P-selectin Expression in a Metastatic Pancreatic Tumor Cell Line (SUIT-2)

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ABSTRACT

The human pancreatic tumor cell line SUIT-2 was derived from a metastatic lesion in the liver of a patient with pancreatic adenocarcinoma. SUIT-2 and clonal cell lines derived from it show spontaneous metastasis to lung and regional lymph nodes from s.c. nude mouse xenografts and were found to express P-selectin mRNA and protein. Surface expression of P-selectin protein was increased by exposure of the pancreatic tumor cells to thrombin, oxygen radicals, and trypsin, suggesting that common cellular mechanisms for regulating P-selectin surface expression exist among platelets, endothelial cells, and these pancreatic tumor cells. The finding that P-selectin is expressed by metastatic pancreatic tumor cells demonstrates that the range of cell types that express these adhesion molecules is broader than believed previously.

INTRODUCTION

The human pancreatic tumor cell line SUIT-2, which was derived from a patient's liver metastasis, is unusual among human tumor cell lines in that it shows spontaneous metastasis to lung and regional lymph nodes from s.c. nude mouse xenografts (1-3). There is one other published report of such spontaneous metastatic activity among human pancreatic tumor cell lines, the FG cell line, which was established by several cycles of injection of normal COLO 357 cells into the spleens and isolation of spontaneous metastases from the livers of nude mice (4). Twenty-eight clonal sublines of SUIT-2 were derived by in vitro methods (5). Two sublines, respectively, were established by sequential cycles of transplanting SUIT-2 cells to s.c. or i.v. (tail vein) sites in nude mice, harvesting metastases from the lungs, and then repeating this selection process. These clonal cell lines have distinct metastatic and histological properties (2, 3, 5). Six cell lines that show distinct morphological and metastatic properties, ranging from poorly differentiated to well differentiated and from rarely metastatic to highly metastatic, were analyzed by differential display to identify genes that are differentially expressed among them.

One gene differentially expressed by some of the metastatic pancreatic tumor cell lines is P-selectin. Selectins are expressed on the surface of endothelia, leukocytes, and platelets and are known to mediate initial adhesion interactions between different cell types (6). Selectins have a unique and characteristic structure that includes an extracellular NH2-terminal domain similar to Ca2+-dependent C-type lectin, a single epidermal growth factor-like domain, a variable number of short consensus repeat units similar to domains found in complement-binding proteins, a transmembrane segment, and a short cytoplasmic tail (6, 7, 8). Three members of the selectin family are currently known. E-selectin (ELAM-1) mediates binding of neutrophils and memory T cells to inflamed vascular endothelium.

E-selectin is not expressed constitutively but is induced on the surface of endothelial cells upon activation by inflammatory cytokines (interleukin 1 or tumor necrosis factor; Refs. 9 and 10). L-selectin (Leu 8 and TQ 1) is expressed constitutively at the cell surface and mediates the specific homing of lymphocytes into murine peripheral lymph nodes as well as the binding of polymorphonuclear neutrophils to endothelium in inflamed tissues (11, 12). P-selectin (PADGEM protein, GMP-140, and CD-62) is located in a granules of resting polymorphonuclear leukocytes and memory T cells to inflamed vascular endothelium. P-selectin is rapidly redistributed to the plasma membrane, where it mediates adhesion of activated platelets to monocytes and neutrophils and adhesion between endothelial cells and neutrophils (15, 19-22). P-selectin is believed to play an important role in leukocyte extravasation during cellular inflammatory responses (18, 23).

It was hypothesized previously that selectins play a role in tumor metastasis by contributing to adhesion of migrating tumor cells to endothelial cells (24, 25), but based on the fact that many tumor cells express on their cell surfaces high levels of ligands for the selectins such as the sialyl Lewis X, sialyl Lewis A, and related carbohydrate structures (26-32). This report is the first to demonstrate expression of P-selectin by human pancreatic adenocarcinoma tumor cells. In addition, it is shown that cell surface expression of P-selectin is increased on SUIT-2 cells following treatment with thrombin, oxygen radicals, and trypsin.

