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# COMMENTARY

# PROSTACYCLIN AND THROMBOXANES

## IMPLICATIONS FOR THEIR ROLE IN TUMOR CELL METASTASIS

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### METASTASIS

Advances in the treatment of primary neoplasms, particularly with surgery and radiation therapy, have left the inhibition of metastasis as the major obstacle to decisive, curative cancer treatment. Metastasis as simply defined is the loss of contiguity between a tumor cell or clumps of tumor cells and the primary lesion and successful transfer of tumor cells to, and growth at, a site spatially separate from the original tumor. It is an inefficient process since only a small subpopulation of cells that detach from the primary tumor possesses the necessary phenotypic characteristics to ensure their eventual survival [1–3]. This process termed "metastatic cascade" involves an intricate series of sequential events:

1. Invasion by the primary tumor into normal tissue.
2. Detachment of tumor cells from the primary tumor.
3. Intravasation into the microvasculature or lymphatics or direct shedding of tumor cells into circulation.
4. Hematogenous dissemination with possible interaction between circulating host platelets and tumor cells.
5. Arrest of a tumor cell–platelet thrombus in the microvasculature or direct adhesion of tumor cells to endothelial or deendothelianized surfaces with subsequent platelet thrombi formation.
6. Extravasation through the vessel wall into tissue or organ of secondary arrest.
7. Immediate growth into a secondary metastatic tumor or dormancy.

### PLATELET–TUMOR CELL INTERACTIONS AND METASTASIS
(for reviews see Refs. 4–7)

Following an initial period of primary tumor growth and invasion of normal tissue, tumor cells penetrate into blood vessels and/or lymphatics and subsequently disseminate to distant organs via the blood or lymph. Carcinomas were once believed to metastasize via the lymphatics, whereas malignant tumors of mesenchymal origin were believed to spread via the hematogenous route. This separation between the two systems of dissemination is probably an oversimplification. Hilgard *et al.* [8] have noted that tumor cells initially injected into the lymphatics will reach the vascular system. Similar conclusions have been reached by others [9, 10], and there is now substantial evidence that malignant cells can pass freely between the lymphatics and blood vessels [11, 12]. Although the presence of tumor cells in the circulatory system does not necessarily indicate a poor prognosis [13], it must be concluded that the use of the hematogenous route for the spread of malignant cells is an event which does occur at some point in the disease.

Once in circulation, tumor cells can, theoretically at least, assume various forms during transport. They can travel singly, in clumps, or enmeshed in a coating of fibrin-like material [14]. They can exist attached to blood platelets, RBCs, leukocytes, etc. All of the above have been observed in one tumor type or another, but it does not necessarily follow that all of the above modes of transport will allow for successful metastasis. One transport form may enhance chances of survival over another.

In any case, it is intuitive that for successful metastasis to occur the metastatic cell must first arrest and adhere to the vascular endothelium or to deendothelianized surfaces and remain intravascular until such time as it can extravasate either by destruction of the endothelium [13, 15] or diapedesis [16]. The actual mechanism for extravasation is unknown and currently a matter of some debate. The fate of the arrested tumor cell is by no means guaranteed. Some cells loosely adherent to the endothelium may become detached, shed into circulation, and die [17]. Numerous studies using [$^{125}$I]deoxyuridine-labeled tumor cells have demonstrated that, although entrapment in the lung from tail vein-injected cells is rapid (<10 min), the majority of cells are rapidly cleared and destroyed, with less than 1% of the injected cells surviving after 24 hr [1, 18, 19]. It has also been observed, both *in vivo* [14] and *in vitro* [20], that tumor cells adhere more readily to damaged endothelium than to intact endothelium. In fact, a zone of reduced oxygenation and overt vascular endothelial damage may preferentially facilitate attachment and tumor cell invasion. Whether or not this is due to a loss of some protective substance produced by intact endothelium, coaggregation with platelets, or some other mechanism is unknown. The role of platelets in tumor cell metastasis has been the subject of debate for several years. Morphologists at the beginning of the century

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described tumor cells enmeshed within a thrombus and adherent to the capillary wall. In a classic study, Wood [17] visualized, via continuous cinemicrography of rabbit ear chambers, the fate of V2 squamous carcinoma cells injected intra-arterially. Small clumps of tumor cells (6–10) were found to arrest, and subsequently become enmeshed, in a clot of fibrin and platelets. Within 3–6 hr the tumor cells had extravasated. Wood concluded that the initial site of arrest was not determined by vessel diameter or rate of blood flow, an idea also supported by the observations of Zeidman [21].

Gasic and co-workers [22] were the first to provide direct experimental evidence for a role of platelets in tumor cell metastasis. They had noted that pretreatment of mice with neuraminidase resulted in formation of fewer lung colonies following injection of TA3 ascites tumor cells. These results were found to be attributable to induction of thrombocytopenia in the host by neuraminidase [22]. Similar antimetastatic effects were observed when thrombocytopenia was induced with antiplatelet antiserum. Additional evidence was provided by the fact that the antimetastatic effects of neuraminidase could be reversed by platelet infusion [22].

In a series of follow-up papers, Gasic and coworkers [23–25] demonstrated that numerous human and animal tumor cells and plasma membrane vesicles shed from tumor cells were capable of aggregating platelets *in vitro* with a concomitant stimulation of the release reaction (measured as [$^{14}$C]serotonin released from prelabeled platelets). Several other laboratories have confirmed the *in vitro* aggregation of platelets by both animal and human tumor cells and the induction of thrombocytopenia following i.v. injection of some tumor lines [26–33]. However, most of these studies only provide circumstantial evidence that host platelets facilitate hematogenous metastasis.

Recent work from Karpatkin, Pearlstein and coworkers [30] provides more direct evidence that a causal relationship exists. Using ten cell lines derived from the polyoma virus-induced PW20 Wister–Furth rat renal sarcoma, they examined the correlation between the ability of the tumor cells to metastasize spontaneously from subcutaneous sites in syngeneic hosts and their platelet aggregating activity *in vitro*. Platelet aggregation was measured in response to a plasma membrane material extracted with 1 M urea [34]. A significant correlation between platelet aggregability and spontaneous metastasis was observed. These workers further correlated the sialic acid content of cell surface glycoproteins with the ability of these ten lines to (1) aggregate platelets and (2) spontaneously metastasize. A high degree of correlation was found between these parameters, suggesting that the sialic acid content of tumor cell surface glycoproteins may increase initial adhesiveness to platelets prior to stimulation of platelet aggregation or enhance arrest in the microvasculature [35].

