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THE ROLE OF PLATELET CYCLOOXYGENASE AND LIPOXYGENASE PATHWAYS IN TUMOR CELL INDUCED PLATELET AGGREGATION

Kenneth V. Honn[1,°,†,§], Bruce W. Steinert[°], Kamiar Moin[°], James M. Onoda[°], John D. Taylor[°,†], and Bonnie F. Sloane[°,†,Δ]

Departments of Radiation Oncology[°], Biological Sciences[†], and Pharmacology[Δ], Wayne State University, Detroit, MI 48202

Gershenson Radiation Oncology Center[§], Harper/Grace Hospitals, Detroit, MI 48201

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Walker 256 carcinosarcoma cells induce the aggregation of rat platelets and concomitant production of eicosanoid metabolites (e.g., 12-hydroxyeicosatetraenoic acid, thromboxane A2). Cyclooxygenase inhibitors, but not lipoxygenase inhibitors, were able to inhibit platelet aggregation induced in vitro by low concentrations of agonists. At high agonist concentrations, neither cyclooxygenase nor lipoxygenase inhibitors affected platelet aggregation; however the combination of both inhibitors resulted in inhibition of aggregation. Also, a low concentration of agonist induced minimal eicosanoid metabolism, whereas a high concentration resulted in increased eicosanoid metabolism. These inhibitors, at the doses tested, did not inhibit protein kinase C activity.

Perhaps the single most important hindrance to improved survival of cancer patients is the formation of metastases. Platelet enhanced tumor cell adhesion has been shown to play an important role in the adhesion of tumor cells to vessel walls during the formation of hematogenous metastasis (1,2), although the exact mechanism has yet to be determined (3). Arachidonic acid metabolism is important for normal platelet function. Prostacyclin, produced by endothelial cells, has potent antiplatelet properties (4), whereas thromboxanes (primarily platelet thromboxane A2) enhance platelet aggregation and platelet adhesion to the vessel wall. The role of platelet lipoxygenase products in platelet aggregation has yet to be confirmed (5-7). We have

[1]To whom correspondence should be addressed.

Abbreviations: 12-HETE, 12-hydroxyeicosatetraenoic acid; TXA2, thromboxane A2; W256, Walker 256 carcinosarcoma; MEM, Minimum Essential Medium; PEG, polyethylene glycol 400; PMSF, phenylmethylsulfonylfluoride; HEPES, N-2-hydroxy-ethypiperazine-N'-2-ethanesulfonic acid

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previously proposed the use of agents that inhibit arachidonic acid metabolism (including antiplatelet/antithrombotic agents) as antimetastatic agents (3,8-12). Recent research on the efficacy of antithrombotic and antiplatelet agents in antimetastatic therapy has resulted in controversy. In the present study we established that both the cyclooxygenase and lipoxygenase pathways in platelets were involved in tumor cell induced platelet aggregation. Thus inhibition of tumor cell induced platelet aggregation or of hematogenous metastasis due to tumor cell induced platelet aggregation or platelet enhanced tumor cell adhesion *in vivo* may require a combination of inhibitors.

Materials and Methods

Tumor Cells. W256 cells were orginally obtained from the DCT Human and Animal Tumor Bank (National Institutes of Health, Frederick, MD) and passaged *in vivo* in Sprague Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN). Prior to use, tumors were excised, minced, and pieces were cultured *in vitro* in MEM (Gibco, Grand Island, NY) supplemented with 1% fetal bovine serum (Gibco), as previously described (13). W256 cells were harvested by agitation of the culture flask, washed 3X with serum free MEM, enumerated with a model ZB1 counter (Coulter Electronics, Hialeah, FL), and adjusted with MEM to 2.5×10^7 cells/ml prior to use. Viability, as determined by trypan blue exclusion, was >80%.

