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Platelet Aggregation Induced by Adenosine Diphosphate Released from Cloned Murine Fibrosarcoma Cells Is Positively Correlated with the Experimental Metastatic Potential of the Cells

Yoshihiro Mogi, Katsuhisa Kogawa, Tetsuji Takayama, Naohito Yoshizaki, Kiyoshi Bannai, Hirohito Muramatsu, Kazuhiko Koike, Yutaka Kohgo, Naoki Watanabe and Yoshiro Niitsu[1]

Department of Internal Medicine (Section 4), Sapporo Medical College, South-1, West-16, Chuo-ku, Sapporo 060

We established five clones (ML-01, ML-02, MH-01, MH-02, MH-03) from murine 3-methylcholanthrene-induced fibrosarcoma A (Meth A), and investigated their experimental metastatic potentials in relation to their platelet-aggregating activities. A clone with a high metastatic potential (MH-02) showed a characteristic biphasic pattern of platelet aggregation, of which the first peak was not present in the aggregation patterns of the clone with low metastatic potential (ML-01). The first peak was eliminated by treatment of the cells with apyrase, indicating that adenosine diphosphate (ADP) was the causative substance of this particular peak. The metastatic potential of clones correlated well with the ADP concentration of the culture media. These results suggest that the increased ADP production and consequential enhancement of platelet-aggregating activity are closely related to the increment of pulmonary metastatic potential of MH-02 clone.

Key words: Platelet aggregation — ADP — Highly metastatic clone

Platelets are involved in blood-borne metastasis by facilitating tumor cell thrombus formation.[1-4] Mechanisms underlying this tumor cell-induced platelet aggregation are not yet completely understood. In particular, with regard to the nature of the platelet-aggregating substances, conflicting results have been obtained. Relatively well defined platelet aggregating factors include membrane-associated sialolipoproteins,[5-7] thrombin-like substances,[8,9] ADP,[10,11] cathepsin B-like proteinase,[12] tissue factor-like substance,[13] and 44 kDa[14] and 18 kDa cell membrane proteins,[15] although direct relationships between the activity of these materials and metastatic potential have not been demonstrated. One of the difficulties of these types of experiments stems from the fact that the tumor cells used in the previous studies were not cloned but were derived from mixed populations. For example, a recent study provided evidence that uncloned high- and low-metastatic tumor cell lines actively released ADP, which caused platelet aggregation, but the platelet aggregation in that study appeared to be unrelated to metastatic potential.[16] On the other hand, cloned variants of murine fibrosarcoma were found to possess platelet-aggregating activity which correlated with their metastatic potential.[17] In the present paper, a positive correlation between metastatic ability of cloned methylcholanthrene-induced murine fibrosarcoma and the concentration of ADP in conditioned medium is demonstrated.

MATERIALS AND METHODS

Reagents Apyrase (Grade I), neuraminidase (type V), collagenase (type VII), phenylmethylsulfonyl fluoride (PMSF) and soybean trypsin inhibitor were obtained from Sigma Chem. Corp., St. Louis; trypsin was from DIFCO. A PGI_2 analogue; (7*R*)-16,17,18,19,20-pentanol-15-cyclopentyl-7-fluoro-PGI_2 sodium salt (PGI_2-TEI 8153)[18] was provided by the Teijin Biomedical Research Institute.

Animals Six-week-old female BALB/c mice weighing 20–25 g were obtained from Clea Japan, Inc., Tokyo.

Tumor cell lines and cloning Methylcholanthrene-induced fibrosarcomas A (Meth A),[2] provided by the courtesy of Dr. N. Sato (Department of Pathology, Sapporo Medical College), were cultured in RPMI 1640 media (GIBCO) containing 10% fetal calf serum (FCS, GIBCO), 100 units/ml penicillin-G and 100 mg/liter streptomycin.[19] Culture flasks (Falcon 3013, 25 cm^2) were maintained in humidified 5% CO_2 at 37°C. High- and low-metastatic clones were established from Meth A as described before.[20] Briefly, 1×10^4/0.1 ml medium of Meth A cells were injected into the caudal vein of BALB/c mice. The cells from pulmonary nodules were also cultured in RPMI 1640 medium supplemented with

[1] To whom correspondence should be addressed.