Despite the above findings, the mechanism by which platelets enhance metastasis remains to be definitively established. Several suggestions appear in the literature and these include: protection against destruction by host cytotoxic macrophages by physical shielding of tumor cells within the emboli and increased ability of tumor platelet emboli to arrest in the microvasculature with possible occlusion of a precapillary sphincter. This would result in distal hypoxia and stasis with possible damage to the endothelium (microinjury hypothesis). Additional possible mechanisms are: greater adhesion of tumor cells to the subendothelial matrix via platelet bridges, protection of attached tumor cells from shear forces in the circulatory system, and enhanced tumor cell survival due to the release of platelet derived growth factors. The relevance of each of the above-suggested mechanisms to platelet enhancement of tumor cell metastasis remains to be established.

## ANTICOAGULANT THERAPY FOR THE TREATMENT OF METASTASIS
(for reviews see Refs. 4, 7 and 36)

The above results suggest that some form of anticoagulant therapy would prove efficacious in the treatment of experimental and human metastasis. Nevertheless, a review of the literature reveals that results to date have been ambiguous. The most consistent results to date have been obtained with coumarin derivatives [36]. The anticoagulant heparin has been reported to both decrease [37, 38] and increase metastasis, especially extrapulmonary [39–41]. The anticoagulants batroxobin, urokinase and dipyridamole have also produced mixed results [42–44]. Aspirin, a potent cyclooxygenase inhibitor and therefore a platelet aggregation inhibitor, has also been reported to both decrease and increase metastasis depending on the tumor used and the dose studied [44–46]. A number of the agents which produced mixed results may have had effects on the tumor cells themselves which would alter an otherwise favorable response. For example, aspirin like indomethacin inhibits prostaglandin synthesis. Indomethacin has been shown to increase metastasis in variants of the B16 melanoma presumably due to the inhibition of an antiaggregatory prostaglandin ($PGD_2$) which is produced by these tumor cells [47]. Aspirin would inhibit platelet aggregation due to its inhibition of thromboxane $A_2$ production [48], yet high doses of aspirin would also inhibit vascular wall production of prostacyclin [49] which has been proposed to function as a natural deterrent to metastasis [19].

## PROSTACYCLIN, THROMBOXANES AND PLATELETS
(for reviews see Refs. 49–55)

The existence of a relationship between plasma lipids and blood coagulation has been acknowledged for the last three decades. Discoveries made during the last ten years have provided a fresh insight into that relationship. Novel compounds derived from arachidonic acid [prostacyclin ($PGI_2$) and thromboxane $A_2$ ($TXA_2$); Fig. 1] have been demonstrated to have profound but possibly not exclusive [56] effects on platelet aggregation. Central to those discoveries was the isolation and characterization of the two labile endoperoxide intermediates ($PGG_2$ and $PGH_2$; Fig. 1) by Hamberg, Samuelsson and coworkers [57, 58]. Prostaglandin $H_2$ occupies a pivotal