Platelet Rich Plasma. Blood was drawn from the inferior vena cava of Sprague Dawley rats anesthesized with sodium pentabarbital (50 mg/kg; Schein, Port Washington, NY) into heparin (5 Units/ml final). Platelet rich plasma and platelet poor plasma were prepared by differential centrifugation of the blood as previously described (13). The platelets were enumerated as described above and adjusted with platelet poor plasma to $0.9-1.2 \times 10^9$ platelets/ml prior to use.

Aggregometry Studies. Ibuprofen was a generous gift of Dr. Lawrence Marnett (Wayne State University, Detroit, MI). Quercetin and PEG were purchased from Sigma Chemical Co.(St. Louis, MO). Ibuprofen and quercetin were dissolved (with sonication) as stock solutions in PEG which would provide the required final concentration of drug without exceeding 0.3% (v/v) PEG in the aggregometry cuvette. PEG in excess of 1.0% (v/v) significantly inhibited platelet aggregation.

Aggregometry studies were performed in Model DP-247E dual channel aggregometers (Sienco, Morrison, CO) as previously described (13). Platelet rich plasma was preincubated (4 min; 37°C) in the aggregometry cuvette prior to the addition of ibuprofen or quercetin, after which, an additional 4 min incubation period elapsed before induction of aggregation. Aggregation was induced by the addition of an appropriate number of W256 cells depending on the experimental design. Aggregation was terminated after 15 min by aspirating the contents of the cuvette into 2 ml of cold (4°C) acetone. Radioimmunoassay of TXB_2 and 12-HETE were performed as previously described (13).

Protein Kinase C Assay. Protein kinase C was measured by a modification of the method by Melloni *et al.* (14). Briefly, rat platelet rich plasma was prepared as described above and washed in Ca^{++} and Mg^{++}-free MEM (GIBCO) as previously described (13). Platelets were resuspended following the final wash in HEPES (10 mM; pH 7.5) buffer containing: 0.25 M sucrose; 5 mM EDTA; 10 mM 2-mercaptoethanol; 0.01% leupeptin; and 2 mM PMSF at a concentration of 8×10^8

platelets/ml. The platelets were disrupted by sonication for 1 min with a model 16-850 cell disrupter (Virtis, Gardner, NY). The assay was conducted in sodium borate buffer (50 mM; pH 7.5) containing $MgCl_2$ (5 mM) and $CaCl_2$ (1 mM). To 100 µl of this buffer was added 50 µl of substrate solution containing histone (type III; 2 mg/ml; Sigma), phosphatidyl serine (0.2 mg/ml; Sigma) and dioleolyl glycerol (25 µg/ml; Sigma). [γ-^{32}P]ATP (300 mM; New England Nuclear, Boston, MA) was added prior to the addition of sonicate. The assay was initiated by the addition of 30 µl of platelet sonicate and was terminated after 10 min incubation at 37°C by the addition of 1 ml 10% TCA. The precipitate was collected on a 2.4 cm glass fiber filter and washed once with 20 ml of cold 10% trichloroacetic acid followed by 95% ethanol washes (2 x 20 ml) and air dried. Radioactivity was determined in an Isocap 300 liquid scintillation counter (Searle Analytic, Chicago, IL) in a toluene based 2,5-diphenyloxazole (0.3%; Kodak, Rochester, NY):p-bis-2,5-diphenyloxazolyl-benzene (0.3%, Baker, Phillipsburg, NJ) counting cocktail.

Results

Effect of Agonist Strength on Aggregation and Eicosanoid Metabolism. Tumor cell induced platelet aggregation was acutely sensitive to agonist (tumor cell) concentration. Aggregation intensity decreased and lag time (before the initiation of aggregation) increased at progressively lower agonist concentrations (Figure 1a-d). W256 cells (2.5 x 10^4 cells/cuvette) failed to induce observable aggregation (Figure 1d) and had near basal (plasma) levels of eicosanoid metabolite production (data not shown). At agonist concentrations less than $1.25x10^5$ W256 cells, the aggregation response was partially reversible (Fig. 1c). The production of TXA_2 (measured by radio-immunoassay as TXB_2) correlated with the decrease in aggregation (Fig. 2).