[2] Abbreviations: Meth A, 3-methylcholanthrene-induced fibrosarcoma A; ADP, adenosine diphosphate; FCS, fetal calf serum; PBS, phosphate-buffered saline; PRP, platelet-rich plasma; PPP, platelet-poor plasma.

10% FCS. After the tumor cells had grown, the cells (ML) were injected into the caudal vein of BALB/c mice as described above. After repeating these steps 15 times, the cell line MH was established. ML and MH cells were further cloned by the method of limiting dilution in 96-well microtiter plates. To minimize the possibility of phenotypic changes, clones were maintained in culture for 4 to 6 weeks after which they were replaced with new frozen stock. Meth A cells and their clones grown as suspension cultures in plastic dishes were washed 3 times in serum-free RPMI 1640 medium, resuspended in phosphate-buffered saline (PSB) and pipetted gently to dissociate all cell clumps for platelet aggregation studies or for experimental pulmonary metastasis.

Experimental pulmonary metastasis Mice were given intravenous injections of $1.5\sim2.0\times10^4$ viable tumor cells suspended in 0.1 ml of PBS via the tail vein. All mice were killed 14 days after injection of the tumor cells, and their lungs were removed, rinsed in saline, and fixed in 70% ethanol. The number of metastatic nodules on the surface of the lungs was determined under a dissecting microscope.[15]

The antiplatelet agent, PGI_2-TEI 8153, which is extremely stable compared to the authentic prostacyclin as described before,[15] was administered intravenously (20 μg) to mice in 0.1 ml of buffer solution one minute prior to the injection of cells.

Platelet aggregation studies Heparinized (10 units/ml) normal human blood was centrifuged at 160g for 10 min at room temperature to prepare platelet-rich plasma (PRP). After the PRP was removed, the blood was further centrifuged at 2,100g for 10 min to obtain platelet-poor plasma (PPP). The platelet aggregating activity of tumor cells was measured by using an aggregometer, NKK Hematracer 1 (Niko Bioscience). The aggregometer was calibrated with PPP to 100% optical transmission and with PRP to 0% optical transmission. Tumor cells (5×10^5) suspended in 0.01 ml of PBS were added to 0.2 ml of PRP. Human platelets have been shown to have aggregating properties similar to those of mouse platelets.[15, 21, 22]

Treatment of tumor cells with enzymes Enzyme solution (10 μl) at the final concentrations shown in Fig. 4 was added to 100 μl of tumor cells suspension (5×10^7/ml); the pH was preadjusted to the optimal value, and the mixture was incubated for 1 h at 37°C. The tumor cells were then washed with serum-free RPMI 1640 medium and 5×10^7 cells/ml were resuspended in the same buffer. The enzyme-treated tumor cells were assayed for platelet-aggregating activity by using the aggregometer as described above.

Preparation of conditioned medium from tumor cells Tumor cells (5×10^7/ml) were incubated in RPMI 1640 medium for 15 min at 37°C and the conditioned media were obtained by centrifugation at 400g for 10 min. Conditioned media obtained by this method were either treated with enzymes including trypsin, neuraminidase and apyrase, or heated at 100°C for 5 min. For the tryspin treatment, the conditioned media was digested with trypsin, 5 mg/ml, pH 8.5, for 30 min at 37°C. The enzymatic activity was then inhibited with soybean trypsin inhibitor, which was added in a ratio of 1:1.5. Neuraminidase treatment was performed with 50 units/ml, pH 6.0, for 1 h at 37°C in the presence of 2 mM PMSF to inhibit any contaminating protease activity. For the apyrase treatment, the culture media was incubated with apyrase, 40 μg/ml, pH 7.0, for 30 min at 37°C. Supernatants of culture media free of membrane vesicles were obtained by centrifugation at 100,000g for 1 h at 4°C. The enzyme- or heat-treated conditioned media and supernatants of the conditioned media were then tested for platelet-aggregating activity.

