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The lack of correlation between experimental metastatic potential and platelet aggregating activity of B16 melanoma clones viewed in relation to tumor cell heterogeneity†

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Two widely used B16 melanoma cell lines of low and high lung colonizing potential (B16-F1 and B16-F10) were compared in their ability to induce platelet aggregation. The results of these experiments showed a reproducible difference in platelet aggregating activity of these two cell lines which directly correlated with their lung colonizing potentials. However, when clones were derived from these heterogeneous cell lines and tested for experimental metastatic potential, platelet aggregating ability and Met-72 expression, no correlation could be attached to the platelet aggregating activity of the clones. Results of these experiments provide direct evidence that platelet aggregation is not an accurate index of experimental metastatic potential of tumor cell clones, nor is it an essential trait of all metastatic cells. The ability of tumor cells to induce platelet aggregation is examined and discussed in the context of cellular heterogeneity.

Introduction

According to our present understanding of the metastatic cascade, a number of blood-borne interactions are believed to occur during the hematogenous spread of malignant cells from the primary tumor to distant anatomical sites [5, 15, 17]. The ability of tumor cells to aggregate platelets and induce thrombus formation has been regarded as an important property of potentially metastatic tumor cell variants, creating a barrier against immune and physical destruction before extravasation [1, 5, 8, 10]. The importance of microthrombus formation in lung colonization after i.v. injection of tumor cells has been documented in several tumor cell systems by inhibiting platelet function. Pretreatment of experimental animals with anticoagulants [8, 9] or antiplatelet [6, 7] agents causes significant reduction in lung colonization after i.v. injection of tumor cells. These results support the concept of a mechanical role for the tumor microthrombi by favoring their arrest in the lung capillaries and subsequent development into metastases. Finally, convincing ultrastructural evidence that some tumor cells attached to the basement membrane of the vascular system do in fact have 'protective' fibrin deposits around them has

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supported the belief that platelet aggregation can be an important event in the hematogeneous spread of malignant tumor cells [2, 17, 18].

The question of whether tumors of different histological origin and predilection for hematogenous versus lymphatic metastatic spread may have a differential dependence on platelet aggregation has been addressed [13]. Despite general trends, exceptions in the predicted route of metastasis for certain solid tumors and their ability to aggregate platelets do exist [13]. Thus, the need for and/or role of platelet aggregation by metastatic tumor cell variants remains unresolved.

In the present study we have examined the platelet aggregating activity of two widely used *in vivo* selected B16 melanoma cell lines and compared their behavior with individual clones derived from them. Simultaneous analyses of platelet aggregating activity, lung colonizing activity and Met-72 expression [11] were performed on the same day using the same preparation of cells. Results of these experiments provide direct evidence that platelet aggregation is not an accurate index of experimental metastatic potential of tumor cell clones, nor is it an essential trait of all metastatic cells. The ability of tumor cells to induce platelet aggregation is viewed and discussed in the context of cellular heterogeneity.

Materials and methods

Animals

C57BL/6 mice were obtained from the Jackson Laboratory, Bar Harbor, Maine and housed in laminar flow containment units in our mouse colony, Department of Pathology. Female mice between 8 and 16 weeks of age were used in these studies.

Cell lines, clones and culture conditions

The C57BL/6 melanoma, B16, and various *in vitro* and *in vivo* selected lines derived from it were obtained from the Division of Cancer Treatment Tumor Bank (EG & G Mason Research Institute, Worchester, Mass.) where they had been deposited by Dr I. J. Fidler. Clones were derived by limiting dilution and micromanipulation from the parent line, B16-F1. Stocks from early passages of these lines and clones were prepared, frozen at $-85°C$, and restarted every 6–7 weeks to limit the possibilities of functional and phenotypic drift. All cell lines and clones were maintained *in vitro* at 37°C in a humidified incubator containing 8·0 per cent CO_2/air. Cells were passaged for optimal growth by subculturing every 4 days in Eagle's Hanks amino acid supplemented medium containing 10 per cent newborn calf serum, penicillin and streptomycin [11]. Cells were detached from standard plastic surfaces using 0·5 mM EDTA in Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS) supplemented with 1·0 g/l glucose [11].