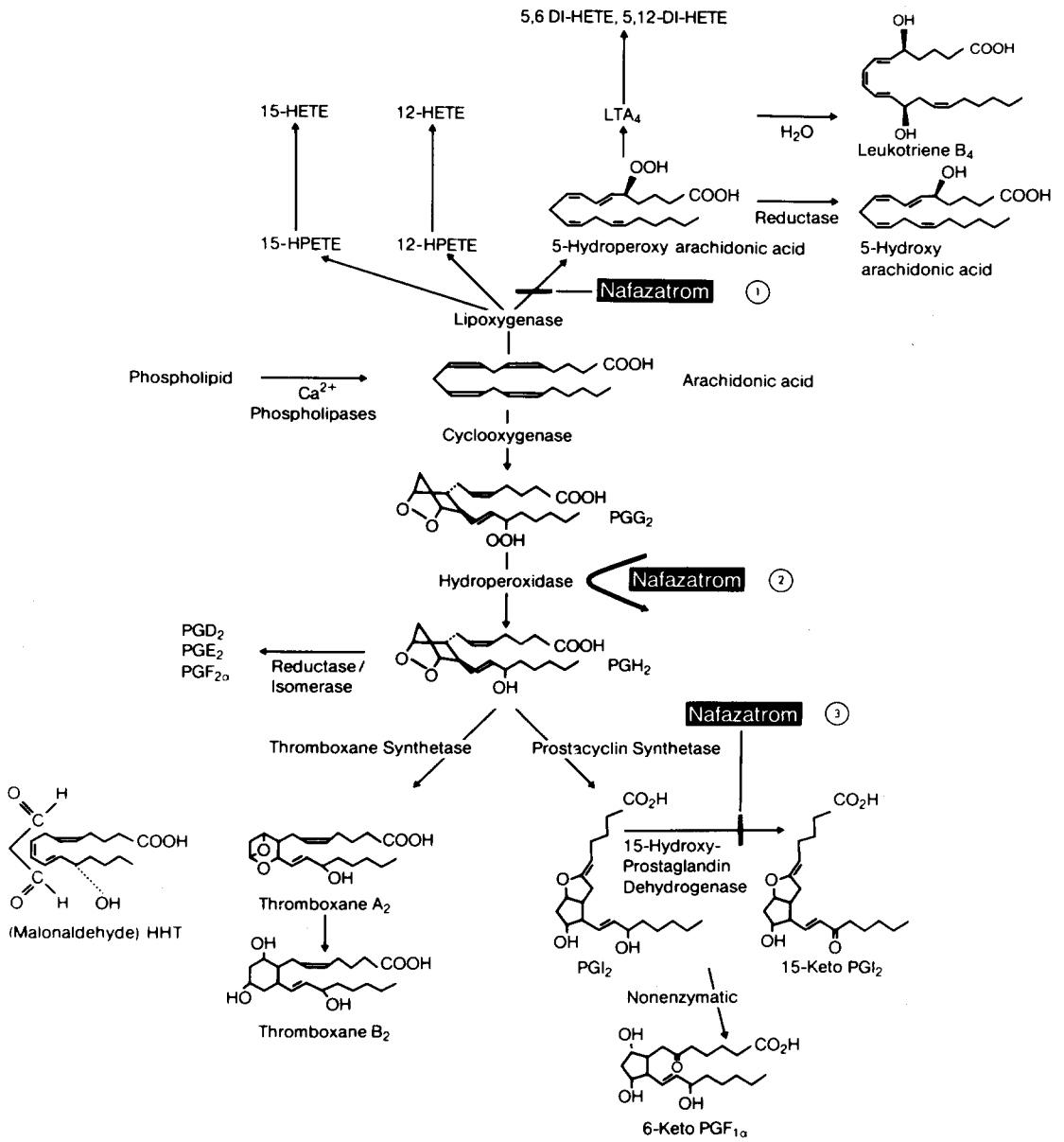


Fig. 1. Arachidonic acid metabolism via the lipoxygenase pathway resulting in hydroperoxy fatty acids and leukotrienes and via the prostaglandin endoperoxide synthetase (cyclooxygenase + hydroperoxidase) pathway resulting in prostaglandins (including prostacyclin) and thromboxanes. Possible sites of action of nafazatrom include: (1) inhibition of lipoxygenase activity; (2) reducing cofactor for the hydroperoxidase of prostaglandin endoperoxide synthetase; and (3) inhibition of prostacyclin ($PGI_2$) degradation by 15-hydroxy-prostaglandin dehydrogenase.

role in the subsequent biosynthesis of prostaglandins and thromboxanes (Fig. 1). In 1975, Hamberg *et al.* [59] demonstrated the formation of $TXA_2$ from $PGH_2$. Thromboxane $A_2$ was shown to have the half-life and biological profile of rabbit aorta contracting substance. Subsequently, platelet $TXA_2$ biosynthesis was found to be stimulated by numerous aggregating agents and was believed to be an absolute requirement for platelet aggregation [50]. This view has been challenged recently by the observations that in some cases the endoperoxide $PGH_2$ can initiate platelet aggregation independent of its conversion to $TXA_2$ [60]. In addition, Benveniste and co-workers [61, 62] have demonstrated that 1-*O*-

alkyl-2-*O*-acetyl-2sn-glyceryl-3-phosphorylcholine (platelet-activating factor, PAF-acether) can initiate platelet aggregation independent of $TXA_2$ production. Low doses of thrombin can initiate platelet aggregation apparently in the absence of $TXA_2$ biosynthesis. Presumably, PAF can function as an alternate mediator of platelet aggregation [56]. It is not clear whether or not all aggregating stimuli can utilize the $TXA_2$ and/or the PAF pathway. As work in this new area continues, stimuli may be discovered which discriminate in their aggregation mechanism utilizing $TXA_2$ and/or PAF.

One year following the discovery of $TXA_2$, Vane and co-workers [63] discovered $PGI_2$ as a transformation product of prostaglandin endoperoxides by a microsomal fraction of pig aorta. Prostacyclin is produced by vascular tissue of all species so far tested [53] and is the main product of arachidonic acid metabolism in isolated vascular tissue. Prostacyclin is the most potent endogenous inhibitor of platelet aggregation yet discovered, being 30–40 times more potent than $PGE_1$ [64] and 1000 times more potent than adenosine [65]. In addition, $PGI_2$ can reverse secondary platelet aggregation *in vitro* [66] and in circulation in man [67]. It has been suggested that $PGI_2$ and $TXA_2$ play an antagonistic and pivotal role in the control of thrombosis centered upon their bidirectional ($PGI_2$ increases, $TXA_2$ decreases) effect on platelet cAMP levels.

Gryglewski *et al.* [68] and Moncada *et al.* [69] have proposed the attractive hypothesis that $PGI_2$ is a circulating hormone continuously released from the lung. The isolated, perfused rat lung releases both $PGI_2$ and $TXA_2$ in a ratio of 5:1 [70]. Prostacyclin release increases in response to platelets or a platelet membrane suspension, an effect which is inhibited in aspirin-treated lungs [71]. These findings and the observation of Wynalda and Fitzpatrick [72] who reported that albumins of several mammalian species significantly prolong the half-life of $PGI_2$ lend credence to the above hypothesis. Nevertheless, this hypothesis has been challenged recently [73–76]. Bioassay of cAMP generation from platelets prelabeled with $^3H$-labeled adenine nucleotides in response to freshly drawn rabbit arterial blood revealed insufficient $PGI_2$ levels to affect platelet function [74]. Physicochemical analysis of human arterial blood $PGI_2$ [75] or the estimation of the rate of $PGI_2$ secretion measured as urinary metabolites [76] produced similar conclusions. Nevertheless, convincing studies report that $PGI_2$ is stabilized by human plasma and whole blood [77, 78]. In addition, rat stomach fundus generated $PGI_2$ can be stabilized by human serum [79]. The protective effect on $PGI_2$-like activity by human plasma seems directed at the $PGI_2$ molecule itself and is not the result of an enzymatic mechanism, i.e. formation of the antiaggregatory agent, 6-keto-$PGF_{1\alpha}$ [80]. Therefore, the biological importance of circulating $PGI_2$ in normal and pathophysiological circumstances remains unresolved. Nevertheless, $PGI_2$ does appear to limit platelet adhesion to the endothelium and subendothelium [81, 82]. The concentration of $PGI_2$ needed to inhibit the primary process of platelet adhesion is, however, higher than the concentration required to inhibit subsequent thrombi formation.

## PROSTACYCLIN, THROMBOXANES AND TUMOR CELL METASTASIS

Honn and co-workers [19, 83] proposed the working hypothesis that the primary tumor, tumor cell shed vesicles, and/or circulating tumor cells disrupt the intravascular balance between $PGI_2$ and $TXA_2$ in favor of platelet aggregation. Based upon the assumptions that platelet–tumor cell and/or platelet–tumor cell–vessel wall interactions are important for tumor cell metastasis, we proposed that (1) the exogenous administration of $PGI_2$ should reduce lung colony formation by tail vein-injected tumor cells, (2) a therapeutic synergism should result from the use of $PGI_2$ with a phosphodiesterase inhibitor (since the effect of $PGI_2$ is mediated by increasing concentrations of cAMP in platelets, it follows that phosphodiesterase inhibitors, by slowing the breakdown of cAMP, should potentiate the antithrombogenic action of $PGI_2$ and thus the antimetastatic effect), (3) prostacyclin acts as a natural deterrent to tumor cell metastasis; therefore, an inhibitor of endogenous $PGI_2$ biosynthesis should enhance metastasis, (4) agents that augment *in vivo* $PGI_2$ biosynthesis or activity should function as antimetastatic agents, and (5) thromboxane synthetase inhibitors should also possess antimetastatic activity.