Analysis of tumor cell growth Tumor cells were plated in 35 mm dishes at a density of 1×10^5 cells per dish in 2 ml of RPMI 1640 medium containing 10% FCS. At the designated time intervals, the number of viable cells was calculated in triplicate by microscopically counting cells capable of excluding trypan blue to determine their growth rates *in vitro*.

Alternatively, 1×10^6 tumor cells were implanted subcutaneously in BALB/c mice. At weekly intervals, tumor volume was calculated from the formula: $V=0.5\times ab^2$ (mm^3), where a is the maximum (lengthwise) diameter of the tumor and b is the minimum diameter perpendicular to a, as described previously.[23]

Measurement of ADP Tumor cells (5×10^7/ml) were washed, incubated in saline for 10 min at 37°C and centrifuged at 400g for 10 min. Concentrations of ADP in the conditioned media were measured by the luciferin-luciferase method by using a Lumiphotometer TD-4000 (Laboscience) as described previously.[24]

RESULTS

Metastatic behavior of a highly metastatic subline derived from Meth A Injection of ML and MH cells into their syngeneic hosts confirmed that the cells retained their different metastatic phenotypes (Table I). MH cells formed approximately 5 times more metastatic nodules per animal than ML cells.

Metastatic properties of cloned Meth A cells Table II shows that all clones formed pulmonary metastatic nodules in all of 5 animals tested. MH-02 retained the most pronounced metastatic ability; four- to seven-fold greater than those of other clones. Accordingly, MH-02, which showed biphasic platelet aggregation and lacked the lag time, had the highest metastatic capacity. On the other hand, all clones had nearly identical growth rates

Table I. Metastatic Potential of MH and ML Cells

Cells	Pulmonary metastases	
	Number of metastatic nodules[a]	Metastatic incidence
MH	188.2±43.8[b]	5/5
ML	41.4±23.4	5/5

Cell suspension (2.0×10^4/0.1 ml/mouse) was injected into the tail vein of BALB/c mice. All mice were autopsied on day 14. The lungs were removed for subsequent microscopic examination.
a) Values represent the mean±SD.
b) Significantly high ($P<0.01$) as compared to ML cells.

Table II. Metastatic Potential of Clones Derived from Meth A

Clone	Pulmonary metastases	
	Number of metastatic nodules[a]	Metastatic incidence
ML-01	24.3±17.4	5/5
ML-02	45.0±8.5	5/5
MH-01	45.2±13.9	5/5
MH-02	170.6±40.6[b]	5/5
MH-03	32.0±8.9	5/5

Cells (1.5×10^4/0.1 ml/mouse) were injected into the tail vein of BALB/c mice. On day 14, the animals were killed and metastatic nodules in the lungs were counted.
a) Values represent the mean±SD.
b) Significantly high ($P<0.01$) as compared to other clones.

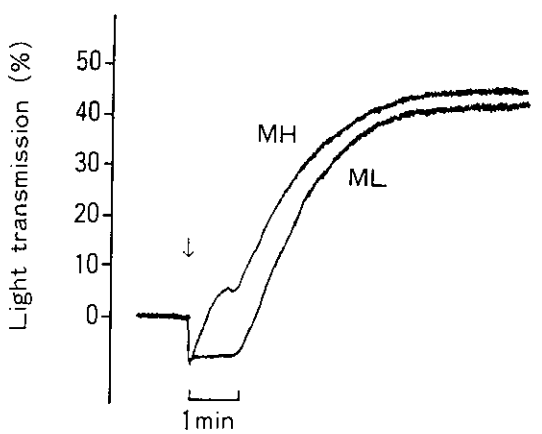

Fig. 1. Platelet aggregation induced by MH and ML cells. MH or ML cells (5.0×10^5) in 0.01 ml of PBS were added to heparinized PRP (0.2 ml). Platelet aggregation was measured photometrically by using an NKK Hematracer 1 (Niko Bioscience). PPP and PRP were incubated in a cuvette at 37°C for 5 min under constant stirring and platelet aggregation curves were recorded. The positions of 0 and 100% represent the light transmissions of PRP and PPP, respectively. Data were taken from one of five experiments with similar results. Arrow, time of addition.

