Correlation between spontaneous metastatic potential, platelet-aggregating activity of cell surface extracts, and cell surface sialylation in 10 metastatic-variant derivatives of a rat renal sarcoma cell line*

(Wistar–Furth rats/polyoma-induced PW20 tumor/platelet)

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ABSTRACT Several properties of 10 cell lines derived from the polyoma-induced PW20 Wistar–Furth rat renal sarcoma have been examined, including the ability of the tumor cells to metastasize spontaneously from subcutaneous sites in syngeneic hosts, the platelet-aggregating activity of material extracted by urea from the surface of cultured cells, the sialic acid content of the platelet-aggregating material, and the degree of sialylation of cell surface glycoconjugates in cultured cells. A correlation has been observed among all of these parameters. The results suggest a possible link between the degree of cell surface sialylation of tumor cells, their ability to aggregate platelets, and their ability to metastasize.

Several lines of evidence suggest that platelets may play a role in the hematogenous spread of certain tumors: (i) tumor cells injected intravenously into experimental animals have been observed to aggregate platelets and induce thrombocytopenia (1–5); (ii) the ability of intravenously injected tumor cells to implant and form tumors and the ability of tumor cells to metastasize spontaneously from primary sites may be reduced by the induction of thrombocytopenia (3); and (iii) a rough correlation has been observed between the ability of intravenously injected tumor cells to form lung nodules and their ability to aggregate platelets *in vivo* and induce thrombocytopenia *in vitro* (3).

Extraction of a platelet-aggregating material (PAM) from SV40-transformed BALB/c 3T3 (SV3T3) cells has been reported (6), which was a sedimental complex of protein and lipid containing sialic acid. The PAM obtained from SV3T3 cells was capable of aggregating platelets at 2 $\mu\text{g}/\text{ml}$ (a similar extract from nontransformed 3T3 cells barely aggregated platelets at 40 $\mu\text{g}/\text{ml}$). Platelet aggregation induced by the PAM required Ca^{2+} and a plasma factor(s) other than fibrinogen and operated via the release reaction. The PAM-containing extract was enriched with membrane vesicles, as determined by electron microscopy,** and thus appeared to be similar to platelet-aggregating material spontaneously shed from tumor cells of other types (7, 8).

In this paper we report the platelet-aggregating activity of the PAM extracted from cells of 10 derivatives of the polyoma-induced PW20 Wistar–Furth rat renal sarcoma cell line, which vary in their ability to metastasize spontaneously from subcutaneous sites of tumor growth (9). The PAM's activity was found to correlate positively with the propensity of the cells to

metastasize. In addition, the PAM's activity correlates with the sialic acid content of the PAM and with the degree of cell surface sialylation of the cultured tumor cells.

METHODS

Cell Lines. The PW20 family of tumor cell lines (Table 1) was derived from a culture of the polyoma virus-induced PW20 Wistar–Furth rat renal sarcoma (obtained from H. O. Sjögren, Univ. of Lund, Sweden) by varying conditions of passage in tissue culture and passage through syngeneic animals (9). The RO(L) and RO(H) cell lines represent low and high tissue culture passages, respectively, of the original cell culture. The R1 cell line was obtained by reestablishing a subcutaneous RO(L) tumor in tissue culture and maintaining the culture for a large number of passages. The R2 cell line was similarly produced by reculturing a subcutaneous R1 tumor and maintaining the resultant culture for a prolonged period. The RO(L)R1, RO(H)R1, R2R1, and R2R2 cell lines were produced by reculturing subcutaneous RO(L), RO(H), R2, and R2R1 tumors, respectively; the R2L1 and R2N1 cell lines were obtained by culturing explanted lung (L) and lymph node (N) metastases that developed from subcutaneous R2 tumors. These last-named lines were all tested at low passage numbers. *In vitro* assays were performed with cells within several passages of those listed in Table 1, obtained either from the same cells used for *in vivo* testing or from an aliquot of identical cells preserved in liquid nitrogen. For the *in vitro* assays, the cell lines were maintained in Dulbecco-modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100–500 units of penicillin and 100 μg of streptomycin per ml, with or without additional nonessential amino acids, and were passaged twice a week.

