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Interactions between Neoplastic Cells with Different Metastasizing Capacity and Platelet Function*

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Abstract—*We have examined the effects on platelet function of two sublines (M_4 and M_9) derived from spontaneous lung nodules of a benzopyrene-induced murine fibrosarcoma (m FS6). The subline M_4 was more metastatic and the subline M_9 less metastatic than the primary tumour. Only the more malignant cells were able to induce irreversible aggregation of human platelets; this effect was concentration-dependent and was associated with the release of serotonin by platelets. Both aggregation and release were inhibited by preincubation of platelets with ASA, not by preincubation of the cells. The supernatants of cell suspensions had no aggregating activity. However, the neoplastic cells in culture media released an activity directly stimulating platelet aggregation and potentiating the platelet response to ADP; again, this activity was higher for the more malignant cells and the effects were inhibited by preincubation of platelets with ASA. These results suggest a role for platelets in the development of tumour metastases.*

INTRODUCTION

IT HAS been shown that the cells from both human and experimental tumours are able to activate platelet function by inducing irreversible aggregation and release of serotonin by platelets; these interactions are potentially important in metastasis formation [1–5].

The ability to stimulate platelet function has been reported to be related in some way to the *in vivo* invasiveness of cancer cells [6]. Recently, different cell lines derived from polyoma-induced rat sarcoma were studied in this respect [7]; the lines with high metastatic potential provided potent platelet aggregating material, while those with low metastatic potential showed very low aggregating activity.

The aim of the present work was to further elucidate this key question by studying the effects on platelet function of two sublines of a benzopyrene-induced murine fibrosarcoma with different metastasizing activity.

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MATERIALS AND METHODS

Cell lines and cell culture

Two sublines (M_4 and M_9) derived from spontaneous lung nodules of the benzo[a]pyrene-induced mFS6 sarcoma were kindly supplied by Dr. Alberto Mantovani, Istituto Mario Negri, Milan, Italy. These tumour sublines were chosen because they differed significantly from the primary tumour, M_4 being more metastatic and M_9 being less metastatic [8, 9].

Cells were grown in tissue culture flasks (3050 Costar, Cambridge, MA) in RPMI-1640 medium (KC Biological Inc., Lenexa, KA) supplemented with 10% foetal calf serum. Cells were harvested by two procedures: by brief exposure (1–5 min) to 0.25% trypsin–0.02% ethylene diamine tetracetic acid (EDTA) in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline (PBS) or utilizing 0.2% collagenase (Sigma type I) in PBS for 10–15 min. In some experiments cell lines were preincubated with acetylsalicylic acid (ASA) (final concentration 10^{-4} M) in RPMI-1640 medium without foetal calf serum for 30 min at 37°C before harvesting.

Tumour cells were washed 3 times and resuspended in Ca^{2+}, Mg^{2+}-free Hank's balanced salt solution (HBSS), counted in a haemocyto-

meter and held at 37°C. Cell viability, determined by trypan blue exclusion, exceeded 80%, was similar for all cell lines and remained constant throughout the experiments.

Platelet function studies

Blood was obtained from 28 apparently healthy subjects aged between 20 and 35 yr by clean venepuncture of the antecubital vein and gently mixed with heparin (final concentration 8 U/ml) in plastic tubes; heparin was chosen as the anticoagulant because the chelation of divalent cations by trisodium citrate or EDTA is known to affect platelet-tumour cell interactions [6].

Platelet-rich plasma (PRP) was obtained by centrifugation at 200 *g* for 20 min at room temperature and platelet-poor plasma (PPP) by centrifugation at 900 *g* for 15 min. PRP was diluted with PPP, when necessary, to give a final platelet concentration between 250,000 and 300,000/μl.

Platelet aggregation assays. Platelet aggregation was investigated by Born's method [10] with an Aggregation and Shape Change Monitor Born/Michal MK IV for the measurement of both transmitted and scattered light [11] and a two-channel Potentiometric Servogor Recorder RE571. It was therefore possible to obtain information on platelet aggregation and shape change at the same time.

In order to evaluate the aggregating activity of the two cell sublines 0.8 ml of PRP was pipetted in a siliconized glass cuvette and placed in the appropriate compartment of the aggregometer at 37°C. After 3 min 0.2 ml was added of one of the following solutions: (1) HBSS; (2) suspensions of the cells of the two sublines in HBSS at different concentrations; (3) supernatants of these suspensions obtained by centrifugation at 1000 *g* for 10 min at room temperature after storing the cells in HBSS up to 3 hr at 37°C; (4) RPMI-1640 medium supplemented with 10% foetal calf serum; (5) culture media collected after 48–72 hr when the cells were at confluence (2–3 × 10^6 cells per flask).