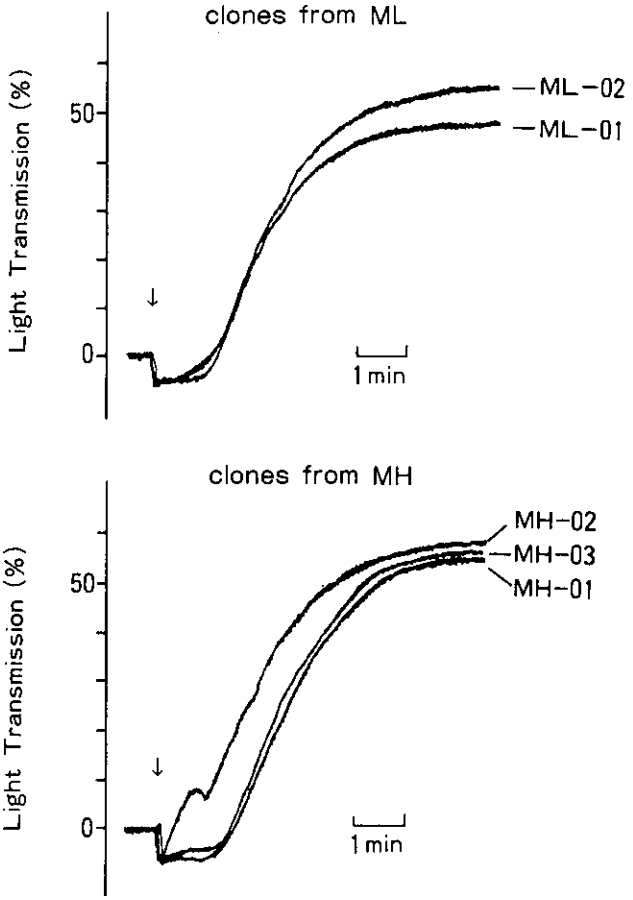

Fig. 2. Platelet aggregation induced by clones derived from Meth A. Cloned Meth A cells (5.0×10^5/0.01 ml) were added to 0.2 ml of human heparinized PRP. Clones MH-01, MH-02 and MH-03 were obtained from MH cells and clones ML-01 and ML-02 from ML cells. Data were taken from one of five experiments with similar results. Arrow, time of addition.

with doubling times of approximately 23.0 to 23.8 h *in vitro*. *In vivo* growth curves of these clones that were inoculated subcutaneously into syngeneic mice were also similar in all cases (data not shown). Therefore, the high metastatic potential of MH-02 was not due to enhancement of cell growth activity.

Platelet aggregation induced by Meth A sublines Both MH and ML cells were capable of inducing platelet aggregation (Fig. 1). The aggregation profile produced by MH cells was characterized by the presence of a small initial peak followed by a second phase of irreversible aggregation. The ML cells lacked the initial peak, and yielded a monophasic aggregation pattern with a short lag time. From five separate experiments, the difference between the maximal aggregations induced by MH and ML was not statistically significant.

Table III. Platelet Aggregation Induced by Cloned Meth A Cells

Clone	Platelet aggregation	
	Lag time[a)]	Maximum aggregation[a)]
ML-01	1.1±0.1	50.7±4.5
ML-02	1.1±0.1	61.7±2.9
MH-01	1.0±0.1	57.3±7.0
MH-02	0	62.3±6.4
MH-03	1.1±0.1	58.3±5.8

a) Values represent the mean±SD of five separate experiments.