Lung colonization assay

The ability of the various B16 clones to form experimental metastases was determined and quantified by the i.v. lung colonization assay [14]. Groups of four to six age- and sex-matched mice were injected i.v. with $3·0 \times 10^5$ melanoma cells and the extent of metastases was determined macroscopically three weeks later.

Relative levels of cell surface Met-72 expression determined using ^{125}I-protein A (^{125}I-pA)

The extent of monoclonal antibody (MoAb) binding to various B16 melanoma clones was measured indirectly with ^{125}I-pA as described [11]. Briefly, 3×10^5 freshly harvested, *in vitro* grown cells were incubated for 1 h with a predetermined excess of anti-Met-72 MoAb (K88·146) [11]. The cells were then washed three times with PBS + 5 per cent fetal bovine serum (FBS) and then incubated for an additional 1 h with 2×10^5 cpm of ^{125}I-pA. Radioactivity bound to the cells was assessed after three washes with PBS + 5 per cent FBS by gamma scintillation counting. Data is presented as the mean of triplicate determinations. Because of potential differences in background binding of ^{125}I-pA to the different B16 melanoma cell lines and clones, Met-72 expression is expressed as a binding index, which was calculated as follows:

$$\text{Binding index} = \frac{\text{cpm } ^{125}\text{I-pA bound with MoAb}}{\text{cpm } ^{125}\text{I-pA bound without MoAb}}$$

In this way, individual differences in background binding are normalized and allow comparison between the different B16 melanoma lines and clones [11, 20].

Platelet aggregation

Platelet stimulatory activity was evaluated by measuring the effect of tumor cells on platelet aggregation. To do this, blood was obtained from normal human volunteers who had not ingested aspirin or other platelet inhibitory drugs within the previous 10-day period. Blood was collected into sodium citrate (3·8 per cent) and centrifuged at 300*g* for 15 min. Tumor cells (2×10^7 cells/ml) were incubated with platelet-rich plasma for 2 min at 37°C in a stirring well of a platelet aggregometer (Sienco Co., Denver, Co). All measurements were performed in duplicate and mean values were used in calculating statistical analyses. The use of human platelet-rich plasma instead of syngeneic murine platelet preparations was justified by the following reasons. First, it is technically difficult to obtain sufficient amounts of platelet-rich plasma from mice for tumor cell-induced platelet aggregation studies. Second, earlier comparative studies were performed in our laboratory and it was found that aggregation of human or mouse platelets induced by B16 melanoma cells is ADP and fibrinogen dependent. Third, high concentrations of thrombin inhibitors failed to inhibit B16 melanoma-induced platelet aggregation. Since human and murine platelets are both aggregated by B16 melanoma cells through the same mechanism, human platelet-rich plasma was used for these studies.

Statistical analyses

Data obtained from analyses of platelet aggregation, Met-72 expression and metastatic activity were subjected to linear regression analyses. Correlation coefficients comparing two parameters are given.

Results

Comparison of functionally selected B16 melanoma lines versus individual clones derived from them

Two widely used, functionally selected lines from the B16 melanoma B16-F1 and B16-F10 [4, 5, 14], were used to represent low and high lung colonizing forms in

platelet aggregation studies. Typical results of these experiments (figure 1) have shown that the degree of platelet aggregation correlates with their respective lung colonizing abilities. Aliquots of the same tumor cells used in the platelet aggregation studies were also examined for experimental metastatic potential. The poorly (B16-F1) and highly metastatic (B16-F10) lines gave respective mean values of 14 versus 276 lung colonies.

The cellular identity of cells capable of platelet aggregation and those giving rise to experimental pulmonary metastases was examined using clones derived from the parental population by limiting dilution procedures. When two well-characterized, functionally stable clones of the B16 melanoma [11] were subjected to similar comparisons of lung colonizing activity and platelet aggregating activity, a reverse correlation was observed. As seen in figure 2, the highly metastatic clone, B16-BL6-10, was approximately half as effective as the poorly metastatic clone B16-F1-7 in inducing platelet aggregation. In this experiment, aliquots of the same cell

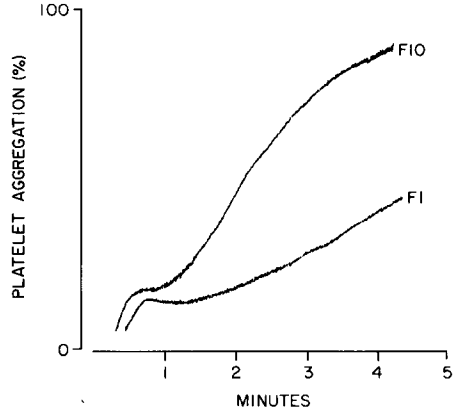

Figure 1. Platelet aggregation induced by the high lung-colonizing B16 melanoma line (F10) compared with the low lung-colonizing B16 melanoma line (F1).