*In vitro*, the addition of elutriated B16 amelanotic melanoma (B16a), Lewis lung carcinoma (3LL) or Walker 256 carcinosarcoma (W256) cells to human platelets results in aggregation accompanied by a concomitant rise in platelet-generated $TXA_2$ (measured as $TXB_2$ by radioimmunoassay) and release of [$^{14}C$]serotonin from prelabeled platelets ([84] and unpublished results). All of these tumor cell-induced events (platelet aggregation, $TXA_2$ biosynthesis, and the release reaction) can be inhibited by thromboxane synthetase inhibitors ([84] and unpublished results). Similarly, tumor cell-induced platelet aggregation is inhibited by $PGI_2$ at doses as low as 10 ng/ml (Fig. 2). The addition of $PGI_2$ (10–100 ng/ml) to platelets aggregating in response to W256 cells (at 50% of maximum response) immediately arrests this process and subsequently dissolves the tumor cell–platelet aggregates (unpublished results) consistent with the ability of $PGI_2$ to reverse secondary platelet aggregation [66, 67]. These *in vitro* observations may explain the ability of $PGI_2$ to dislodge tumor cells trapped in the lung after i.v. injection into mice (see below).

We have reported previously that bolus i.v. injection of $PGI_2$ into mice reduces lung colony formation from tail vein-injected B16a cells by greater than 70% [19]. The combination of $PGI_2$ with a phosphodiesterase inhibitor (theophylline) further inhibits metastasis to greater than 93% ([19, 85] and Table 1). Prostaglandins $E_2$, $F_{2\alpha}$ and the stable hydrolysis product of $PGI_2$, 6-keto-$PGF_{1\alpha}$, are ineffective in reducing metastasis (Table 1). Prostaglandin $D_2$ is also antimetastatic as reported by Stringfellow and Fitzpatrick [47]; however, it is less than one-third as effective as $PGI_2$ ([19] and Table 1). The antimetastatic effects of $PGI_2$ are not due to its pulmonary vasodilatory effects ([19] and unpublished results). In this regard, $PGI_2$ does not significantly alter tumor

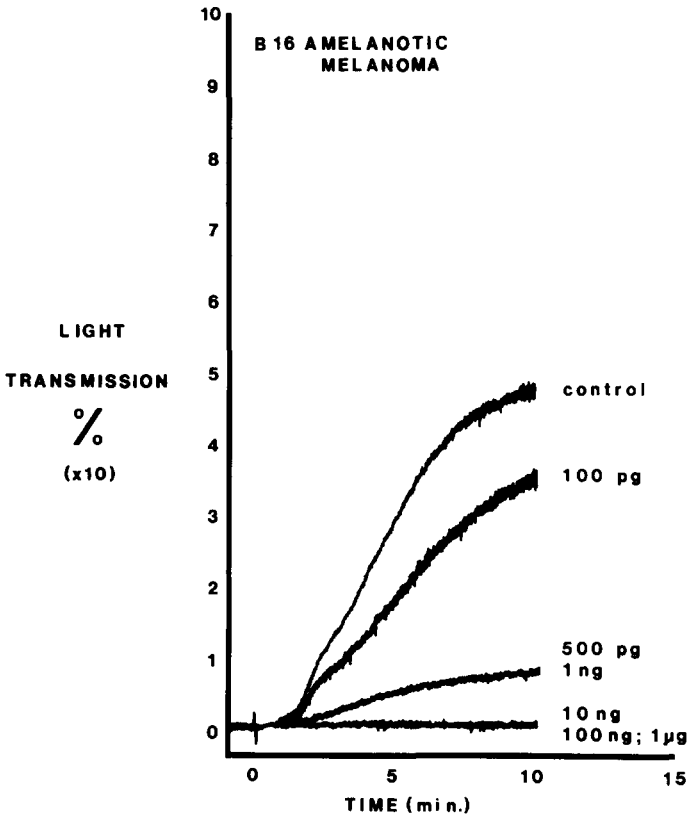


Fig. 2. Inhibition of B16a-induced human platelet aggregation by $PGI_2$. Prostacyclin was added in 2 µl of glycine buffer (pH 10.5) to 250 µl of human platelet rich plasma ($2.8 \times 10^8$ platelets/ml) 1 min prior to the addition of $2.2 \times 10^6$ B16a cells. Aggregation was monitored with a Sienco dual channel aggregometer.

cell distribution patterns. Using [$^{125}$I]deoxyuridine-labeled tumor cells, we demonstrated [19] that, although some alteration of cell distribution may occur, these cells once released from the lung are not retained in the organ of secondary arrest (liver, spleen, etc.). In addition, there is no effect of $PGI_2$ on the initial entrapment of tumor cells in the lung following i.v. injection [19, 85] which might be expected if the $PGI_2$ effect were due to vasodilation. The lack of a $PGI_2$ effect on initial tumor cell arrest raises the question of whether tumor cell-induced platelet aggregation occurs while the tumor cell is in circulation or alternatively whether upon arrest and attachment to the endothelium the critical aggregation event occurs. It is clear from our studies that $PGI_2$ significantly increases organ clearance of tumor cells [85]. Marcum *et al.* [86] utilized rabbit aortic segments in a standard Baumgartner perfusion chamber to study tumor cell (HUT 20)–platelet–endothelial interactions. Tumor cells and platelets were perfused in the presence of $PGE_1$ (30 µg/ml) or $PGI_2$ (50 ng/ml). Both agents totally inhibited tumor cell-induced platelet aggregation and the deposition of both tumor cells and platelets on the vascular surface. Although the above studies demonstrate a role for platelets in metastasis, the question of whether the critical platelet aggregation occurs prior to, or post, tumor cell arrest has not been established definitively.