In other experiments 8 μl of ASA (final concentration 10^{-4} M) were added to PRP during preincubation and before addition of the cell suspensions or culture media. The presence of a potentiating effect on ADP-induced aggregation in the media after 48–72 hr of culture was assayed as follows: 0.8 ml of PRP and 0.2 ml of culture media or of RPMI-1640 medium supplemented with 10% foetal calf serum was pipetted into the cuvette and 3 min later ADP at the final concentration of 2 μM was added. The following parameters were evaluated on the aggregation curves: maximal aggregation, expressed as the maximal percentage change in light transmission, the aggregation velocity, measured by taking the tangent to the steepest slope of the aggregation tracing and expressed in mm/min, and the platelet shape change, which was measured as the initial velocity of the decrease in scattered light and also expressed in mm/min.

In other experiments 10 μl of ASA (final concentration 10^{-4} M) was preincubated with PRP and culture media before adding ADP.

Serotonin release assays. [^{14}C]-5-Hydroxytryptamine ([^{14}C]-5HT) (Sorin, France), with a specific activity of 48 mCi/mM, was dissolved in 70% ethanol to a radioactivity of 10 μCi/ml and stored at -20°C. Before each experiment this solution was diluted 1:5 (v/v) with distilled water. The uptake and release of [^{14}C]-5HT by platelets were studied according to Jerushalmy and Zucker [12]: 0.8 ml of PRP was put in the aggregometer cuvette at 37°C for 3 min, then 10 μl of the solution of [^{14}C]-5HT was added (final concentration 0.4 μM). Preliminary experiments showed that at these 5HT and platelet concentrations maximum uptake was reached after 3–5 min. Therefore the time of 5 min was chosen for all the experiments. After this time 0.2 ml of tumour cell suspensions or of HBSS was added and the aggregation was recorded. After 4 min samples were centrifuged for 30 sec in an Eppendorf centrifuge at 11,000 *g* to obtain platelet-free plasma (PFP).

Table 1. *Effect of ASA* (10^{-4}M) *on the activity of culture media of* M_4 *and* M_9 *cells in potentiating ADP-induced aggregation*

		% increase (means ± S.E.)		
		Without ASA	With ASA	*P*
Line M_4	Maximal aggregation	62.83 ± 7.58	24.73 ± 16.88	<0.01
	Aggregation velocity	46.85 ± 11.88	1.92 ± 9.23	<0.01
	Platelet shape change	32.02 ± 8.78	-7.87 ± 7.47	<0.05
Line M_9	Maximal aggregation	50.20 ± 10.68	6.40 ± 16.42	<0.05
	Aggregation velocity	19.40 ± 11.30	29.38 ± 25.18	N.S.
	Platelet shape change	16.52 ± 2.94	-23.35 ± 8.34	<0.05

One hundred microlitres of radioactive samples were added to 1 ml of a mixture of soluene and isopropyl alcohol (1:1 v/v) in counting vials and mixed well; 10 ml of Instagel were added and radioactivity was determined in a Packard liquid scintillation counter with an external standard for quenching corrections.

The release of 5HT was calculated with the following formula:

$$\% \text{ release} = \frac{\text{cpm}_{\text{sup 1}} - \text{cpm}_{\text{sup 2}}}{\text{cpm}_{\text{PRP}} - \text{cpm}_{\text{sup 2}}} \times 100,$$

where $\text{cpm}_{\text{sup 1}}$ is the radioactivity of PFP after addition of cell suspensions, $\text{cpm}_{\text{sup 2}}$ the radioactivity of PFP after addition of HBSS and cpm_{PRP} the total radioactivity of PRP after addition of HBSS and before centrifugation.

RESULTS

Effects of neoplastic cells on platelet function

The cells of the M_4 subline were able to induce irreversible platelet aggregation after a short lag time with no significant shape change of platelets before aggregation, while the cells of M_9 subline sometimes induced only reversible aggregation (Fig. 1).