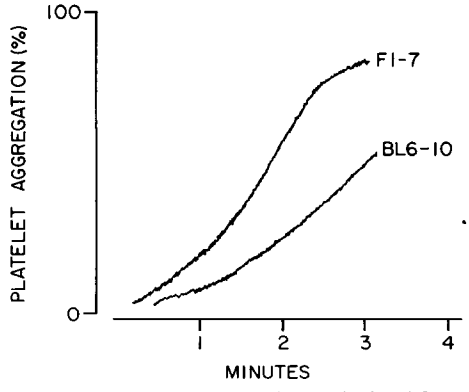

Figure 2. Platelet aggregation induced by two clones derived from the B16 melanoma. F1-7 and BL6-10 are the respective low and high lung-colonizing clones derived from the B16 melanoma.

suspensions were used to quantify the lung-colonizing activity of the clones. Three hundred thousand cells from each of the clones were injected i.v. into four age- and sex-matched C57BL/6 mice, and the extent of experimental pulmonary metastases was quantified three weeks later. Results of this analysis showed the predicted, stable difference in metastatic activity of the clones as reported previously [11]. Cells from the B16 melanoma clones F1-7 and BL6-10 produced a mean of 16 and >250 lung colonies, respectively. In this experiment, as with previous measurements [11], no extrapulmonary metastases were observed.

Lack of correlation between the experimental metastatic potential and platelet aggregating activity of B16 melanoma clones

A number of other B16 melanoma clones were derived from the parent melanoma, B16-F1, to examine more rigorously the relationship between lung-colonizing activity and platelet-aggregating activity. For these studies, only those melanoma clones exhibiting stable lung-colonizing potential during several months of *in vitro* culture were used in attempts to minimize functional variations in phenotypic drift of cloned tumor cells [15]. Clones which produced small numbers of large pulmonary colonies or animals with variable degrees of extrapulmonary metastases were excluded from these analyses. Only those clones which produced lung colonies of uniform size were counted and included in this study. As a result, data obtained from 16 out of the original 20 B16 melanoma clones were subjected to a linear regression analysis to obtain correlation coefficients comparing the expression of the cell-surface metastatic marker, Met-72 [11], lung-colonizing potential, and platelet-aggregating activity. Representative results of several experiments comparing all three of these parameters are shown in the table and individually examined below.

High-density Met-72 expression and lung-colonizing potential

Results documenting the high degree of correlation between experimental metastatic activity and cell surface expression of a 72 kd glycoprotein, Met-72, have been reported [11, 20, 21]. The 16 new clones isolated and developed for this study were similarly subjected to correlative analysis between these parameters to compare with platelet-aggregating activity. Anti-Met-72 MoAb (K88·146) was used to measure Met-72 surface expression on the individual melanoma clones tested for lung-colonizing activity. Results of these experiments are given in the table and the linear regression analysis of their correlation is shown in figure 3. The correlation coefficient of $r=0{\cdot}83$ for these 16 new clones compares well with a previously reported correlation coefficient of 0·94 for 17 different B16 melnoma clones [11].

Lack of correlation between platelet-aggregating activity, Met-72 expression and lung-colonizing potential of B16 melanoma clones

Aliquots of the same cell suspensions described in the preceding section were used to determine their platelet-aggregating activity. These simultaneous analyses permitted comparisons with the values obtained for cell-surface Met-72 expression and lung-colonizing potential (table). As shown in the table, levels of platelet aggregation ranged from 2·5 to 40 per cent with the various clones. It is noteworthy that three clones giving the lowest value of platelet aggregation (2·5 per cent for clones 6, 13, BL6-10) had the largest variation in lung colonies (1 versus 215

Platelet aggregation, Met-72 expression and metastatic potential of B16 melanoma clones.