Effect of Inhibitors on Tumor Cell Induced Platelet Aggregation and Eicosanoid Metabolism. Platelet aggregation by cells was examined in the presence or absence of the cyclooxygenase inhibitor, ibuprofen, and the lipoxygenase inhibitor, quercetin (15). Aggregation was induced following a 4 min preincubation of the platelets with either or both compounds. Ibuprofen or quercetin did not inhibit aggregation induced by $5x10^5$ W256 cells; however, partial inhibition was observed with ibuprofen at lower ($<1.25x10^5$) cell numbers (Figure 1a-c). The aggregation of platelet rich plasma by $5x10^5$ W256 cells was significantly inhibited by ibuprofen and quercetin in combination (Fig. 3). At the concentrations tested, quercetin preferentially inhibited 12-HETE production (compared to TXB2), whereas ibuprofen preferentially inhibited TXB2 production (compared to 12-HETE). The combination of quercetin

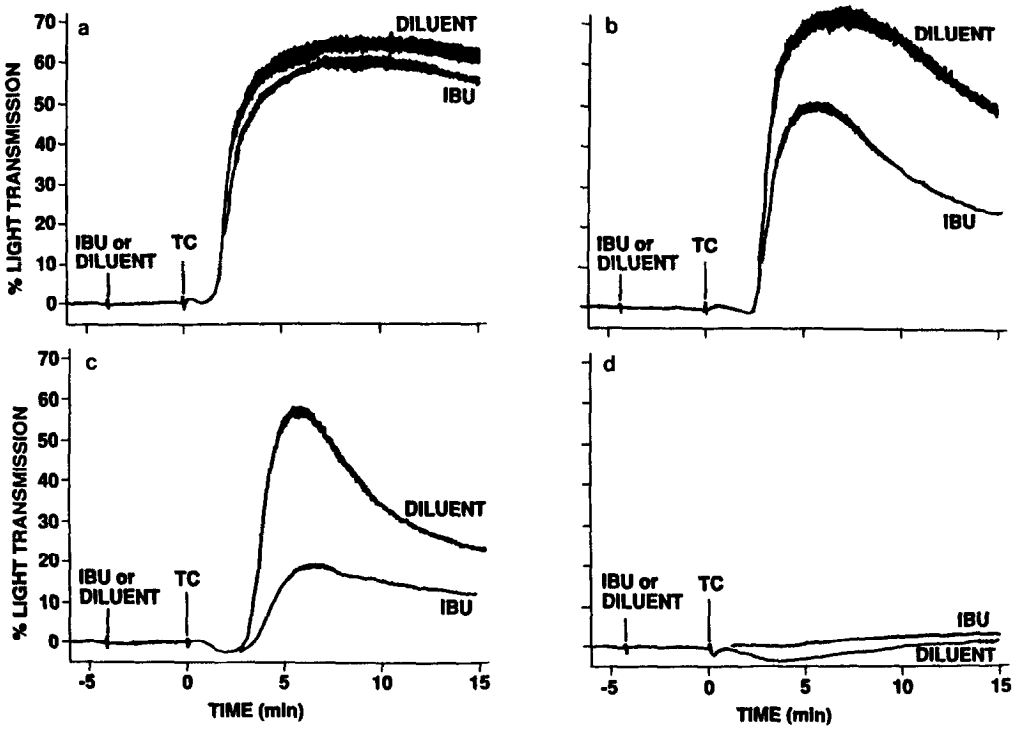

Fig. 1. Effect of Agonist Strength and Ibuprofen (250 μM) on Tumor Cell Induced Platelet Aggregation. Aggregation was induced by addition of W256 cells (TC) at $5x10^5$ (a), $1.25x10^5$ (b), $8.75x10^4$ (c), or $2.5x10^4$ (d) cells/cuvette. Ibuprofen (IBU) or PEG (DILUENT) were added 4 minutes prior to the induction of aggregation.