The time frame for $PGI_2$ effectiveness is not limited to events immediately preceding or coincident with tumor cell arrest [85]. Since $PGI_2$ has been reported to reverse secondary platelet aggregation [66, 67], it is possible that its effectiveness in reducing tumor metastasis may extend into a critical period after tumor cell arrest but before extravasation. The period until extravasation is complete is variable with tumor type but is generally considered to be on the order of hours instead of minutes [87]. Therefore, we examined the effects of post-administration of $PGI_2$ on the retention of [$^{125}$I]deoxyuridine-labeled B16a cells. Prostacyclin was administered either 15 min prior to tumor cell injection or 1 hr post tumor cell injection. Animals were then killed 3, 8 and 20 hr post tumor cell injection, and the lungs were removed and counted. Both treatment regimens were equally effective in reducing tumor cell retention in the lung and subsequent tumor colony formation [85], indicating that arrested, intravascular, tumor cells can be dislodged by $PGI_2$ presumably, but possibly not exclusively, due to disaggregation of adherent platelet–tumor cell thrombi.

The above results with exogenous $PGI_2$ demonstrate its efficacy as an antimetastatic agent. We have

Table 1. Effects of $PGI_2$ on pulmonary and extrapulmonary metastasis following injection of B16a melanoma cells*

| Treatment | Macroscopic tumor colonies (mean ± S.E.M.; N = 7) Lungs | Liver | Kidney | Spleen | Brain |
|---|---|---|---|---|---|
| MEM control | 144 ± 18† | 13 ± 5 | ND‡ | 1 ± 0.5 | ND |
| Tris control | 130 ± 9 | 8 ± 3 | ND | 3 ± 1 | ND |
| Ethanol control | 127 ± 14 | 9 ± 5 | ND | ND | ND |
| $PGI_2$ (25 μg) | 95 ± 10 | 2 ± 0.5 | ND | ND | ND |
| $PGI_2$ (50 μg) | 77 ± 5 | ND | ND | ND | ND |
| $PGI_2$ (100 μg) | 43 ± 13 | ND | ND | ND | ND |
| $PGI_2$ (200 μg) | 15 ± 7 | ND | ND | ND | ND |
| 6-Keto-$PGF_{1\alpha}$ (50 μg) | 138 ± 15 | ND | ND | ND | ND |
| 6-Keto-$PGF_{1\alpha}$ (200 μg) | 100 ± 14 | 3 ± 1 | ND | ND | ND |
| Theophylline (100 μg) + $PGI_2$ (100 μg) | 10 ± 5 | ND | ND | ND | ND |
| Theophylline (100 μg) | 119 ± 8 | 7 ± 3 | ND | ND | ND |
| $PGE_2$ (100 μg) | 139 ± 39 | 13 ± 6 | ND | ND | ND |
| $PGF_{2\alpha}$ (100 μg) | 121 ± 15 | 7 ± 3 | ND | 2 ± 0.5 | ND |
| $PGD_2$ (100 μg) | 102 ± 8 | ND | ND | ND | ND |
| 15-HPETE§ (100 μg) | 380 ± 40 | 60 ± 21 | ND | 17 ± 6 | ND |
| 15-HPETE (200 μg) | >500 | 85 ± 33 | ND | 22 ± 8 | ND |
| 15-HPETE + theophylline (100 μg) + $PGI_2$ (100 μg) | 39 ± 16 | 3 ± 2 | ND | ND | ND |

* Part of this table was published in Honn *et al.*, *Science* **212**, 1270 (1981). Copyright 1981 by the American Association for the Advancement of Science.

† Number of metastatic colonies on lung surface (bilateral).

‡ No detectable tumor colonies observed with Bausch and Lomb stereo zoom microscope.

§ 15-Hydroperoxyeicosatetraenoic acid (15-HPETE) was synthesized from arachidonic acid with soybean lipoxygenase and purified by silicic acid column chromatography.

also proposed that the production of $PGI_2$ by the vascular endothelium is a natural deterrent to metastasis [19]. To test this hypothesis we perfused mice with a lipoxygenase product of arachidonic acid [15-hydroperoxyeicosatetraenoic acid (15-HPETE); Fig. 1] prior to tail vein injection of B16a tumor cells. Hydroperoxy fatty acids in general are potent inhibitors of prostacyclin synthetase [88]. The production of these lipoxygenase products by endothelial cells has been suggested as a natural feedback inhibitor of $PGI_2$ synthesis [89]. Trapped blood elements within the decidua of the pregnant rat uterus generate lipoxygenase-derived HPETEs which inhibit myometrial $PGI_2$ biosynthesis [90], suggesting that HPETEs could regulate $PGI_2$ production in normal as well as pathological conditions. Mice pretreated with 15-HPETE develop 300–500% more metastatic lesions than untreated mice ([19, 91] and Table 1). The administration of $PGI_2$ following 15-HPETE treatment reduces metastatic tumor colony formation (Table 1). Collectively, these results suggest a role for endogenous $PGI_2$ production in the inhibition of tumor metastasis.

Recent work from Donati and co-workers [92] suggests another exciting possibility for $PGI_2$ control of metastasis. They examined arachidonic acid metabolites produced by several sublines developed from the murine FS6 sarcoma [93]. These cell lines were reported to produce $PGI_2$, $TXA_2$, $PGD_2$, $PGE_2$ and $PGF_{2\alpha}$ [92]. In addition, these lines differed widely in their abilities to successfully metastasize. A comparison between arachidonic acid metabolites and metastatic potential revealed an inverse correlation between tumor cell $PGI_2$ production and metastatic potential. Thromboxane $A_2$ production was elevated in the highly metastatic cells [92]. These authors suggested that the balance between $PGI_2$ and $TXA_2$ was shifted towards a proaggregatory condition in the highly metastatic cells [92]. We speculate that treatment of the highly metastatic lines with compounds which enhance $PGI_2$ production (e.g. nafazatrom, see below) would reduce their colonizing abilities. This $PGI_2$ effect could be due to decreasing the ability of tumor cells to aggregate platelets or decreasing tumor cell adhesiveness to vascular endothelium as suggested by Fantone *et al.* [94]. A new proposal for the prevention of tumor metastasis by $PGI_2$ is the use of agents which stimulate endogenous $PGI_2$ biosynthesis or prolong its half-life. One such compound which may represent the prototype of such a new class of pharmacologically active agents is nafazatrom (Bay g 6575; 2,4-dihydro-5-methyl-2-[2-(2-napthyloxy)ethyl]-3*H*-pyrazol-3-one*). Nafazatrom has been reported to