MATERIALS AND METHODS

Cells and RNA Preparation. A human pancreatic tumor cell line (SUIT-2), which was derived from a liver metastasis, shows spontaneous metastasis to lung and regional lymph nodes after s.c. injection of tumor cells into the lateral flank of nude mice. Twenty-eight sublines were cloned from SUIT-2 by culture in soft agar and characterized for their morphological and metastatic properties (1-3). Four sublines were selected for further study (5): S2-007, moderately differentiated tubular adenocarcinoma, highly metastatic; S2-020, poorly differentiated tubular adenocarcinoma, moderately metastatic; S2-013, well differentiated tubular adenocarcinoma, moderately metastatic; and S2-028, papillary-tubular adenocarcinoma, rarely metastatic. Two additional metastatic cell lines were established by sequential selection of lung colonies in nude mice following tail vein injection (S2-VP10, moderately differentiated tubular adenocarcinoma, highly metastatic) or spontaneous metastasis to the lung following s.c. injection (S2-CP9, moderately differentiated tubular adenocarcinoma, highly metastatic). S2-VP10 and S2-CP9 are cell lines established after 10 and 9 cycles of selection, respectively. All of these cell lines have distinct metastatic and morphological properties. They are maintained in DMEM supplemented with 5% FBS3, ECV304, a cell line derived from spontaneously transformed human umbilical endothelial cells, was obtained from American Type Culture Collection (Rockville, MD) and maintained in a 1:1 mixture of DMEM and Ham's F-12 (DMEM/F12) with 5% FBS. One human pancreatic tumor cell line, HPAC, was obtained from Dr. William Gower (University of South Florida) and another, HPAF, was obtained from Dr. Richard Metzgar, Duke University Medical Center. The human pancreatic tumor cell lines COLO 357, Capan-1, and ASPC-1; human colon

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3 The abbreviations used are: FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
cancer cell lines LS 180, HT-29, and SW480; human breast tumor cell lines SK-BR-3, RT-20, and MCF7; human gastric tumor cell line MS; human duodenal tumor cell line HuTu 80; human malignant melanoma cell line SK-MEL-28 were obtained from American Type Culture Collection. The lung cancer cell line, HAT-1, was established in our laboratory. HPAF, HPAC, LS180, HuTu 80, and HAT-1 were grown in DMEM/F12 with 5% FBS. COLO 357, Capan-1, ASPC-1, HT-29, SW480, SK-BR-3, BT-20, MCF7, MS, and SK-MEL-28 were grown in DMEM with 5% FBS.

RNA was isolated by using the acid guanidinium thiocyanate-phenol-chloroform extraction method (33). Cells at 80–90% confluence were dissolved in solution D (4 m guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, and 0.1 m 2-mercaptoethanol), followed by the addition of sodium acetate (pH 4.0) to 0.2 m. The solution was extracted with phenol (saturated 100 mM Tris, pH 6.0), chloroform (1:0:1:1:0:2), and RNA was precipitated by adding 2.5 volumes ethanol, washed in 75% ethanol, and redissolved in diethylpyrocarbonate-treated water at 4 mg/ml.

**Differential Display.** The differential display procedure used here was modified from the original method described by Liang and Pardee (34) and Liang et al. (35). Anchored oligonucleotide primer pairs (T<sub>c</sub>, CT) were synthesized by the Molecular Biology Core Facility of The Eppley Institute for Research in Cancer and Allied Diseases. Arbitrary 10-residue oligonucleotides were purchased from Operon Biotechnology, Inc. (Alameda, CA). In separate reactions, 1.0 and 4.0 μg of total RNA from the indicated cell lines were reverse transcribed in a reaction containing 1 μM T<sub>c</sub>, CT, 10 mM DTT, 0.5 unit/μl RNase inhibitor, 200 μM deoxynucleotide triphosphates, 1 unit/μl Stratascript reverse transcriptase (Stratagene, La Jolla, CA), 1× RT buffer (Stratagene) in a 40-μl reaction volume. One μl of each reaction was used for PCR in 1 μM T<sub>c</sub>, CT, 1 μM OPA-04 (AATCGGGCTC), 1.5 mM MgCl<sub>2</sub>, 100 μM deoxynucleotide triphosphates, 0.025 μM/μl of [α<sup>32</sup>P]dATP, 1× PCR buffer, and 0.125 unit/μl Taq DNA polymerase in 10 μl reaction volume. The cycling parameters were: 4 cycles of 94°C, 30 s; 40°C, 2 min; 72°C, 2 min; then 36 cycles of 94°C, 30 s; 44°C, 30 s; and 72°C, 1 min. Taq DNA polymerase was purchased from Life Technologies, Inc. (Grand Island, NY). [α<sup>32</sup>P]dATP was purchased from Amersham International (Buckinghamshire, England). The amplified cDNAs were separated on 5.0 and 3.0% denatured polyacrylamide gels at 2000 V for 3 h, and radioactivity was quantified and visualized on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Cloning of Differential Display Products and DNA Sequencing. Differentially displayed cDNA fragments were recovered from denaturing polyacrylamide gels at 2000 V for 3 h, and radioactivity was quantified and visualized on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

cDNA fragments that gave evidence of differential mRNA expression by Northern blot analysis were sequenced in both directions (Sequenase kit version 2.0; U.S. Biochemical, Cleveland, OH) by using oligonucleotide primers for M13 reverse and T7. Sequences of cDNA fragments were compared to known sequences by searching the GenBank data base with the FASTA program (Genetics Computer Group, Madison, WI).