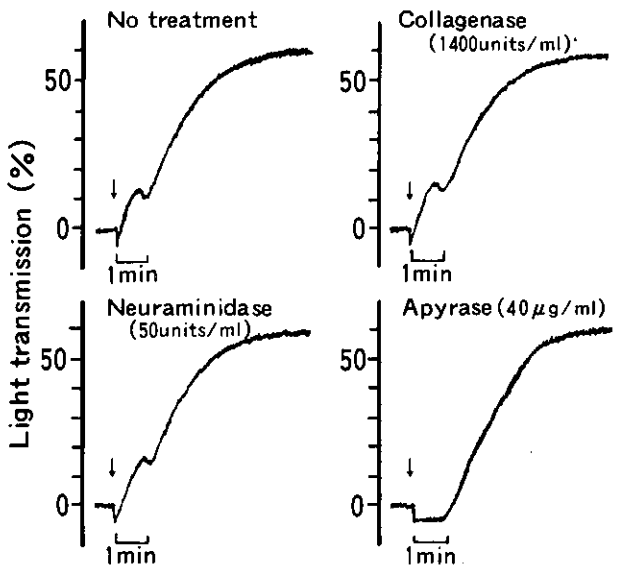

Fig. 3. Effects of enzyme treatments on MH-02-induced platelet aggregation. MH-02 cells were pretreated with collagenase (1,400 units/ml, pH 7.0), neuraminidase (50 units/ml, pH 6.0), or apyrase (40 μg/ml, pH 7.0) for 1 h at 37°C and then washed with RPMI 1640 medium. Cell suspensions (5.0×10^5/10 μl) were added to PRP as indicated by the arrows. Data were taken from one of five experiments with similar results.

Platelet aggregation induced by cloned Meth A cells Two clones, ML-01 and ML-02, were obtained from ML cells and three clones, MH-01, MH-02 and MH-03, from MH cells. These clones were tested for platelet-aggregating activity. As shown in Fig. 2, ML-01, ML-02, MH-01 and MH-03 induced platelet aggregation in a manner essentially similar to that of ML, whereas platelet aggregation by MH-02 showed a similar profile to that of MH with a small shoulder and no lag time. From five separate experiments, the difference between the maximal aggregations occurring with the various clones was not statistically significant (Table III).

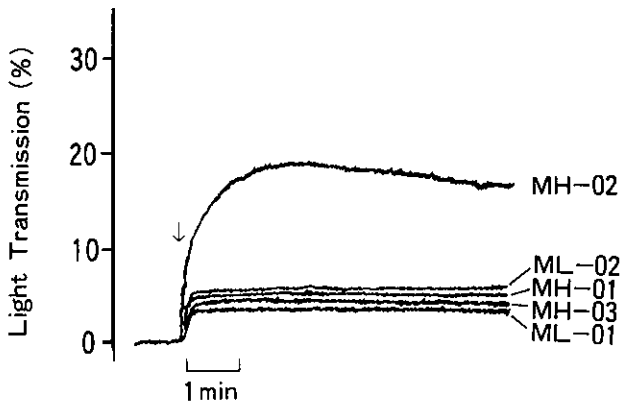

Fig. 4. Platelet aggregation induced by supernatants of culture media of clones with different metastatic potentials derived from Meth A. The supernatants of culture media of different clones (10 μl) were added to PRP as indicated by the arrows. Data were taken from one of five experiments with similar results.