* L. J. Marnett, P. H. Siedlik, R. Ochs, K. V. Honn, R. Warnock, B. Tainer and T. E. Eling, unpublished results.

possess significant antithrombotic activity in model systems of experimental thrombosis [95]. Thrombus formation in the femoral arteries of rabbits was inhibited at a minimal effective dose of 1 mg/kg, p.o. Nafazatrom also possesses significant thrombolytic properties similar to urokinase [95]. The mechanism of action for these antithrombotic effects may be related to the ability of the drug to stimulate $PGI_2$ production by the vascular wall [96]. Nafazatrom significantly increased bioassayable $PGI_2$ release from aortic rings obtained from normal and diabetic rats [97]. In addition, plasma, obtained from human volunteers after ingestion of a single dose (1.2 g) of nafazatrom, stimulated $PGI_2$ release from slices of rat aorta [96]. Nafazatrom also stimulates the biosynthesis of $PGI_2$ from arachidonic acid by ram seminal vesicle microsomes [98 and *], cultured porcine endothelial cells and B16a tumor cells (K. V. Honn, L. J. Marnett and T. E. Eling, unpublished observations). It is unlikely that the actions of nafazatrom are due to a single biochemical effect since this compound has been found to have multiple effects on arachidonic acid metabolism (Fig. 1 and *). Nafazatrom has been found to inhibit the cytosolic lipoxygenase of B16a cells [99] as well as that of human and rabbit neutrophils [100]. Inhibition of lipoxygenase-produced HPETEs could contribute to protection of $PGI_2$ synthetase (Fig. 1). Second, nafazatrom functions as a reducing cofactor for the hydroperoxidase activity of prostaglandin endoperoxide synthetase and stimulates $PGI_2$ biosynthesis by reduction of hydroperoxy fatty acids such as $PGG_2$ (Fig. 1; [98 and *]). Finally, nafazatrom has been found to inhibit the 15-hydroxy-prostaglandin dehydrogenase catalyzed inactivation of $PGI_2$ (Fig. 1; [101]).

Nafazatrom has been evaluated for its antimetastatic activity against tail vein-injected elutriated B16a and 3LL tumor cells and found to inhibit lung colony formation by >80% (Fig. 3; [83]). In addition, this compound significantly reduces spontaneous pulmonary metastasis from subcutaneous B16a and 3LL tumors [83]. Collectively, these results point to significant antimetastatic properties of this $PGI_2$ enhancing agent.

Finally, we have proposed that alteration of platelet thromboxane levels with selective thromboxane synthetase inhibitors would prevent tumor cell metastasis. We have reported that a series of endoperoxide analogues [102] was effective in reducing metastasis from tail vein-injected elutriated B16a cells [85]. In addition, it was determined that these antimetastatic effects were not peculiar to the endoperoxide structure. A structurally unrelated thromboxane synthetase inhibitor also prevented metastasis [85]. Unlike $PGI_2$, thromboxane synthetase inhibitors are not effective when administered post tumor cell injection [85].

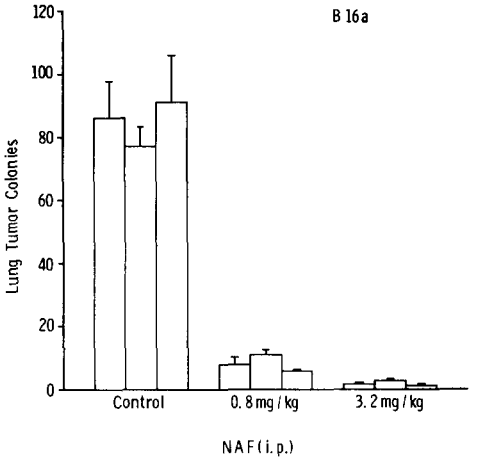


Fig. 3. Nafazatrom (NAF) inhibition of lung tumor colony formation following tail vein injection of elutriated B16a cells. NAF was administered 1 hr prior to tumor cells by intraperitoneal injection at doses of 0.8 and 3.2 mg/kg. Each bar represents results of a single experiment expressed as mean ± S.E.M.; N = 10.

## RELATIONSHIP OF CATHEPSIN B TO $TXA_2$-DEPENDENT PLATELET AGGREGATION INDUCED BY TUMOR CELLS

Lysosomal proteinases such as cathepsin B, a cysteine proteinase, could act at several steps in the metastatic cascade and have been implicated in the ability of tumor cells to invade normal tissue at primary and secondary or metastatic sites [103]. In this regard, cathepsin B can degrade two major components of the extracellular matrix, proteoglycans [104] and native collagen [105]. Cathepsin B may also act as a regulatory enzyme in collagen degradation by activating procollagenase [106]. Since cathepsin B can degrade pericellular proteins at pH 7.1 [107], this enzyme might also be responsible for modifications in the cell surface of tumor cells which enhance the detachment of tumor cells from a solid primary tumor, modify the adherence of tumor cells to glass or plastic, and prevent contact-inhibition of growth in tissue culture. Cathepsin B may also act in the metastatic cascade by mediating tumor cell-induced platelet aggregation.

Of particular relevance to metastasis is the increasing body of literature on release of cathepsin B from human and animal tumors. Poole and co-workers [108, 109] have shown that up to eleven times more cathepsin B is released from malignant human breast tumors than from normal breast tissue or nonmalignant tumors. In contrast, release of a lysosomal aspartic proteinase, cathepsin D, did not differ among the three breast tissues. Other groups have reported elevation of cathepsin B levels in pancreatic fluid of patients with pancreatic cancer [110] and in serum of women with diverse invasive neoplastic diseases [111, 112] including vaginal adenocarcinomas [113]. We have shown that tumor cells isolated from solid murine tumors and grown in monolayer culture will release cathepsin B into the culture medium [114, 115].