Evaluation of Metastatic Properties. The spontaneous metastatic behavior of the tumor cell lines was evaluated in syngeneic Wistar–Furth rats of both sexes obtained from Microbiological Associates (Bethesda, MD). Animals were injected subcutaneously in the loose skin of the midback with tumorigenic doses of cultured cells. Tumors were measured at regular intervals after injection and were excised by using a closed surgical technique when they weighed 2–6 g. The animals were then observed for the development of palpable regional lymph node metastases and clinical signs suggestive of internal me-

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Abbreviation: PAM, platelet-aggregating material.

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** G. J. Stewart and G. J. Gasic, personal communication.

Table 1. Derivation of PW20 tumor cells lines

Derivative lines	Number of animal passages*	<i>In vitro</i> subcultures tested†
RO(L)	0	11–19
RO(H)	0	276–283
RO(L)R1	1	18
RO(H)R1	1	3
R1	1	88–92
R2	2	80–84
R2R1	3	6–16
R2L1	3	5–7
R2N1	3	5, 6
R2R2	4	6

* Number of animal passages since receipt of the original PW20 cell line.

† The number of the subcultures used for testing of metastatic properties after establishment of each new derivative line. For the RO(L) and RO(H) cell lines, the numbers listed represent the number of subcultures used for testing after receipt of the original cell lines.

tastases to the lung or other organs. Animals with large external metastases or with signs of visceral metastatic growth were sacrificed, and a thorough search was conducted for macroscopic metastases to regional lymph nodes and to intrathoracic and intraabdominal sites. Asymptomatic animals were similarly sacrificed after a prolonged period of observation. In some experiments, the lungs were injected with India ink and fixed according to the method of Wexler (10) to facilitate visualization of the metastatic nodules.

Extraction of PAM. The PAM was extracted from adherent cell cultures with 1M urea in veronal buffer, dialyzed, and concentrated as described (6). Viability of the adherent cells recovered after extraction was greater than 90%, judged by trypan blue exclusion.

Platelet Aggregometry. Platelet aggregation was measured turbidometrically with a Bio-Data aggregometer (Bio-Data, Willow Grove, PA) as described (6, 11) by using rabbit platelet-rich plasma. The velocity of platelet aggregation was determined by measuring the greatest slope of the aggregation curve and was expressed as the change in percent transmission per min. The velocity of aggregation produced by PAM extracted from each cell line was determined at 3–5 different concentrations of PAM (from 10 to 100 μ g of protein per ml) on 2–5 different occasions, with independently prepared PAM extracts used on each occasion. The aggregation velocity observed among the different concentrations of PAM tested on each occasion was averaged over the different occasions, and the maximum average aggregation velocity was obtained.

Total Sialic Acid Content of PAM. The sialic acid content of the PAM was determined by the thiobarbituric acid method of Warren (12) with *N*-acetylneuraminic acid (Sigma) as a standard.

Protein Content of PAM. The protein content of the PAM was determined by the method of Lowry *et al.* (13) with bovine serum albumin as a standard.

Percent Sialylation of Cell Surface Glycoconjugates. The percent sialylation of the exposed cell surface glycoconjugates was determined by using the galactose oxidase/sodium boro[3 H]hydride-labeling technique as described (14). This procedure results in the tritiation of the terminal galactosyl (Gal) and *N*-acetylgalactosaminyl (GalNAc) groups on exposed cell surface carbohydrates. Gal and GalNAc groups that are further substituted with sialic acid are protected from labeling. It is therefore possible to determine the percentage of the exposed

terminal Gal and GalNAc groups that are sialylated by labeling cells with and without prior incubation with neuraminidase, which cleaves terminal sialic acid residues from the saccharide chains. Galactose oxidase was obtained from A. B. Kabi (Stockholm, Sweden), *vibro cholerae* neuraminidase was obtained from Calbiochem-Behring, and sodium boro[3 H]hydrides (4–6 Ci/mmol; 1 Ci = 3.1×10^{10} becquerels) was obtained from Amersham/Searle.

