spreading depression can disclose the functional state of a brain.

The use of the multiple organ recording was started when Chance *et al.* (*1*) monitored NADH fluorescence in brain and kidney simultaneously. This approach was essential in the initial studies of organ anoxia by the fluorescence method. Our present study expands their idea and gives a strong justification to the use of surface fluorometry–reflectometry.

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Tumor Cell–Platelet Aggregation: Induced by Cathepsin B–Like Proteinase and Inhibited by Prostacyclin

Abstract. *The ability of tumor cells to metastasize may be related to their ability to promote aggregation of host platelets. The use of inhibitors of cysteine proteinases resulted in parallel inhibition of B16 amelanotic melanoma–induced platelet aggregation and of cathepsin B activity. The antimetastatic agent prostacyclin inhibited platelet aggregation induced by the tumor cells and by papain, a cathepsin B–mimicking agent.*

Abnormalities in blood coagulation are common in patients with advanced malignant disease (*1*). Tumor cells have platelet aggregating activity (*2*) as well as procoagulant activity (*3*). A tumor cell procoagulant activity responsible for alterations in the fibrin-fibrinogen system was shown to result from a cysteine proteinase (*3*). The ability of tumor cells to enhance aggregation of platelets in vitro correlates positively with their metastatic potential in vivo (*2*), and manipulation of host platelet levels by induced thrombocytopenia decreases metastatic tumor colony formation in some systems (*2*). Prostacyclin (PGI_2), the most potent agent known for preventing platelet aggregation, significantly inhibits metastasis of the murine B16 amelanotic melanoma (B16a) (*4*).

Local proteolysis by proteinases released into the extracellular matrix of tumors has been implicated in tumor invasiveness and metastasis (*5*). A cathepsin B–like cysteine proteinase has been shown to be released from tumors (*6, 7*), and cathepsin B levels are elevated in the serum of patients with several varieties of cancer (*7*). We have found that tumor cell levels of a cathepsin B–like cysteine proteinase correlate positively with the metastatic potential of the B16 melanotic melanoma (*8*) and that both the B16a and the B16 melanotic melanoma in culture release cathepsin B into the media (*8, 9*). We now describe studies designed to determine whether a tumor cell–derived cathepsin B–like cysteine proteinase is responsible for B16a tumor cell–induced platelet aggregation and whether this effect can be inhibited by PGI_2.

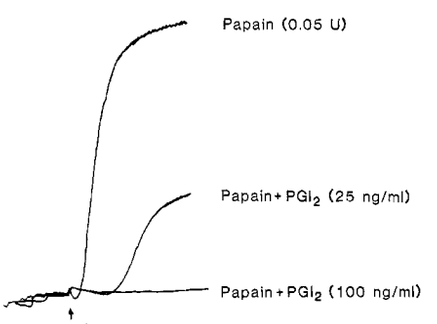

Fig. 1. Inhibition of papain-induced aggregation of washed human platelets by prostacyclin. Aggregometer tracings of human platelet aggregation. The upward deflection of the pen indicates an increase in light transmission due to platelet aggregation. The time scale from the arrow to the end of the tracing is 5 minutes. Washed platelets were prepared from human platelet-rich plasma by the method of Hamberg *et al.* (*21*). Platelets were counted (Coulter counter, model ZBI), and the final count was adjusted to $5.0 \pm 0.5 \times 10^8$ platelets per milliliter. To the aggregometer cuvette were added 250 μl of the washed platelet suspension, 5 μl of 0.1*M* $CaCl_2$, and 5 μl of PGI_2 or its diluent (0.025*M* glycine and 0.025*M* NaCl, *p*H 10.5). One minute after addition of PGI_2 or diluent, 5 μl of papain was added, as indicated by the arrow. The aggregation was determined at 37°C with a dual-channel aggregometer (Sienco) at a stirring speed of 800 rev/min. Prostacyclin (100 ng/ml) completely inhibited papain-induced aggregation.

Subcutaneous B16a tumors were maintained in syngeneic C57BL/6J mice (Jackson Laboratory). Tumors were disaggregated, and the tumor cells were purified by centrifugal elutriation (*8*). Elutriated tumor cells (>90 percent viability) were used for platelet aggregation studies or were homogenized and assayed fluorometrically for cathepsin B activity and protein (*8*). Cathepsin H was assayed with L-Arg-4-methoxy-β-naphthylamine (L-Arg-4-OMe-β-NA) (Arg, arginine) or L-Arg-aminofluorocoumarin (L-Arg-AFC; Enzyme Systems Products, Livermore, California) as substrate, at *p*H 6.8 (*10*). Enzyme assays were standardized to ensure linearity.