Figure 2 shows that the lag time and the extent of aggregation induced by the M_4 cells were dependent upon the concentration of neoplastic cells. This aggregating activity was partially inhibited by preincubation of platelets with ASA and was much greater than that of the cells of the M_9 subline. There was also a qualitative difference in the activity of the two lines, the aggregation induced by the M_4 cells being almost invariably irreversible while that induced by the others was almost always reversible. Neither HBSS nor the supernatants of the two cell lines had any activity.

The results of the experiments with cells of the two sublines at different concentrations are reported in Fig. 3, which confirms the much higher activity of the more malignant cells (M_4).

Figure 4 reports means and standard errors of maximal platelet aggregation and the simultaneous serotonin release induced by the M_4 cells, by these cells on platelets pretreated with ASA and by these cells pretreated with ASA and by the M_9 cells. The cells of the metastasizing line induced significantly higher aggregation and release than those of the non-metastasizing line. Aggregation and release were significantly reduced when ASA was preincubated with platelets, not when it was preincubated with the cells.

These results suggest that the aggregation was not due to the release of prostaglandins and thromboxane by the cells; interestingly, in this respect, the supernatants of cell suspensions never had any aggregating activity (Fig. 2). Furthermore, no difference in aggregation response was observed when the neoplastic cells were harvested with collagenase.

Effects of culture media on platelet function

As the supernatants of the suspensions of neoplastic cells never had any activity we

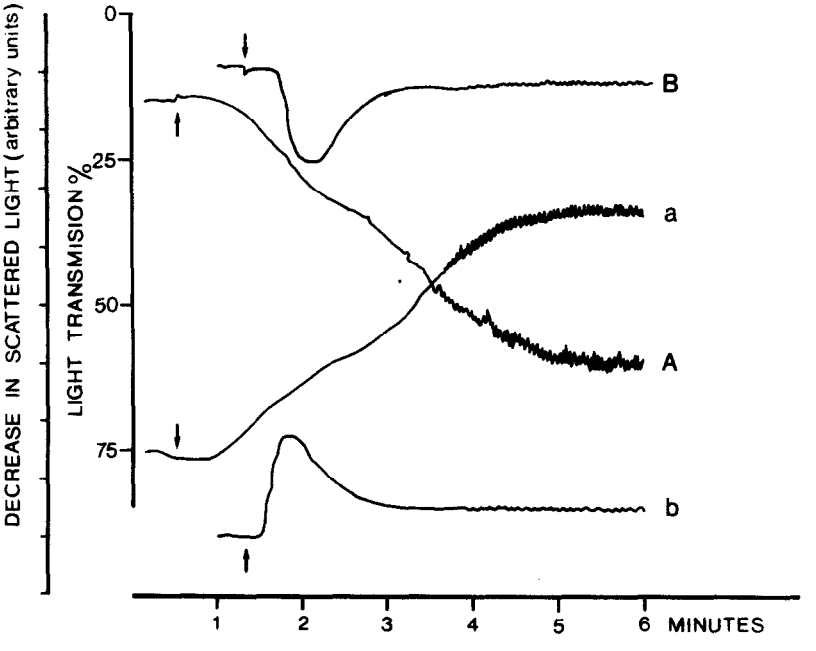

Fig. 1. Aggregating activity of the two cell lines. The final concentration of neoplastic cells was 400,000/ml. *(A) Aggregation and (a) shape change with M_4 cells; (B) aggregation and (b) shape change with M_9 cells. The cell suspensions were added to PRP where indicated by the arrows.*