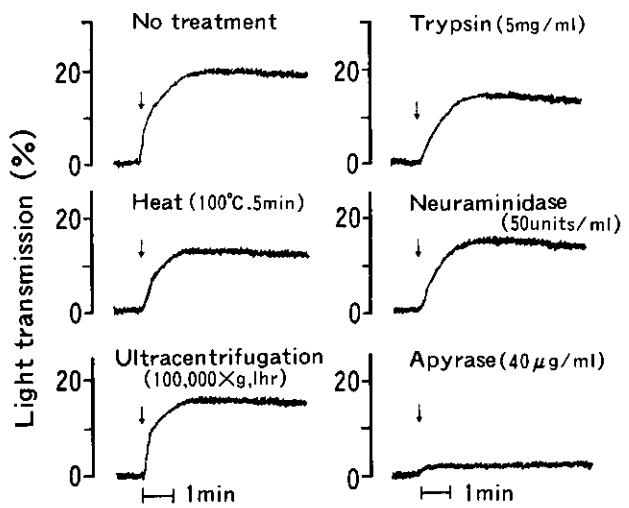

Fig. 5. Effect of physical and chemical modifications of conditioned medium from MH-02 cells on the induced platelet aggregation. Conditioned medium from 5×10^7/ml of MH-02 cells was treated with trypsin (5 mg/ml), neuraminidase (50 units/ml) or apyrase (40 μg/ml) at 37°C for 30 min, or heated at 100°C for 5 min prior to platelet aggregation assay as described in the text. Data were taken from one of five experiments with similar results. Arrow, time of addition.

Effects of enzyme treatments of MH-02 on its platelet-aggregating capacity To characterize further the platelet-aggregating material associated with tumor cells, MH-02, which showed the highest metastatic potential, was treated with the various enzymes shown in Fig. 3. Collagenase and neuraminidase had no effect on platelet

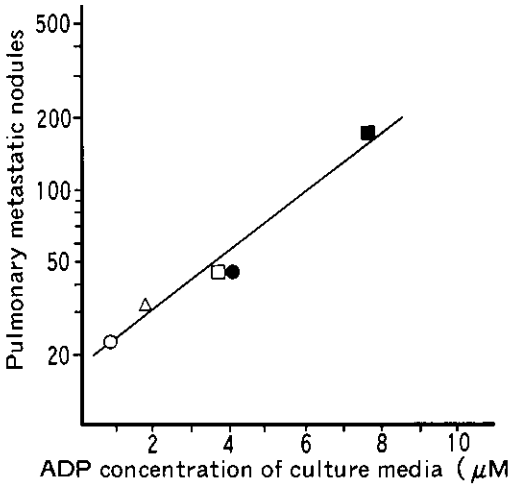

Fig. 6. Correlation of ADP concentration in culture media with pulmonary metastatic potential of Meth A clones (r= 0.93). ○, ML-01; ●, ML-02; □, MH-01; ■, MH-02; △, MH-03.

aggregation. Apyrase which is an ADP scavenger, however, eliminated the early phase of aggregation without influencing the later phase of aggregation (Fig. 3).

Platelet aggregation induced by conditioned media of cloned Meth A cells As can be seen in Fig. 4, conditioned media from only MH-02 were found to contain substance(s) that cause prominent platelet aggregation (20% maximal aggregation) with no lag time. This is compatible with the fact that MH-02 showed a characteristic biphasic aggregation profile.

Effects of physicochemical modification of conditioned medium from MH-02 on its platelet-aggregating capacity Conditioned media of MH-02 were subjected to a series of physical and chemical medifications. As shown in Fig. 5, incubation at 100°C for 5 min, ultracentrifugation, trypsin digestion and neuraminidase treatment did not abolish the platelet-aggregating activity of the conditioned media. However, apyrase treatment completely nullified platelet aggregation induced by the conditioned medium.

Relationship between ADP production and metastatic potential of Meth A clones ADP was detected in all conditioned media of the five clones in the concentration range between 0.8 and 7.7 μM. As can be seen in Fig. 6, the concentration of ADP appeared to correlate well with the metastatic potential of each clone (r=0.93). The highest concentration was observed in the conditioned medium of the highly metastatic clone, MH-02.

Effect of a PGI_2 analogue on artificial metastases of cloned Meth A cells The antimetastatic effect of a PGI_2 analogue, TEI 8153, was studied by using a weakly metastatic clone, ML-01, and a highly metastatic clone, MH-02, as target cells. As shown in Table IV, the number of pulmonary metastatic foci in mice treated with the analogue decreased for both ML-01 and MH-02. However, the reduction of pulmonary metastasis of MH-02 was significantly greater than that of ML-01, indicating that the metastatic ability of MH-02 is more dependent on its platelet-aggregating activity.