The suggestion that elevated lysosomal enzyme activities in tumors may be due to the presence of necrotic tumor cells or of invading macrophages [116, 117] does not appear to be valid for elevated cathepsin B activities. As early as 1957, Sylven and Malmgren [118] reported that the youngest and most

* L. J. Marnett, P. H. Siedlik, R. Ochs, K. V. Honn, R. Warnock, B. Tainer and T. E. Eling, unpublished results.

rapidly growing tumors had the highest cathepsin B activity. However, solid tumors contain a multiplicity of cell types (macrophages, lymphocytes and stromal cells as well as tumor cells), any of which could account for cathepsin B activity. By using the technique of centrifugal elutriation [119, 120] to separate tumor cells from other cell types dispersed from a solid tumor, we were able to demonstrate definitively that cathepsin B activity in metastatic variants of the murine B16 melanoma is a property of the viable tumor cells [121]. Eighty to ninety percent of the total cathepsin B activity (based on DNA) in dispersed and elutriated cells from solid tumors of B16 metastatic variants [114], B16 amelanotic melanoma, Lewis lung carcinoma and Walker 256 adenocarcinoma (unpublished observations) is found in the viable tumor cells. We also demonstrated that cathepsin B activity correlates positively with increased metastatic potential [114, 121]. In contrast, only 50% of cathepsin D activity is found in the viable tumor cells and the activity does not correlate with metastatic potential of the variants tested [114].

Tumor cells seem to have both a platelet aggregating activity [6, 30, 34, 122] and a procoagulant activity [123, 124]. Gordon and co-workers [123, 124] have demonstrated that the procoagulant activity of tumor cells may be a cysteine proteinase. Platelets can be stimulated to aggregate *in vitro* by addition of such exogenous proteinases as the plant cysteine proteinase, papain [125]. Recent work in our laboratories has demonstrated that one principle responsible for tumor-cell-induced platelet aggregation is cathepsin B [126]. Proteinase inhibitors of varying specificity for cysteine and serine proteinases were tested for their abilities to inhibit B16a-induced aggregation of washed human platelets. The most effective inhibitors are leupeptin, antipain and iodoacetic acid (IAA); the least effective are soybean trypsin inhibitor (SBTI) and aprotinin (Fig. 4; [126]). The abilities of the proteinase inhibitors to reduce platelet aggregation paralleled their abilities to inhibit cathepsin B activity in the B16a tumor cells (Fig. 5).

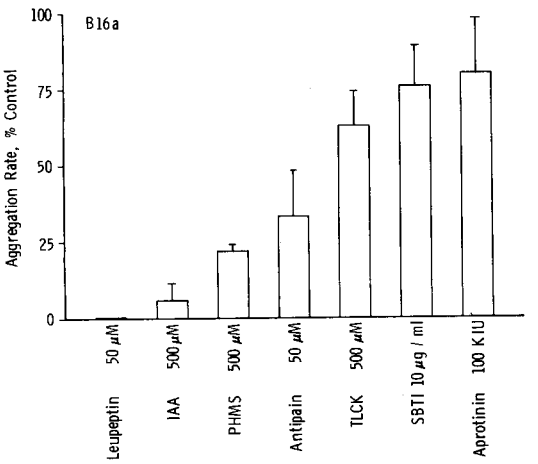


Fig. 4. Effect of proteinase inhibitors on platelet aggregation induced by elutriated B16a tumor cells. Platelet aggregation is expressed as percentage of control aggregation rate with 20,000 B16a tumor cells. Abbreviations: TLCK, *N*-α-*p*-tosyl-L-lysinechloromethylketone; PHMS, *p*-hydroxy-mercuriphenylsulfonate; IAA, iodoacetic acid; and SBTI, soybean trypsin inhibitor.

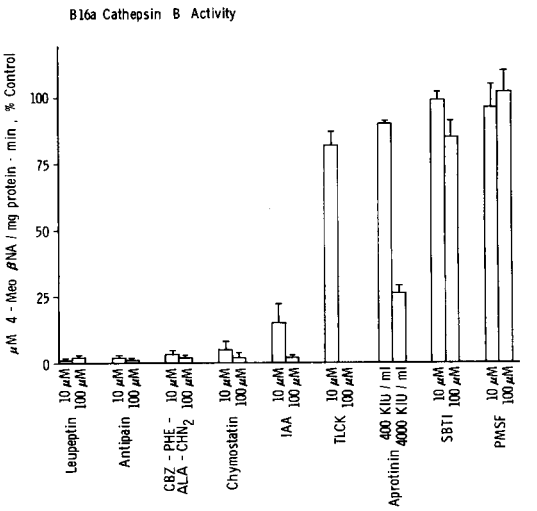


Fig. 5. Effects of proteinase inhibitors on cathepsin B activity in homogenate of B16a tumor cells. Cathepsin B activity is expressed as μM 4-methoxy-β-napthylamine formed per mg protein per min as percentage of control activity ($2.3 \times 10^5$ tumor cells). Abbreviations: IAA, iodoacetic acid; SBTI, soybean trypsin inhibitor; CBZ-Phe-Ala-$CHN_2$, a peptidyl diazomethylketone specific for cathepsin B at $10^{-6}$ M (CBZ = carbobenzyloxy, and $CHN_2$ = diazomethylketone); and PMSF, phenylmethylsulfonyl fluoride.

To mimick the proaggregatory activity of cathepsin B, we utilized papain, a commercially available cysteine proteinase [126]. Papain has been shown to have an extremely high sequence homology to cathepsin B. Ten residues surrounding the active site cysteine in the light chain are identical and seven residues forming the active site groove in the heavy chain are identical [127]. Addition of papain (0.02 units) to washed human platelets induced aggregation. As with the B16a tumor cells, the most effective proteinase inhibitors against both papain-induced platelet aggregation and papain-induced activity in a specific fluorometric assay for cathepsin B were leupeptin and antipain (0.5 to 1.0 μM; 99 ± 1% inhibition). The least effective were SBTI (10–100 μg/ml; 0 ± 0% inhibition) and aprotinin (400–1000 kI.U.; 0 ± 0% inhibition). Preliminary results suggest that cathepsin B may also induce platelet aggregation by Lewis lung carcinoma cells and by 15091A mammary adenocarcinoma cells [25] obtained from Dr. G. J. Gasic (unpublished observations).

