-renewal, limited lineage differentiation, and other properties that define cancer stem cells cannot be attributed to any single element, and the acquisition of these properties is dynamic in nature [7,16]. Our observation that after depletion of SSEA-1$^+$ cells, a subset of SSEA-1$^-$ cells upregulated SSEA-1 to the membrane is consistent with this notion. While this could be interpreted to suggest that SSEA-1 expression is important to support cancer stem cell function in canine tumors, the dynamic regulation of its expression suggests that expression of SSEA-1 alone at a single time is probably not a suitable marker to identify these cells. The mechanisms that regulate the steady-state expression of SSEA-1, as well as its biological function and clinical importance in canine cancers will require further investigation.

In summary, our results demonstrate, for the first time, that SSEA-1 expression is heterogeneous in a multiplicity of canine cancer lines, but when it is present, SSEA-1 expression seems to be regulated in a stable steady state. Further studies to investigate the pathophysiological functions of SSEA-1 in canine cancer seem justified.

Acknowledgments

Conflict of Interest

The authors declare no conflicts of interest.

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