Proteinase inhibitors of varying specificity for both cysteine and serine proteinases (*11*) were tested for their ability to inhibit B16a-induced aggregation of washed human platelets. The most effective inhibitors of B16a-induced aggregation of washed human platelets were leupeptin and antipain (Sigma) (Table 1). Umezawa (*12*) showed that leupeptin inhibits the cysteine proteinase activity of cathepsin B and papain at a median inhibitory dose (ID_{50}) of 1 μ*M*; the ID_{50} for inhibition of thrombin activity is 20 m*M*. Iodoacetic acid (IAA; Sigma) and *p*-hydroxymercuriphenylsulfonate (PHMS; Sigma), specific inhibitors of cysteine proteinases, both inhibited B16a-induced platelet aggregation. *N*-

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α-*p*-Tosyl-L-Lys-chloromethyl ketone (Lys, lysine) (TLCK; Sigma) a serine proteinase inhibitor with activity against cysteine proteinases, partially blocked aggregation. However, the serine proteinase inhibitors, soybean trypsin inhibitor (SBTI; Worthington) and aprotinin (Bayer AG, West Germany) were not effective against B16a-induced aggregation.

Papain, a plant cysteine proteinase, has an extremely high sequence homology to cathepsin B, especially at its active site (*13*). The ten residues surrounding the active-site cysteine in the light chain of papain and cathepsin B are identical, and the seven residues forming the active site groove in the heavy chain are identical (*13*). The proteolytic activities of cathepsin B closely resemble those of papain (*14*). Since purified papain is commercially available, but cathepsin B is not, we used papain (Sigma) to mimic the action of cathepsin B. Papain (>0.02 U) induced aggregation of washed human platelets in vitro. Leupeptin, antipain, and the specific cysteine proteinase inhibitors IAA and PHMS were the most effective inhibitors of papain-induced aggregation (Table 1).

The effect of proteinase inhibitors on cysteine proteinase activity in homogenates of the B16a tumor cells and on papain activity was measured with CBZ-Arg-Arg-4-OMe-β-NA (CBZ, carbobenzyloxy) (Enzyme Systems Products), a fluorescent substrate highly specific for cathepsin B (*11*). Inhibition of enzyme activity by the proteinase inhibitors paralleled the inhibition of platelet aggregation (Table 2). Leupeptin and antipain were the most effective inhibitors of both cysteine proteinase activity and of platelet aggregation. Aprotinin and SBTI were less effective in inhibiting cysteine proteinase activity than in inhibiting platelet aggregation, suggesting that aprotinin and SBTI may have a direct effect on a serine proteinase in the platelet membrane. The organic-soluble inhibitors, chymostatin and the peptidyl diazomethyl ketones CBZ-Phe-Ala-CHN_2 and CBZ-Phe-Phe-CHN_2 (Phe, phenylalanine; Ala, alanine) (Enzyme Systems Products) (*15*), could not be tested for inhibition of platelet aggregation, since the organic solvents directly inhibited platelet aggregation. However, these inhibitors decreased the cysteine proteinase activity of both the B16a tumor cells and of papain.

The cysteine proteinase activity of the B16a tumor cells could be due to cathepsins B, H, L, or an as yet unidentified cysteine proteinase (*10, 14, 16, 17*). The other well-known cathepsins, D and G, are aspartic and serine proteinases, respectively (*11*). We used selective inhibitors of cysteine proteinases, peptidyl diazomethyl ketones (*15, 18*), which can differentiate between cathepsins B, H, and L at specific concentrations (*18*). These inhibitors do not inhibit serine proteinases and require copper ion to inhibit aspartic proteinases (*18*). At $10^{-9}M$, CBZ-Phe-Phe-CHN_2 inhibits cathepsin L, but not B, and at $10^{-6}M$, CBZ-Phe-Ala-CHN_2 preferentially inhibits cathepsin B, but not H or L (*18*). The cysteine proteinase activity of B16a was inhibited less than 20 percent by CBZ-Phe-Phe-CHN_2, but more than 95 percent by CBZ-Phe-Ala-CHN_2. These results indicate that approximately 80 percent of the B16a cysteine proteinase activity is a cathepsin B–like proteinase.