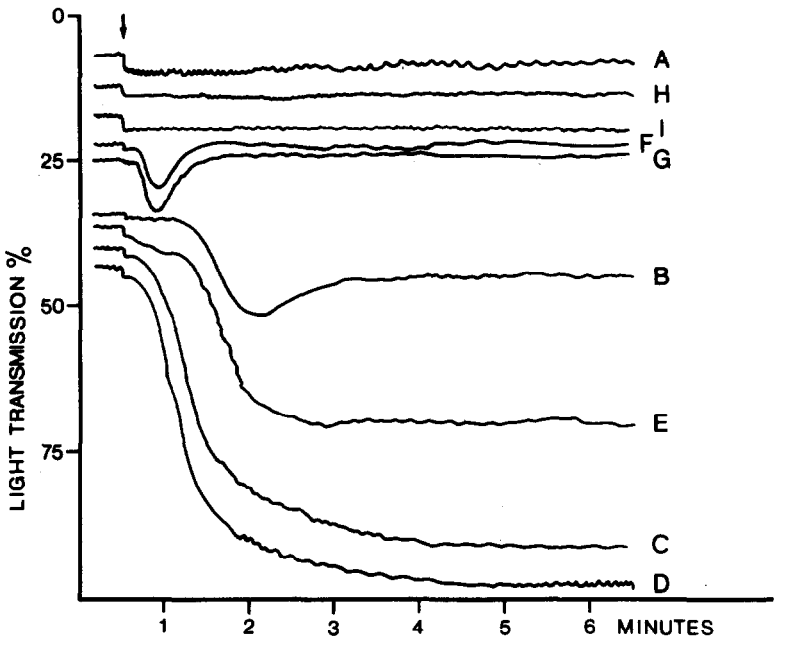

Fig. 2. Platelet aggregation responses with different concentrations of neoplastic cells. To 0.8 ml *of PRP were added (arrow): (A)* 0.2 ml *of HBSS; (B)* 0.2 ml *of M_4 cell suspension (final conc.* 274,000/ml); *(C)* 0.2 ml *of M_4 cell suspension (final conc.* 548,000/ml); *(D)* 0.2 ml *of M_4 cell suspension (final conc.* 822,000/ml); *(E)* 8 μl *of ASA* 10^{-2} M *(final concentration* 10^{-4} M) *for* 3 min *and then as (D); (F)* 0.2 ml *of M_9 cell suspension (final conc.* 450,000/ml); *(G)* 0.2 ml *of M_9 cell suspension (final conc.* 800,000/ml); *(H)* 0.2 ml *of the supernatant of the M_4 cell suspension; and (I)* 0.2 ml *of the supernatant of the M_9 cell suspension.*

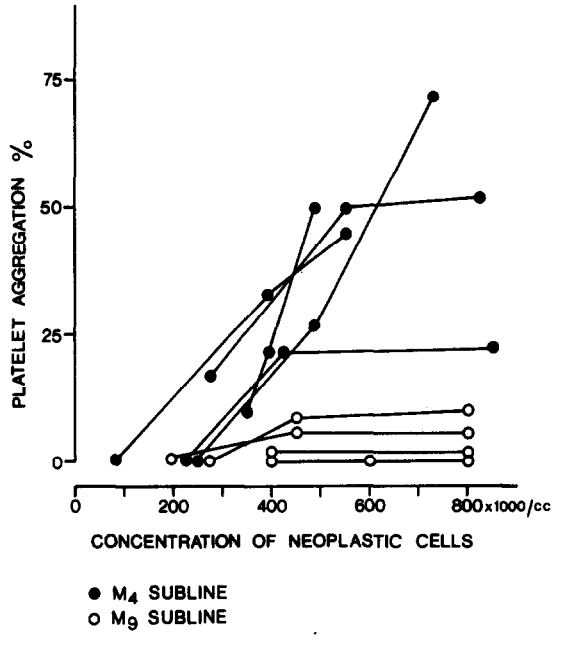

Fig. 3. Maximal aggregation induced by the M_4 and M_9 cells at difference concentrations.

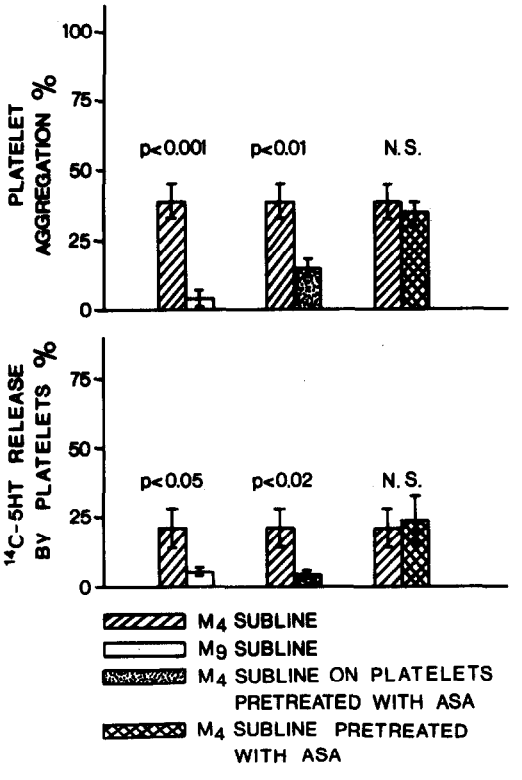

Fig. 4. Effect of ASA on platelet aggregation and serotonin release induced by the neoplastic cells (400,000/ml).

investigated whether the cells released it with time in culture media. This was found to be the case for the M_4 cells but not for the M_9 cells (Fig. 5). Furthermore, the culture media of both cell lines increased ADP-induced platelet aggregation and shape change (Fig. 6); this activity was also higher in culture media from the more malignant cells. The medium itself had no aggregating effect.