Statistical Methods. Correlation coefficients were calculated by linear correlation analysis (15). All PAM extractions, PAM sialic acid determinations, and platelet aggregometry were performed on coded samples.

RESULTS

The results of the various assays are summarized in Table 2.

Metastatic Properties. The percentage of animals developing one or more spontaneous metastatic lesions after primary tumor excision ranged from 0% to 100%, with lesions appearing in the lung, mediastinal lymph nodes, abdominal viscera, and external lymph nodes.

Platelet Aggregation. The maximum average velocity of platelet aggregation induced by the PAM ranged from 1.5 to 100 units of light transmission per min. A significant correlation was observed between the platelet-aggregating activity of the PAM and the metastatic potential of the tumor cell lines from which the PAM was derived (correlation coefficient, $r = 0.68$, probability, $P < 0.03$).

Sialic Acid Content of PAM. The sialic acid content of the PAM ranged from 1.5 to 24.7 mg per 100 mg of protein. PAM preparations from the three least metastatic cell lines, which were the least active with respect to platelet-aggregating ability, contained the lowest levels of sialic acid. A good correlation was observed between the platelet-aggregating activity of the PAM and its sialic acid content ($r = 0.60$, $P = 0.06$), and between the metastatic potential of the cell lines and the sialic content of the PAM ($r = 0.69$, $P < 0.03$).

Sialylation of Cell Surface Glycoconjugates. The percent sialylation of the exposed cell surface Gal and GalNAc residues ranged from 13% to 77%, with the three lowest values observed in the three least metastatic cell lines. A significant correlation was observed between cell surface sialylation and metastatic potential ($r = 0.83$, $P < 0.003$), cell surface sialylation and the platelet-aggregating activity of the PAM ($r = 0.85$, $P < 0.002$), and cell surface sialylation and the sialic acid content of the PAM ($r = 0.74$, $P < 0.02$).

DISCUSSION

Gasic and co-workers (16) first demonstrated that platelets may play a role in promoting hematogenous tumor cell metastasis. Induction of thrombocytopenia significantly decreased the number of lung tumors found after intravenous injection of TA3 ascites tumor cells in mice—an effect which could be reversed by the reinjection of fresh platelets. A rough correlation was observed between the ability of tumor cells to produce lung tumors after intravenous injection and their ability to aggregate platelets *in vitro* and produce thrombocytopenia *in vivo* (4).

Our present data extend these observations in spontaneously metastasizing variant tumor cell lines, using the PAM extracted from the tumor cell surface. Significant correlations were observed between the metastatic potential of the cell lines, the ability of the PAM to aggregate platelets, the sialic acid content of the PAM and the degree of sialylation of the intact tumor cell surface. The correlation between the activity of the PAM and its sialic acid content is in accord with our recent report that the activity of the PAM obtained from SV3T3 cells is abolished by

Table 2. Metastatic properties, platelet-aggregating activity of cell surface extracts (PAM), sialic acid content of PAM, and sialylation of cell surface glycoconjugates of metastatic variant PW 20 tumor cell line

Metastases from primary subcutaneous tumors				Platelet aggregating material (PAM)		Cell surface sialylation, Gal and GalNAc, % [†]
Derivative line	Incidence*	Location [†]	Percent	Velocity of aggregation, $\Delta T/\text{min}$ [‡]	Sialic acid content, mg/100 mg protein [§]	
R2N1	13/13	T, A, N	100	73 $\pm$ 3	7.1	66 $\pm$ 7
R2L1	13/13	T, A, N	100	34 $\pm$ 5	16.4	77 $\pm$ 7
R1	45/49	T, A, N	92	70 $\pm$ 4	15.7	75 $\pm$ 3
R2	56/64	T, A, N	88	69 $\pm$ 5	24.7	73 $\pm$ 5
R2R2	5/7	T, A, —	71	9 $\pm$ 2	14.5	36 $\pm$ 2
RO(L)R1	4/6	T, —, N	67	63 $\pm$ 4	21.4	70 $\pm$ 6
RO(L)	6/9	T, A, N	67	100 $\pm$ 5	15.8	72 $\pm$ 4
R2R1	3/13	T, —, —	23	4 $\pm$ 1	5.4	13 $\pm$ 2
RO(H)	1/19	—, A, —	5	8 $\pm$ 1	5.4	31 $\pm$ 7
RO(H)R1	0/5	—, —, —	0	2 $\pm$ 1	1.5	30 $\pm$ 10

* Number of animals developing one or more metastases following primary tumor excision/number of animals surviving surgery.