Clone number	Platelet aggregation (%)[a]	Number of pulmonary metastases[b]	Met-72 expression[c]
1	29·5	167	19·4
2	12·0	2	13·0
3	10·0	4	6·1
4	17·5	11	6·2
5	22·0	162	16·5
6	2·5	5	6·9
8	33·0	192	20·8
9	39·0	14	11·8
10	15·5	2	7
12	37·5	1	10·4
13	2·5	1	6·6
14	37·0	148	14·1
15	40·0	9	13·8
16	29·0	29	5·6
17	36·0	3	9·2
BL6-10	2·5	215	31·8

[a] Mean percentage platelet aggregation of duplicate determinations. In all cases the S.E. was less than 10 per cent of the mean.

[b] Values represent the mean number of pulmonary metastases from six mice three weeks after i.v. injection of 3×10^5 melanoma cells.

[c] Binding index was calculated as described in the Materials and methods.

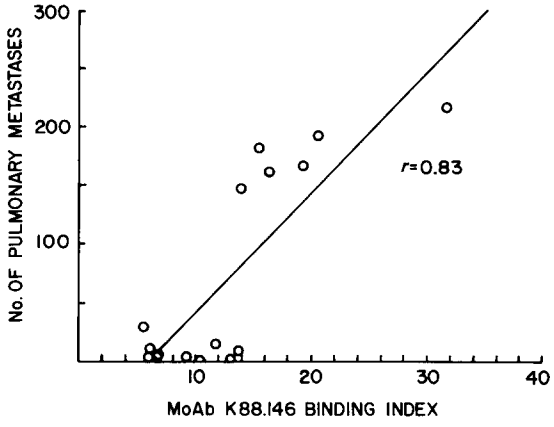

Figure 3. Linear regression analysis of the correlation between the lung-colonizing activity and Met-72 expression of sixteen B16 melanoma clones derived by cloning procedures.

pulmonary colonies). Linear regression analysis of the data examining the correlation between platelet aggregation and lung-colonizing activity is shown in figure 4, where $r = 0{\cdot}16$. Similarly, as seen in the table and figure 5, no positive correlation could be established between the metastatic cell-surface marker, Met-72 and platelet aggregating activity. In the latter comparison, the correlation coefficient was $r = 0{\cdot}06$.

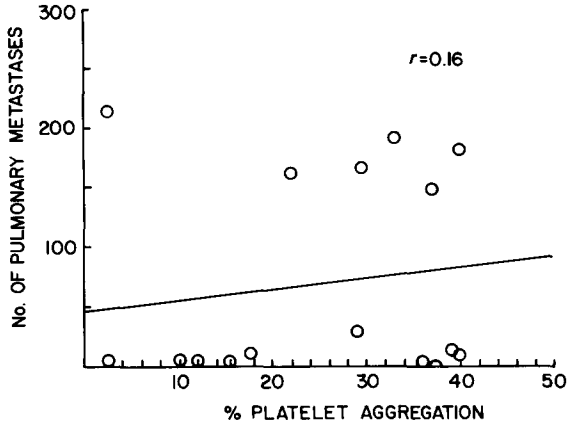

Figure 4. Linear regression analysis of the correlation between the lung-colonizing and platelet-aggregating activity of sixteen B16 melanoma clones.

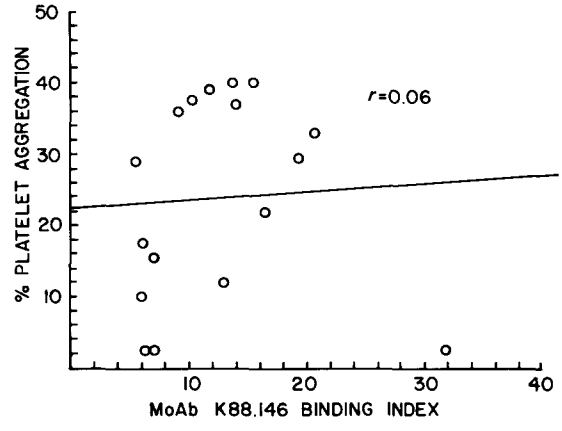

Figure 5. Linear regression analysis of the correlation between Met-72 expression and platelet-aggregating activity of sixteen B16 melanoma clones.