DISCUSSION

In previous studies, we demonstrated that Meth A cells which had a membrane protein of 18,000 daltons specifically bound to platelets and induced platelet aggregation in a dose-dependent manner with a short lag time.[15] We also showed that Meth A cells were capable of forming pulmonary metastasis, and that this metastasis was prevented by using an antiplatelet agent, TEI 8153 (a prostacyclin analogue). However, those observations were based on experiments using cell populations which were heterogeneous in terms of both metastatic potency and platelet-aggregating activity. In the present investigation, therefore, attempts were made to elucidate the relationship between platelet-aggregating activity and the metastatic ability of clones selected from high- and low-metastatic sublines of Meth A.

The clone with the highest metastatic ability (MH-02) exhibited a characteristic platelet-aggregating profile; a

Table IV. Antimetastatic Effect of PGI_2 Analogue on Pulmonary Metastases of High- and Low-metastatic Clones Derived from Meth A

Clone	Number of pulmonary metastatic nodules		% reduction of pulmonary metastases by PGI_2 analogue
	Saline	TEI 8153 (20 μg/mouse)	
ML-01 (n=10)	41.4±17.6	23.0±6.4	44.4
MH-02 (n=10)	252.0±67.3	35.3±25.9	86.0

PGI_2 analogue (TEI 8153, 20 μg/mouse) was injected into the tail vein of BALB/c mice, and tumor cells (2.0×10^4/0.1 ml/mouse) were injected 1 min after.

biphasic aggregation pattern without a lag time. In contrast, platelet aggregation with the other four clones was monophasic with a distinct lag time. The first peak of this biphasic aggregation is likely to be induced by the ADP released from tumor cells, because this peak could be eliminated by apyrase treatment. The action of apyrase was considered to inhibit new release of ADP from tumor cells. The close relationship between ADP production by the tumor cells and their metastatic ability was further suggested by the observations that the ADP concentrations in conditioned media of Meth A clones paralleled the numbers of pulmonary metastatic nodules. The later phase of platelet aggregation was seen with all of the cloned cells, suggesting that this aggregation was induced by a factor such as 18 kDa cell membrane protein identified in parental Meth A cells.[15] We previously reported similar membrane proteins with platelet-aggregating activity in various human cell lines.[25]

Similar biphasic platelet aggregation induced by a highly metastatic clone of Meth A was recently reported by Mahalingam *et al.*,[17] although they have not identified ADP as the causative substance.

On the other hand, Grignani and Jamieson[16] claimed that generation of ADP by tumor cells (human urinary tract carcinoma, 253J and B16 murine melanoma) was unrelated to metastatic potential. The apparent discrepancy between their findings and the present results may be due to the fact that the present results were obtained by employing cloned tumor cells.

Platelet aggregations without lag times, such as those induced by ADP, may contribute more to tumor thrombus formation and successive lodging at peripheral vessels than corresponding platelet aggregation with lag times, because the former type of aggregation can induce tumor thrombus formation more readily in a rapidly flowing circulation than the latter. Moreover, the number of pulmonary metastases was reduced by a PGI_2 analogue in the clone with high metastatic potential (MH-02) rather than the clone with low metastatic potential (ML-01), supporting a positive correlation between the metastatic ability of tumor clones and their ADP production. Nevertheless, the highly metastatic phenotype of MH-02 cells may not be attributed solely to the platelet-aggregating ability, because the antiplatelet agent did not completely nullify the metastatic potential. Additional mechanisms such as adhesion and/or invasion factor(s) may also be involved in the high metastatic potential of the MH-02 clone, and further detailed studies are in progress.

In conclusion, we demonstrated that the increased ADP production and consequential enhancement of platelet-aggregating activity are closely related to the increment of the pulmonary metastatic potential of tumor clones in an experimental metastasis model.

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