Since others have shown that proteinase substrates (>40 μ*M*) will competitively inhibit (by at least 50 percent) platelet aggregation induced by enzymatic or nonenzymatic agents (*19*), we tested the inhibitory ability of CBZ-Arg-Arg-4-OMe-β-NA, a synthetic substrate for cathepsin B. Both B16a- and papain-induced platelet aggregation were completely inhibited by 3 μ*M* substrate, whereas aggregation induced by thrombin or by adenosine diphosphate (ADP) was not inhibited by 3 μ*M* substrate. In addition, leupeptin at a dose that completely inhibited B16a-induced platelet aggregation (50 μ*M*) did not inhibit aggregation induced by thrombin (0.08 U/ml) or ADP (100 μ*M*). When B16a-induced platelet aggregation was totally blocked by leupeptin (50 μ*M*), the platelets were still able to respond to an aggregatory dose of thrombin (0.08 U/ml).

In mice, intravenous administration of PGI_2, before B16a cells are injected into the tail vein, inhibits lung colony formation by 97 percent (*4*), whereas inhibition in vivo of prostacyclin synthetase with hydroperoxy fatty acids increases metastasis (*4*). Since PGI_2 inhibits B16a-induced thromboxane A_2–dependent platelet aggregation at a median effective dose (ED_{50}) of 4 ng/ml (*20*), we tested the ability of PGI_2 to inhibit papain-induced (0.05 U) aggregation of washed human platelets. Platelet aggregation was com-

Table 1. Effect of proteinase inhibitors (listed in order of effectiveness) on platelet aggregation induced by B16a tumor cells and papain. Percentages are means ± standard errors of three experiments. KIU, Kallikrein inactivator units.

Inhibitor	Tumor cell-induced aggregation (20,000 cells)		Papain aggregation (0.05 U)	
	Concentration	Aggregation rate* (%)	Concentration	Aggregation rate (%)
None	0	100 ± 0	0	100 ± 0
Leupeptin	50 μ*M*	0 ± 0	0.5 μ*M*	0 ± 0
Antipain	50 μ*M*	33 ± 15	0.5 μ*M*	0 ± 0
IAA	500 μ*M*	6 ± 5	50 μ*M*	0 ± 0
PHMS	500 μ*M*	22 ± 2	50 μ*M*	0 ± 0
TLCK	500 μ*M*	63 ± 11	50 μ*M*	36 ± 15
SBTI	10 μg/ml	76 ± 13	100 μg/ml	100 ± 0
Aprotinin	100 KIU	80 ± 18	1000 KIU	100 ± 0

*Aggregation rate was computed as the percentage change in transmission per unit of time.

Table 2. Effect of proteinase inhibitors on cystein proteinase activity. Percentage inhibition is expressed as the mean ± standard error for three experiments run in triplicate and calculated as micromoles per liter of 4-OMe-β-NA per milligram of protein per minute, with the control activity in the absence of inhibitor taken as 0 percent inhibition. In this assay the cysteine proteinase activity of a total homogenate of 2.3×10^5 B16a tumor cells is equivalent to the cysteine proteinase activity of 0.025 U of papain; KIU, Kallikrein inactivator units.

Inhibitor	B16a tumor cells (2.3×10^5 cells)		Papain (0.2 U)		
	Concentration	Inhibition (%)	Inhibitor	Concentration	Inhibition (%)
Leupeptin	1 μ*M*	99 ± 1	Leupeptin	1 μ*M*	99 ± 1
Antipain	1 μ*M*	98 ± 1	Antipain	1 μ*M*	99 ± 1
CBZ-Phe-Ala-CHN_2	1 μ*M*	97 ± 2	Chymostatin	1 μ*M*	99 ± 1
Chymostatin	1 μ*M*	95 ± 4	CBZ-Phe-Ala-CHN_2	1 μ*M*	81 ± 1
IAA	100 μ*M*	98 ± 1	IAA	10 μ*M*	94 ± 6
CBZ-Phe-Phe-CHN_2	1 n*M*	18 ± 0	CBZ-Phe-Phe-CHN_2	1 n*M*	0 ± 0
Aprotinin	400 KIU	10 ± 1	Aprotinin	400 KIU	0 ± 0
SBTI	10 μg/ml	1 ± 3	SBTI	10 μg/ml	0 ± 0

pletely inhibited by PGI_2 at a dose of 100 ng/ml (Fig. 1). The ED_{50} for PGI_2 inhibition of papain-induced aggregation was 18 ng/ml or roughly equivalent to the ED_{50} for PGI_2 inhibition of B16a-induced aggregation (3×10^6 tumor cells per milliliter) (*20*). Neither cathepsin B activity nor papain activity was directly inhibited by PGI_2 in the fluorometric enzyme assay.