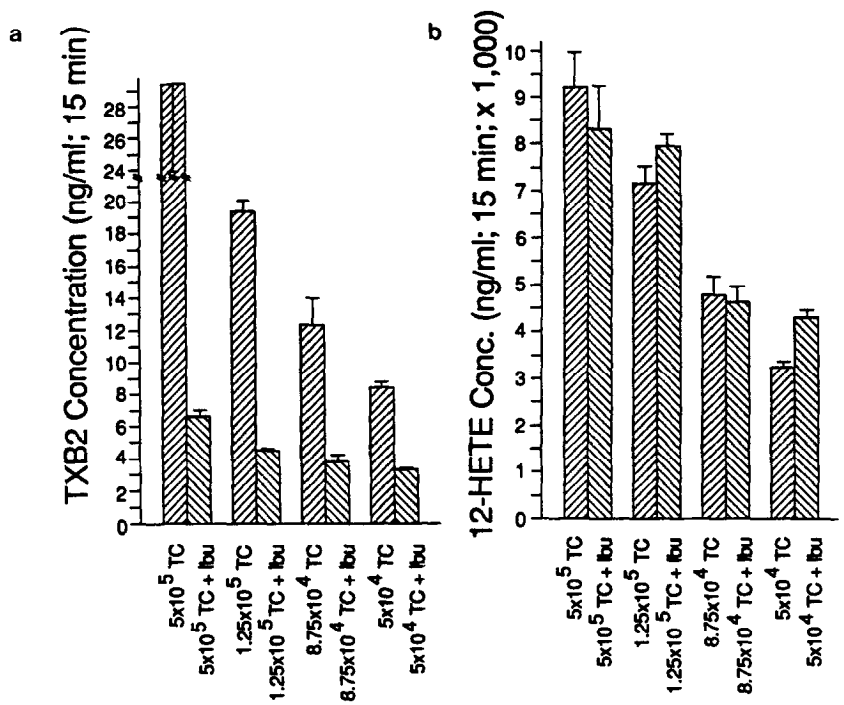

Fig. 2. Effect of Agonist Strength (TC) and Ibuprofen (Ibu; 250 μM) on Endogenous Production of TXA_2 and 12-HETE. TXA_2 (TXB_2; a) and 12-HETE (b) production were determined by RIA analysis of extracted contents of the aggregometer cuvettes.

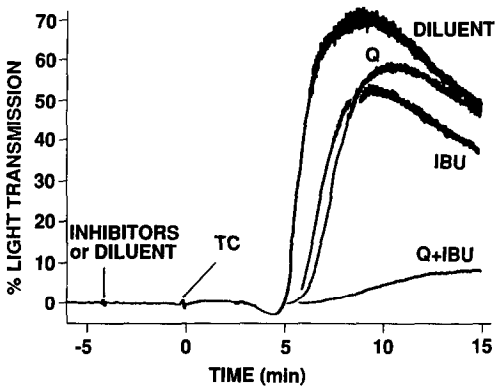

Fig. 3. Effect of Ibuprofen and Quercetin on Tumor Cell Induced Platelet Aggregation. Ibuprofen (IBU; 1mM) and/or Quercetin (Q; 0.5 mM) were preincubated with platelet rich plasma 4 min prior to the induction of aggregation by $5x10^5$ W256 cells.

and ibuprofen resulted in greater inhibition of eicosanoid metabolism than was observed with either inhibitor alone (Table 1).

Effect of Inhibitors on Protein Kinase C Activity. The involvement of protein kinase C in platelet aggregation has recently been determined (see 16 for review). The cyclooxygenase and lipoxygenase inhibitor BW755c (Burroughs Wellcome, Research Triangle Park, NC) has also been shown to affect PKC activity. However, neither ibuprofen (0.25 and 1 mM) or quercetin (0.5 mM) inhibited PKC activity (data not shown).