**Northern Blots.** A cDNA for P-selectin (bp 383-1110) obtained from the differential display reaction was used to probe Northern blots. A cDNA insert from out-of-date platelets were used as a positive control for detecting P-selectin in these experiments. Western blotting was performed using techniques reported previously (36). In summary, SDS-PAGE was performed with a 3% stacking gel and a 5% running gel; molecular weight markers were the Multimark multi-colored standards (Novex); the samples were electrophoresed for 600 V-h under reducing conditions and were then transferred to a polyvinylidene difluoride membrane (Millipore) by electroblotting for 350 V-h at 5°C. The polyvinylidene difluoride membranes were blocked with 10% evaporated milk in PBS at 5°C for 4 h. Purified rabbit anti-human CD62P (P-selectin) polyclonal antibody (1:500; Pharmingen) was incubated with the membranes for 1 h at 25°C. The primary antibody was removed, and the membrane was washed four times with an excess of PBS(–). Following a 1-h incubation with goat anti-rabbit IgG (H+L) horseradish peroxidase-labeled antibody (1:1000; Southern Biotechnology, Birmingham, AL), the membrane was washed with PBS(–) four times, incubated for 8 min with SuperSignal CL-horseradish peroxidase enhanced chemiluminescent substrate (Pierce), and exposed to Enhanced-ECL film (Amersham Corp., Arlington Heights, IL) for 1–60 min.

**Induction of Surface Expression of P-selectin.** S2-013, S2-020, ECV304, and MCF7 cell lines were used for studies of induction of P-selectin mRNA or surface expression of P-selectin protein. Five thousand cells in 100 μl of DMEM/F12 with 5% FBS were placed in 96-well microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA). After 4 days of culture, at 90–100% cell confluence, all of the cell lines were exposed to 0.5 unit/ml of human thrombin (Sigma) dissolved with DMEM/F12 with 5% FBS, washed twice with DMEM, then incubated with 100 μl of DMEM/F12 with 5% FBS. At times prior to thrombin exposure and 10, 20, 30, 45, 60, and 90 min, and 2, 3, 4, 5, 6, 8, 12, and 24 h after treatment with thrombin, the cells were washed with PBS(–) twice, then fixed with 2% paraformaldehyde in PBS(–). In separate experiments, S2-013 cells were exposed to thrombin for 20 min or continuously for 24 h and then fixed as described above or used to purify total RNA. In other experiments, S2-020, ECV304, and MCF7 were exposed to thrombin for 10 min and evaluated in a similar manner until 8 h after exposure to thrombin. In another set of experiments, the indicated cells were treated for 1 or 10 min with thrombin, 1 min with 0.25% trypsin in PBS(–), or 1 h with 250 mM of hydrogen peroxide in DMEM/F12 with 5% FBS. The cells were fixed as described above at the indicated time points up to 3 h after treatment.

Fixed cells were washed twice with PBS(–), 200 μl of 5% skim milk were added to each well, and the plates were incubated at room temperature for 3 h. After discarding the milk, 50 μl of anti-P-selectin antibody solution (0.5 mg/ml in PBS(–)) were added to each well. Plates were incubated at 37°C for 2 h and washed gently with PBS(–) with 0.05% Tween 20. Ten thousand cpm of [125I]-labeled goat antimouse immunoglobulin (Cappel, Durham, NC) in PBS(–) were added to each well, and plates were incubated at room temperature for 1 h. Following three times washes with PBS(–), bound antibody was quantified in a gamma counter (Clingamma; LKB-Wallac, Turku, Finland). Cells cultured on a chamber slide (as described above) were treated with thrombin (0.5 unit/ml, in DMEM/F12 with 5% FBS) for 20 min, washed twice with DMEM, then incubated in 200 μl of DMEM/F12 with 5% FBS. Before exposure and 30 and 60 min and 2, 3, 4, and 6 h after exposure, the cells were washed twice with PBS(–), fixed with 2% paraformaldehyde in PBS(–), and evaluated for P-selectin expression by using the immunoperoxidase method described above.