Our results indicate that cathepsin B could be one of the factors responsible for induction of platelet aggregation by tumor cells. We have previously shown that tumor cathepsin B is a property of viable tumor cells and not of host stromal elements, macrophages, or nonviable tumor cells (*8*). There is a positive correlation between cathepsin B activity and metastatic potential (*8*), as well as a positive correlation between the ability of tumor cells to promote platelet aggregation and metastatic potential (*2*). The release of cathepsin B from a variety of human and murine tumors (*6–9*) may facilitate tumor metastasis, and the ability of PGI_2 to act as an antimetastatic agent may result from its ability to inhibit tumor cell cathepsin B–induced platelet aggregation by an as yet unidentified mechanism.

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17. Cathepsin H unlike cathepsins B and L has activity against unblocked amino acid substrates as well as against blocked substrates and thus has been described as an endoaminopeptidase (*10*). Cathepsin L is an endopeptidase with strong activity against natural proteins but little against synthetic substrates (*16*). Up to 50 percent of lysosomal proteolytic activity has been ascribed to cathepsin L (*16*). In an assay of cathepsin H activity with an unblocked substrate, less than 5 percent of the B16a cysteine proteinase activity was cathepsin H when either the L-Arg-4-OMe-β-NA substrate or the L-Arg-AFC substrate, which is about 100 times more sensitive, was used [(*10*); R. Smith, personal communication].
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Inhibition of Steroid Glycoalkaloid Accumulation by Arachidonic and Eicosapentaenoic Acids in Potato

Abstract. *Eicosapentaenoic and arachidonic acids extracted from the fungus* Phytophthora infestans *elicit the accumulation of fungitoxic sesquiterpenoid stress metabolites and inhibit the accumulation of steroid glycoalkaloids in potato tubers. This dual activity, which did not occur with other saturated and unsaturated fatty acids tested, corresponds to the activity of incompatible races of* Phytophthora infestans *and crude elicitor preparations from* Phytophthora infestans *that contain bound forms eicosapentaenoic and arachidonic acids. Arachidonic acid applied to potato slices, which had been aged for various time intervals, elicited the accumulation of sesquiterpenoid stress metabolites and concomitantly inhibited the accumulation of steroid glycoalkaloids.*

The accumulation of steroid glycoalkaloids in potato tuber slices is inhibited when the tissues are inoculated with incompatible races of the late blight fungus *Phytophthora infestans* (Mont.) de Bary or treated with cell-free sonicates of either compatible or incompatible races of the fungus. Inhibition of steroid glycoalkaloid accumulation is associated with the accumulation of sesquiterpenoid stress metabolites (SSM), including rishitin and lubimin (*1–3*). Two polyunsaturated fatty acids in *P. infestans*, *cis*-5,8,11,14,17-eicosapentaenoic and *cis*-5,8,11,14-eicosatetraenoic (arachidonic), are responsible for eliciting SSM accumulation (*4*, *5*). We examined the effect

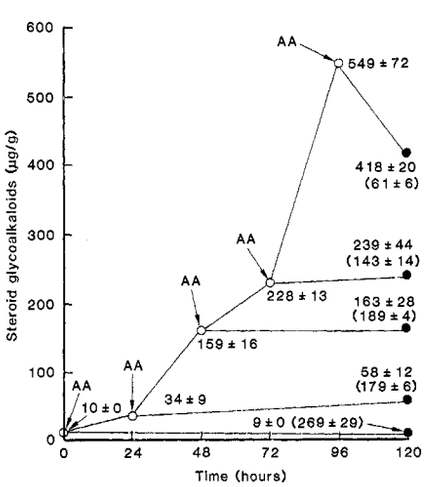

Fig. 1. (●) The effect of arachidonic acid (*AA*) on the accumulation of steroid glycoalkaloids and sesquiterpenoid stress metabolites (in parentheses) in Kennebec potato tuber slices. Values are given as micrograms per gram (fresh weight). Slices were aged for 6 hours, treated with water or with arachidonic acid (100 μg per slice) 0, 24, 48, 72, and 96 hours later, and extracted 120 hours after the initial application (time 0). (○) Steroid glycoalkaloids present in the tissues at the time of arachidonic acid application. Values are the means ± standard error (S.E.) of three replicates containing eight slices per replicate and two determinations per replicate.

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