Figure 7 compares the aggregating activities of the culture media from the two sublines; the activity released by the M_4 cells was always higher and the differences were significant for aggrega-

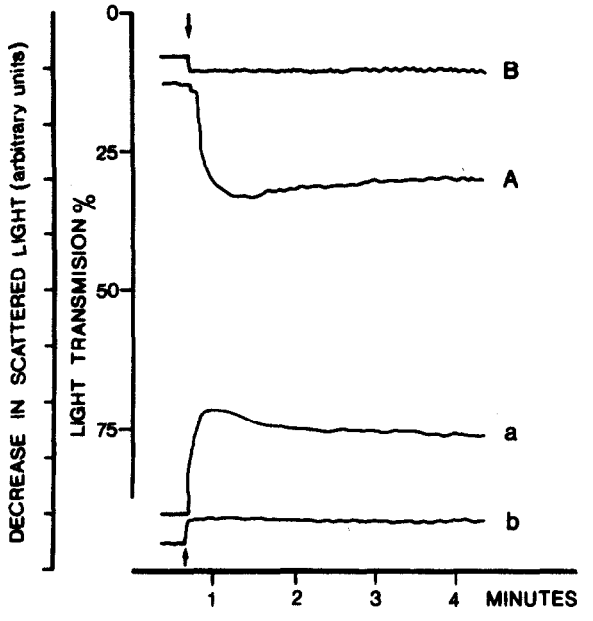

Fig. 5. Aggregating activity of the culture media from the cells of the two sublines. To 0.8 ml *of PRP* 0.2 ml *of culture medium was added (arrow): (A) aggregation and (a) shape change with the M_4 cells; (B) aggregation and (b) shape change with the M_9 cells.*

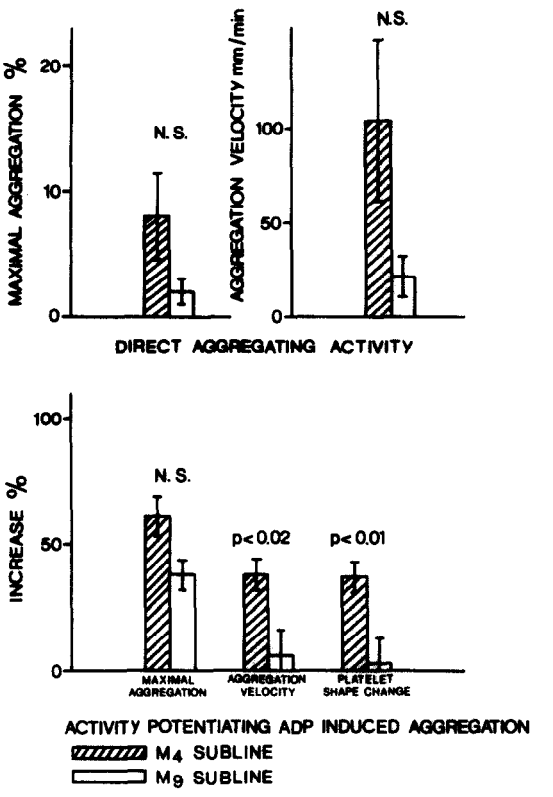

Fig. 7. Activities stimulating platelet function from culture media of the two sublines.