Discussion

Considerable controversy has arisen regarding the role of the cyclooxygenase pathway in platelet aggregation. Previous studies have shown that cyclooxygenase inhibitors do not inhibit tumor cell induced platelet aggregation (8). We have established that inhibition of tumor cell induced platelet aggregation by one cyclooxygenase inhibitor, ibuprofen, was highly

Table 1
Effect of Quercetin and Ibuprofen on Platelet Eicosanoid Production

Conditions[a]	12 HETE (% of control)	TXA_2 (% of control)
Control (PEG)	100	100
Quercetin	14	52
Ibuprofen	115	26
Quercetin + Ibuprofen	17	16

[a]0.5 mM quercetin; 1 mM ibuprofen.

dependent on agonist strength, whereas inhibition of both the cyclooxygenase and lipoxygenase pathways resulted in inhibition of tumor cell induced platelet aggregation, even at high agonist strength. We conclude therefore, that the strength of the inducing agonist, as well as products of both the platelet cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism play important roles in the control of tumor cell induced platelet aggregation. In turn both of these may be involved in platelet facilitation of metastasis.

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References

1. Jamieson, G.A. (ed., 1982) In: Interaction of Platelets and Tumor Cells. Alan R. Liss, New York.
2. Honn, K.V. and Sloane, B.F. (eds., 1983) In: Hemostatic Mechanisms and Metastasis. Martinus-Nijhoff, The Hague.
3. Honn, K.V., Menter, D., Moilanen, D., Cavanaugh, P.G., Taylor, J.D. and Sloane, B.F. (1983) In: Protective Agents in Cancer. (D.C.H. McBrian and T.F. Slater, ed), pp. 57-79, Academic Press, New York.
4. Moncada, S. and J.R. Vane. (1977) In: Biochemical Aspects of Prostaglandins and Thromboxanes. (N. Kharasch and J. Fried, eds.), pp. 155-177, Academic Press, New York.
5. Maclouf, J., Levy-Toledano, S., Savariau, E., Hardistry, R., and Caen, J.P. (1984) Prostaglandins **28**:383-398.
6. Herrmann, K.S. (1985) Prostaglndins **28**:459-465.
7. Coene, M.-C., Bult, H., Claeys, M., Laekeman, G.M., and Herman, A.G. (1986) Thromb Res **42**:205-214.
8. Honn, K.V., Menter, D.G., Onoda, J.M., Taylor, J.D., and Sloane, B.F. (1984) In: Cancer Invasion and Metastasis: Biologic and Therapeutic Aspects. (G.L. Nicolson and L. Milas, eds), pp. 361-388, Raven Press, New York.
9. Honn, K.V., Cicone, B., and Skoff, A. (1981) Science **212**:1270-1272.
10. Honn, K.V., and Dunn, J.R. (1982) FEBS Lett. **139**:65-68.
11. Honn, K.V. (1983) Clin. Exp. Metastasis **1**:103-114.
12. Menter, D.G., Onoda, J.M., Taylor, J.D., and Honn, K.V. (1984) Cancer Res **44**:450-456.
13. Menter, D.G., Steinert, B.W., Sloane, B.F., Taylor, J.D., and Honn, K.V. (1987) Cancer Res (In Press).
14. Melloni, E., Pontremoli, S., Michetti, M., Sacco, O., Sparatore, B., Salamino, F. and Hoercker, B.L. (1985) Proc. Natl. Acad. Sci. USA **82**:6435-6439.
15. Welton, A.F., Tobias, L.D., Fiedler-Nagy, C., Anderson, W., Hope, W., Meyers, K., and Coffey, J.W. (1986) Prog. Clin. Biol. Res. **213**:231-242.
16. Nishizuka, Y. Science **233**:305 (1986).