[†] T, intrathoracic (lung and/or mediastinal lymph nodes); A, intra-abdominal (mesenteric lymph nodes, kidney, adrenal gland, ovary and/or liver); N, superficial lymph nodes (inguinal, axillary, cervical).

[‡] Maximum average velocity of platelet aggregation induced by PAM tested at 3–5 concentrations ranging from 10–100 $\mu\text{g}/\text{ml}$. Mean $\pm$ SE of values obtained from 2–5 independent experiments that measured the change of transmission per min.

[§] Mean obtained from two independent determinations that were very similar.

[†] Mean $\pm$ SE of values obtained from 3–7 independent experiments.

treatment with neuraminidase (6), and the correlation suggests that the sialic acid content of the PAM is important in determining its activity. The correlation between the sialic acid content of the PAM and the degree of cell surface sialylation is consistent with the evidence suggesting that PAM is composed of membrane vesicles. Interestingly, the total sialic acid content of the cells (data not shown) correlates poorly with the other parameters shown in Table 2. This finding suggests that the degree of sialylation of the cell surface, which is reflected in the sialic acid content of the extracted PAM, may be a more important determinant of metastatic behavior and platelet-aggregating properties than the overall sialic acid content of the cell.

One cell line (R2R2) appears to be an exception in that it shows relatively low values of PAM activity and cell surface sialylation for its degree of metastasis. Of interest is the observation that this cell line demonstrated *in vivo* a transient incomplete regression of the initially formed tumors, followed by delayed progressive growth prior to excision. Therefore, it is possible that an immunoselection of highly sialylated cells (9) with enhanced metastatic potential may have taken place *in vivo* from a heterogeneous population of predominantly non-metastatic, poorly sialylated R2R2 cells. In this regard, the reduced *in vitro* adhesiveness and motility of R2R2 cells is also characteristic of the poorly metastatic cell line (17). If this line were eliminated from the correlation, it would raise the correlation coefficients by an average of 0.04 (range 0–0.08).

The correlation between the spontaneous metastatic behavior of murine tumor cells and their cell surface sialylation *in vitro* has been noted (14, 18) and now has been observed in a total of 32 murine tumor cell lines (unpublished data). One mechanism which might be proposed to account for the observed correlation is that elevated levels of sialic acid on the cell surface may reduce the immunogenicity of the tumor cells (9, 19), thereby increasing their ability to evade immune destruction and establish metastatic foci (20). Differences in cell surface sialic acid also may bring about differences in the adhesive properties of the cells (17, 21), thereby altering their patterns of arrest and implantation at secondary sites (22, 23). Another mechanism,

which is suggested by the results of the present study, is that increased sialylation of the tumor cell surface may enhance the ability of these cells to aggregate platelets, either prior to or after their arrest in the microvasculature. The resulting aggregates may prolong the survival of the tumor cells at the site of arrest (1–3). The release of vasoactive substances from the aggregated platelets (6, 24) may facilitate passage of the tumor cells through the vessel wall by promoting endothelial cell damage and enhanced cell adhesion to the subendothelial matrix.

The mechanisms of tumor cell metastasis are inherently complex (25). The extent to which platelets may be involved in promoting tumor cell metastasis remains to be determined. The variant cell lines employed in this study provide an excellent opportunity to study the role of platelets in tumor cell metastasis. Our data are consistent with the hypothesis that platelets play a role in certain tumor cell metastases and lend support to the therapeutic possibility that certain tumor metastases may be reduced by agents capable of eradicating that property of platelets that promotes metastasis.

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