**RESULTS**

Isolation of a Differentially Expressed cDNA by Differential Display. Differential display was used to identify genes differentially expressed among six clonal cell lines derived from the human pancreatic adenocarcinoma cell line SUIT-2 that show distinct properties.
of morphology (cellular differentiation) and metastasis. The results of one set of differential display reactions that used primers T12CT and OPA-04 are shown in Fig. 1. One cDNA fragment (CT04B1, arrow) was detected only in the cell lines S2-007 and S2-013. This cDNA fragment was recovered, reamplified, and cloned using the pCR II vector. An insert of approximately 700 bp was obtained, which was similar in size to that seen in the differential display reaction.

Northern Blot Hybridization. Fig. 2 shows the results of probing a Northern blot of RNA from several tumor cell lines and the SUIT-2 cell lines with the CT04B1 cDNA fragment. SUIT-2, S2-007, and S2-013 show high levels of expression, and S2-028 shows very low levels of expression of this mRNA (difficult to see in the figure). Three sublines derived from SUIT-2 (S2-020, S2-CP8, and S2-VP10) and 19 human cell lines from pancreatic, colon, and lung adenocarcinomas do not show expression of this mRNA.

Sequencing of cDNA Fragments. Sequence analysis of the CT04B1 cDNA in both directions revealed that both the 3' and 5' ends of CT04B1 cDNA fragment contained the OPA-04 primer sequence. The cloned insert showed greater than 98% identity in sequence to the cDNA for P-selectin including bp 383-1110 [GenBank; release 91.0 (10/95)].

Immunohistochemical Staining. Expression of P-selectin protein in SUIT-2 and cell lines derived from it was investigated by using an immunoperoxidase technique with antibody AC.1 (Becton Dikinson), which is specific for P-selectin. Consistent with the results of the Northern blotting experiments, the S2-013 cell line showed expression of P-selectin protein (Fig. 3), whereas no expression was detected in ECV304, PANC-1, and MCF-7 (data not shown). Antibody reactivity at the perimeter of the cells is consistent with the interpretation that P-selectin is expressed at high levels on the cell surface of S2-013 cells (Fig. 3B).

Western Blotting. The molecular properties of P-selectin detected in cell lines derived from SUIT-2 was investigated by Western blotting. P-selectin expressed by S2-013 cells showed the same mobility in SDS-PAGE as P-selectin expressed by platelets, with an apparent molecular mass of approximately 148,000 Da (Fig. 4). P-selectin was not detected in lysates of the HT29 colon adenocarcinoma cell line (Fig. 4).

Induction of Surface Expression of P-selectin. Cell surface expression of P-selectin on S2-013 cells before and after treatment with thrombin, which is known to induce surface expression on platelets and endothelial cells, was quantified by RIA. Cell surface expression of P-selectin on S2-013 cells was increased following 20 min of exposure to thrombin, and maximal levels were attained between 30 and 60 min (Fig. 5). At periods between 1 and 3 h after treatment, levels of P-selectin decreased almost 2-fold compared to levels detected prior to thrombin exposure (Fig. 5). Thereafter, cell surface P-selectin increased during the period from 3 to 12 h after thrombin exposure to levels not significantly different from the pretreatment steady-state levels (Fig. 5). Continuous exposure to thrombin for 24 h did not result in a significant superinduction of P-selectin expression above that seen with 20 min exposure (Fig. 5).

Northern blot experiments were used to determine if treatment of S2-013 cells with thrombin affected the steady-state levels of P-selectin mRNA. The results of these experiments (Fig. 6) show that steady-state levels of P-selectin mRNA by S2-013 cells undergo subtle but reproducible fluctuations following treatment with thrombin. There is a 2-fold decrease in steady-state P-selectin mRNA by 180 min, followed by an increase to levels 50% higher than steady-state levels by 360 min, and a return to steady-state levels by 480 min. Thus, fluctuations in steady-state mRNA are a mirror image (inverse) of fluctuations in the levels of surface expression of P-selectin following treatment with thrombin (Fig. 5); the alterations in mRNA
levels are similar in magnitude and duration, and they occur at times 1 h later than fluctuations in levels of expression of surface protein. Thrombin treatment did not induce detectable surface expression of P-selectin on three tumor cell lines (S2-020, ECV304, and MCF7; Fig. 7). Surface expression of P-selectin by S2-013 cells was induced to levels similar to those seen following thrombin exposure by treatment with oxygen radicals or trypsin; likewise, a similar pattern of increase, decrease, and recovery was observed (Fig. 8). The results of immunohistochemical analysis were also consistent with the finding of induction of surface expression of P-selectin by these agents (data not shown).