To discriminate in B16a tumor cells between three known lysosomal cysteine proteinases (cathepsins B, H and L), we assayed for cathepsin H activity using a selective substrate [128]. Cathepsin H activity in the B16a tumor cells was 20-fold less than cathepsin B activity [126]. To discriminate between cathepsins B and L we utilized new specific inhibitors [129, 130] of cysteine proteinases, peptidyl diazomethylketones. All of our results indicate that the major lysosomal cysteine proteinase activity in B16a tumor cells is cathepsin B and that cathepsin B induces aggregation of platelets by the B16a tumor cells.

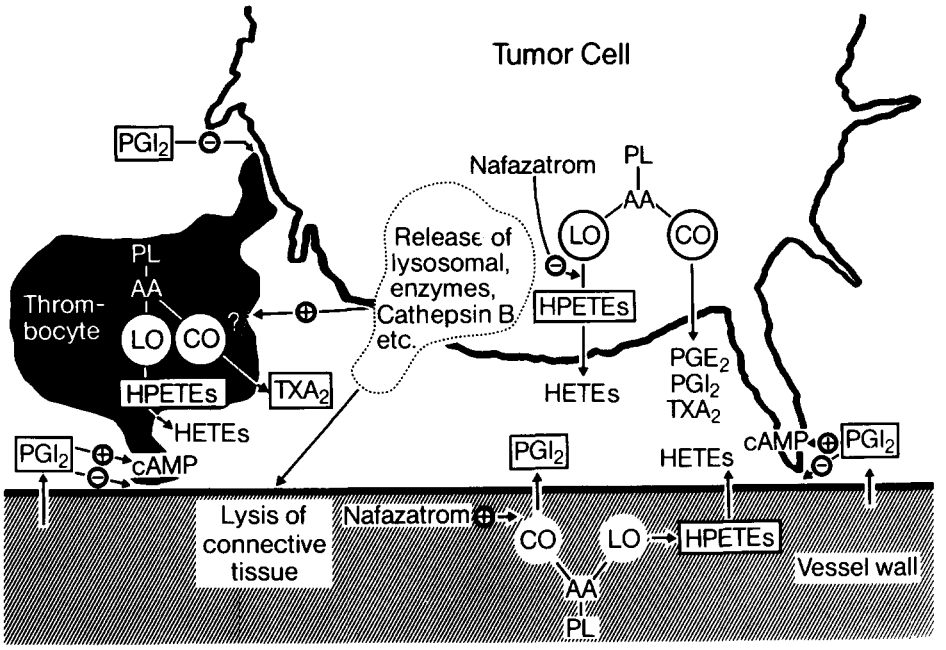


Fig. 6. Interactions among tumor cells, platelets (thrombocytes) and blood vessel wall. Direct interactions are possible between the platelet and the vessel wall, the tumor cell and vessel wall, and the platelet(s) and tumor cell(s). Any one or all of the above may enhance tumor cell arrest and metastasis. Prostacyclin may prevent attachment of tumor cell(s) to platelet(s) or to vessel wall. Abbreviations: PL, phospholipids; AA, arachidonic acid; CO, cyclooxygenase; LO, lipoxygenase; HPETEs, hydroperoxyeicosatetraenoic acids; HETEs, hydroxyeicosatetraenoic acids; $PGI_2$, prostacyclin; and $TXA_2$, thromboxane $A_2$

B16a-induced aggregation of platelets resulted in production of $TXB_2$ [84] and could be inhibited by $PGI_2$ (Fig. 2) and by leupeptin (unpublished results). Using papain to mimick the activity of cathepsin B, we have demonstrated that papain (0.05 units)-induced platelet aggregation also occurs concomitantly with production of $TXB_2$ (unpublished observations). Thromboxane synthetase inhibitors [102] prevented both generation of $TXB_2$ and platelet aggregation in response to papain (unpublished observations). $PGI_2$ inhibited papain-induced platelet aggregation with an $ED_{50}$ of 18 ng/ml [126]. The ability of $PGI_2$ to act as an antimetastatic agent may be linked to its ability to inhibit tumor cell cathepsin B-induced platelet aggregation.

## HYPOTHESIS

It is intuitive that for hematogenous metastasis to occur the tumor cell must arrest in the microvasculature and attach to the vessel wall prior to extravasation and growth into a metastatic foci. Considerable yet indirect evidence supports the concept that tumor cells interact with platelets during this process as discussed above. We propose that the metabolism of arachidonic acid in the tumor cell, the platelet and the vessel wall plays an essential role in the sum total of these interactions (Fig. 6). For example, the production of $PGI_2$ by the vessel wall and of $TXA_2$ by the platelet is thought to play a key (although possibly not an exclusive) role in thrombosis.

We propose that exogenous $PGI_2$ functions as an antimetastatic agent by inhibiting the association of the tumor cell with the platelet (thrombocyte), of the platelet with the vessel wall, and/or of the tumor cell with the vessel wall (Fig. 6). The interactions between the vessel wall and tumor cells or platelets may be inhibited by exogenous $PGI_2$ stimulating platelet cAMP or tumor cell cAMP [94]. Similarly, vessel wall $PGI_2$ serves as a natural deterrent to metastasis by inhibiting these interactions (Fig. 6). Compounds which have the ability to stimulate endogenous $PGI_2$ production and/or prolong the half-life of $PGI_2$ (e.g. nafazatrom) are proposed as a new class of antimetastatic agents.

We further postulate that one tumor cell platelet aggregating principle is an isoenzyme of the cysteine proteinase cathepsin B. Possible problems with the hypothesis that tumor cell-derived cathepsin B plays a role in tumor cell-induced platelet aggregation are the pH optimum and stability of cathepsin B and the presence in serum of $\alpha_2$-macroglobulin. Poole and co-workers [108, 109, 131] have shown repeatedly that tumor-derived cathepsin B is a higher molecular weight isoenzyme of liver cathepsin B which retains activity at alkaline pH [132]. Although $\alpha_2$-macroglobulin is an inhibitor of all classes of proteinases, its inhibition is incomplete in that cathepsin B retains activity against substrates of low molecular weight [133]. The sites of action of cathepsin B are still unknown, but our results suggest that tumor cell cathepsin B can induce aggregation of washed human platelets by a mechanism dependent on generation of $TXA_2$ and inhibited by selective TX synthetase inhibitors, $PGI_2$ and leupeptin. The above hypotheses (and results) suggest that selective manipulation of the arachidonic acid cascade could be used in the control of hematogenous tumor metastasis.

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