Discussion

A number of observations in the literature have pointed to an association between tumor metastasis and blood coagulation. These include the frequent occurrence of thrombi around tumor cell emboli, intravascular coagulation, and decreased incidence of metastasis during thrombocytopenia [1, 2, 7, 8, 12, 17, 19]. These observations have led to the assumption that thromboplastic and procoagulant activities of tumor cells are important, and perhaps essential, properties of metastatic cells. When tumor cells of varying metastatic potential were tested for their ability to cause platelet aggregation *in vitro*, a positive correlation has been reported in some [6, 16] but not all systems [3, 18]. In the studies presented here, we have used the i.v. lung colonization assay to focus on the potential variables relating to the role of tumor cell-induced platelet aggregatory activity in the arrest and extravasation portion of the metastatic cascade.

Much of the confusion and discrepancy regarding the role of platelet aggregation in the metastatic cascade stems from the tacit assumption that the same cells which cause platelet aggregation are in fact the metastatic variants which seed and give rise to metastatic lesions. Ultrastructural evidence of tumor cells surrounded by either platelets or fibrin [2, 17, 18] has suggested, but does not prove, that these cells initiate the thrombus formation and are the successful beginnings of future metastases. In the present study, several *in vivo* selected cell lines of the B16 melanoma which cause little or extensive experimental pulmonary metastases were compared with individual clonal components derived from them. Such studies allowed us to determine more accurately whether the individual clonal abilities of inducing platelet aggregation quantitatively paralleled their experimental metastatic potential. Our results show that selected clones of the B16 melanoma vary in their experimental metastatic potential and that this variability is not directly related to their platelet stimulatory activity (figures 2, 4 and the table). A significant finding to emerge from these studies was that the strong correlation between lung-colonizing activity and platelet aggregation of B16 melanoma lines was lost when these parameters were compared with individual B16 melanoma clones. Previous studies from our laboratory have shown a strong correlation between the experimental metastatic activity of B16 melanoma clones and levels of Met-72 expression on their cell surfaces [11, 20, 21]. In the present studies, this cell surface marker of experimental metastatic potential was included as an additional parameter to test its relationship to platelet aggregating activity. As shown in figure 3 and the table, Met-72 shows a strong correlation ($r = 0{\cdot}83$) with the lung-colonizing potential of the clones specifically developed for this study, and no correlation ($r = 0{\cdot}06$, figure 5) with platelet aggregation.

The reason our melanoma clones did not exhibit parallel metastatic potential and platelet-stimulatory activity in this study may relate to several factors. First, cloned cells represent a population of limited cellular heterogeneity, unlike selected cell lines such as B16-F1 and B16-F10. Our results are consistent with the possibility that tumor cells may require the presence of other cell types for full expression of platelet-aggregatory activity. Alternatively, processing of cells *in vivo*, as occurs when tumor cells are selected after serial selection of cell lines [4, 5], may cause full expression of platelet-stimulatory activity and experimental metastatic potential. This *in vivo* processing effect may be lost when single cell clones are isolated. Such a concept does not diminish the potential significance of platelet aggregation during the blood-borne phases of implantation, extravasation and survival of metastatic variants. Our results along with those of previous reports [3, 18] do show, however, that high platelet-aggregating activity of non-metastatic tumor cell clones is not sufficient for the establishment of experimental or spontaneous metastases.

Consideration of our results may explain why investigators have been unable to agree experimentally on the correlation between platelet aggregation and metastatic potential of tumor cells in different systems. Proponents of a positive correlation between platelet aggregation and metastatic potential have all used tumor cell *lines* either maintained and passaged *in vitro* or *in vivo* [6, 16], while those unable to show a correlation have all used *clones* of presumed limited cellular heterogeneity [3, 18].

Finally, an important technical consideration is that measurement of light transmission through platelet-rich plasma may not accurately measure platelet-tumor cell thrombus formation. Although light transmission increases after platelet aggregation, this may occur due to platelet–platelet, platelet–tumor, or tumor–tumor cell clumping. The results of preliminary morphologic evaluation of aggregates

formed under these conditions have shown that tumor cells are associated with most platelet clumps. Further studies are in progress which will more precisely define the impact of tumor–tumor cell interaction on platelet aggregation.

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