DISCUSSION

Previous examination of the normal tissue distribution of P-selectin showed that it is expressed only on the surface of activated platelets and endothelial cells and not on other normal epithelial of the pancreas or other organs (14). It has been reported previously that P-selectin is found on 37% of primary breast cancers examined by immunohistochemical techniques (37, 38), although it was not conclusively established that the tumors produced P-selectin. The data presented in this report show unequivocally that P-selectin mRNA and protein is constitutively expressed by the human pancreatic tumor cell line SUIT-2, and that modulation of surface expression of P-selectin on these pancreatic tumor cells is regu-
Fig. 6. Quantification of P-selectin mRNA expression by S2-013 cells following exposure to thrombin. S2-013 cells were exposed to thrombin (0.5 unit/ml) for 20 min and then used to quantify mRNA levels for P-selectin. Time was measured after exposure to thrombin. The intensity of a P-selectin probe on Northern blots (similar in quality to those shown in Fig. 2) was quantified on a Fujix bioimaging analyzer BAS1000-MacBAS (Fuji Film Co., Tokyo, Japan). The same Northern blot was stripped and rehybridized with a GAPDH probe. Quantified values of signal strength for the P-selectin probe were divided by values obtained for the GAPDH probe. Mean values (bars, SD) of the P-selectin:GAPDH ratio for three independent experiments are presented. Statistical significance was determined by Student’s t test.

Fig. 7. Induction of surface expression of P-selectin in S2-020, ECV304, and MCF7 cells following thrombin (0.5 unit/ml) exposure for 10 min. RIA of P-selectin expression on the indicated cell lines following treatment with thrombin (0.5 unit/ml) for 10 min. Time was measured after exposure to thrombin. Surface expression of P-selectin was not induced on S2-020, ECV304, and MCF7 cells. Bars, SD.
Lewis A (1). The expression of both P-selectin and its ligand by SUIT-2 and clonal lines derived from it raises the possibility that these molecules play a role in adhesion properties between individual tumor cells.

One characteristic that distinguishes SUIT-2 from the other pancreatic tumor cell lines evaluated here is that SUIT-2 shows spontaneous metastatic activity when xenografted s.c. into nude mice (1–3). Constitutive expression of P-selectin may contribute to the unusual spontaneous metastatic activity of this cell line. Consistent with this hypothesis are two findings: three clonal cell lines derived from SUIT-2, which show moderate to well-differentiated morphology (S2–007 and S2–013) and spontaneous metastatic activity to lung and lymph nodes, express P-selection; and one clonal cell line that is well differentiated and rarely metastatic (S2–028) expresses very low levels of P-selectin (Fig. 2). There is not an absolute correlation between the metastatic properties of all cell lines derived from SUIT-2 and P-selectin expression; however, because two cell lines produced by several cycles of in vivo selection for lung colonization (by spontaneous metastasis, S2-CP9, or from tail vein injections, S2-VP10) did not express P-selectin (Fig. 2).

P-selectin expression on the surface of platelets and other cells plays a significant role in their aggregation and adherence during episodes of coagulation and inflammation. P-selectin is not constitutively expressed on the plasma membrane of endothelial cells and platelets; it is stored in granules. Surface expression is induced by external stimuli, such as exposure to thrombin (13, 45, 46) or oxygen radicals (16). The finding that surface expression of P-selectin protein by S2-013 cells is increased by exposure of the cells to thrombin, oxygen radicals, and trypsin suggests that common cellular mechanisms exist among platelets, endothelial cells, and these pancreatic tumor cells for regulating surface expression of P-selectin. This supports the hypothesis that P-selectin expressed by tumors is responsive to natural factors that regulate its biological activity. Patients with pancreatic tumors frequently present with thrombotic lesions during the course of their disease (47), and the patient from which SUIT-2 was derived had evidence of disseminated thromboses (1). This leads us to propose the hypothesis that expression of P-selectin by adenocarcinoma cells contributes in part to the formation of thromboses in patients with pancreatic adenocarcinoma.

In summary, the SUIT-2 pancreatic adenocarcinoma cell line expresses P-selectin and is responsive to natural factors (thrombin, oxygen radicals, and trypsin) that modulate the surface expression of this adhesion molecule. Additional studies should be performed to establish the incidence of P-selectin expression by adenocarcinomas and to determine if its expression contributes to the development of metastatic and thrombotic lesions.

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\[4\] T. Iwamura, personal observation.
REFERENCES


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