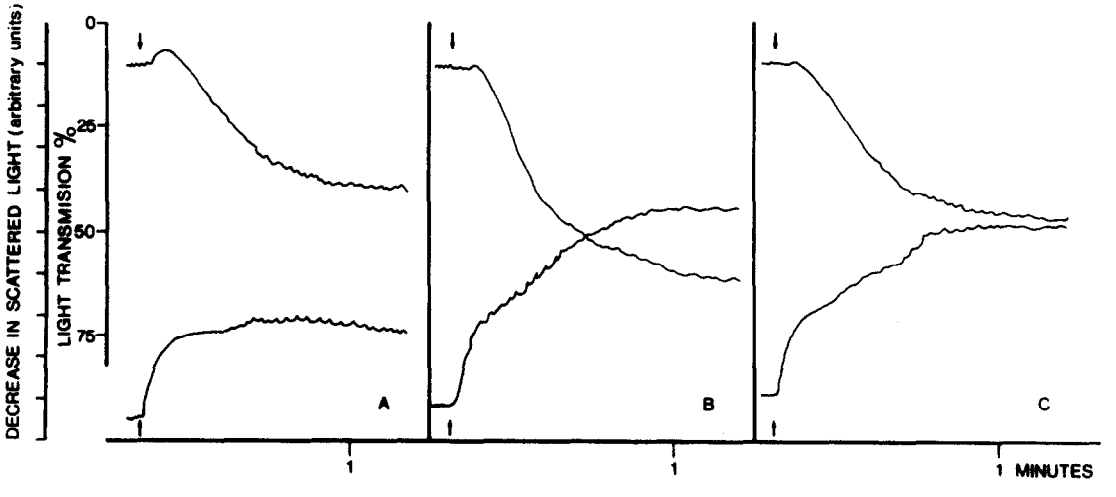

Fig. 6. Effect of culture media from the two cell lines on ADP-induced platelet aggregation and shape change. PRP (0.8 ml) *was preincubated for* 3 min *with: (A)* 0.2 ml *of RPMI-*1640 *medium supplemented with foetal calf serum; (B)* 0.2 ml *of culture medium from M_4 cells; (C)* 0.2 ml *of culture medium from M_9 cells. Then* 20 µl *of ADP* 10^{-4} M *(final conc.* 2 µM*) were added to PRP (arrows).*

tion velocity and platelet shape change. The potentiating effect of culture media on ADP-induced platelet aggregation was inhibited by ASA (Table 1) for all the parameters considered using the M_4 cells and for all the parameters except aggregation velocity using the M_9 cells. Furthermore, the significance of the effects of ASA was generally higher for the more malignant cells.

DISCUSSION

Our study has shown that in this experimental model the *in vivo* invasiveness of neoplastic cells is correlated to their ability to stimulate platelet aggregation and release reaction. In other experimental models murine tumours did not aggregate human platelets [13], but the different nature of the neoplastic cells may well explain

this difference. It is noteworthy that human platelets showed in this study an aggregation response very similar to those of mouse platelets with the same cells [14].

In our experiments there was variability in the individual response of platelets to the malignant cells, as already observed in other studies [15]; this aggregating effect was partially inhibited by ASA and appears to be a property closely linked to the cell surface, as the supernatants of cell suspensions had no activity and preincubation of the cells with ASA had no effect on platelet aggregation induced by them.

It is of interest to note that we used intact cells and not cell extracts as did Pearlstein *et al.* [7], who reported a similar correlation between malignancy and platelet-stimulating activity. Our system seems in some way more physiological and could indeed mimic what in some circumstances can happen *in vivo*. The aggregating activity of the cells we studied seems related to a stimulation of the release reaction, as indicated by the associated release of serotonin and by the inhibition of both aggregation and release by preincubation of platelets with aspirin. This activity is not present in the supernatants of cell suspensions but is spontaneously released by the cells in culture media with time. This last activity, which is more evident for the more malignant cells, greatly increases ADP-induced platelet aggregation and shape change, indicating that early events in platelet activation are involved.

In other experimental models the aggregating activity of the neoplastic cells was associated with membrane vescicles and shed from the cells growing in culture. These cells had a ruffled surface consisting of many microvilli [1]. Something similar could happen for the cells we used, which likewise showed many microvilli on their surface (data not shown).

Our results are potentially interesting because there are many possible mechanisms through which a neoplastic cell with high platelet-stimulating activity can give more metastases than a cell with no or poor such activity: the aggregates of platelets and neoplastic cells can facilitate the arrest and survival of the cells in the microvasculature [16–20]; furthermore, the release of vasoactive substances from the aggregated platelets may facilitate passage of the tumour cells through the vessel wall [2, 21] and for some tumours is even associated with the release of a growth-promoting factor for the neoplastic cells [22–24].

Several important points remain open, such as the nature and mechanism(s) of the cellular component(s) responsible for this activity; furthermore, the species differences between tumour cells and platelets prompt us to consider these results with care. However, they do support the hypothesis that, at least for some tumours, the ability of cancer cells to stimulate platelets is of major importance among the different factors which can lead to metastases formation.

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