

Human Rhabdosarcoma Cell-induced Aggregation of Blood Platelets¹

Gesina L. Longenecker, Barbara J. Beyers, Rebecca J. Bowen, and Timothy King

Departments of Biomedical Sciences and Pharmacology, University of South Alabama, Mobile, Alabama 36688

ABSTRACT

The ability of tumor cells shed into the circulation to cause adhesion and aggregation of blood platelets may be involved in successful metastasis of primary tumors. Rhabdosarcoma is a rare, early metastasizing tumor previously uncharacterized for ability to alter platelet function. It was found that human rhabdosarcoma cells (American Type Culture Collection) dose dependently induce biphasic aggregation of human blood platelets in heparinized platelet-rich plasma; aggregation responses could also be elicited in citrated plasma. Aggregation caused by rhabdosarcoma can be inhibited by apyrase treatment of either rhabdosarcoma or platelets, and by pretreatment of platelets with prostacyclin, cilostamide, inhibitors of thromboxane A₂ production, or TMB-8; only apyrase and prostacyclin inhibited both phases of aggregation. Tumor cell supernatant contained only enough ADP to cause a negligible, reversible aggregation response. Hirudin, verapamil, and triazolam do not inhibit rhabdosarcoma-induced aggregation. Aggregation of platelets by rhabdosarcoma cells thus appears to involve ADP, from tumor cells and/or platelets, and platelet calcium mobilization and thromboxane A₂ synthesis and release.

INTRODUCTION

A role for platelets in tumor metastasis has been suggested based on several types of observation: *in vivo* injection of tumor cells with high metastasis rates causes significant decreases in platelet counts; isolated tumor cells can cause activation (adhesion, aggregation, release reaction) of platelets *in vitro*; sensitivity of platelets to individual tumor cell types correlates with metastasis capability *in vivo*; drugs that interfere with platelet function or induced thrombocytopenia can limit metastasis production, etc. (for review, see Ref. 1). Tumor cell activation of platelets is thought to protect tumor cells from immune surveillance, to make tumor cell adhesion to gaps in the vascular endothelium more likely and more effective for subsequent extravasation, and possibly even to provide metabolic stimulation (for review, see Ref. 2).

Mechanism of induction of platelet activation by tumor cells varies among tumors of different types. Some tumor cells cause activation through thrombin-like entities or by initiating formation of thrombin itself, evidenced by interference with their activation of platelets by thrombin antagonists such as hirudin (for reviews, see Refs. 2 and 3). Some tumor cells secrete or release cathepsin B (4) or the nucleotide ADP (5), evidenced by inhibition of tumor cell-induced platelet activation by cysteine proteinase inhibitors or ADP-scavenging systems such as apyrase, respectively. Other mechanisms include interaction between tumor cells and platelets via a trypsin-sensitive protein on the tumor cells (6), and the formation of a sialolipoprotein complex possibly participates in the mechanism of interaction between tumor cells and platelets (7).

Platelet activation by several agonists may be accompanied by the release reaction (for review, see Ref. 8). This has also been observed in platelets activated by tumor cells, which may release both their granule contents as well as metabolites of arachidonic acid, *e.g.*, thromboxane A₂ (2). Platelets have been

identified clearly as the source of released thromboxane A₂ subsequent to exposure of platelets to tumor cells. Thromboxane A₂ released appears to correlate with the metastatic capability of the tumor (9). Inhibitors of the enzyme cyclooxygenase, which decrease production of thromboxane A₂ by limiting synthesis of its intermediates, indeed do inhibit tumor cell activation of platelets, although *in vitro* use of specific thromboxane synthase inhibitors has yielded equivocal effects (10), possibly due to limited ability of *in vitro* systems to mimic complete *in vivo* systems.

Rhabdosarcoma (rhabdomyosarcoma) is a rare but aggressive, highly malignant tumor in both the adult and juvenile forms. Metastases are an early accompaniment of rhabdosarcoma and are often found in multiple locations (11). Whether rhabdosarcoma cells induce aggregation of platelets and by what mechanism(s) have not been previously reported. Studies of the ability of a human juvenile rhabdosarcoma to activate human platelets were therefore undertaken and are reported in the following sections.

MATERIALS AND METHODS

Rhabdosarcoma Cultures. Human embryonal RD² (CCL 136) (12) cells were obtained from the American Type Culture Collection (Rockville, MD), established in culture in standard 25-cm² tissue culture flasks (Corning Glass Works, Corning, NY), and maintained in a humidified 5% CO₂ atmosphere at 37°C. Medium 199, supplemented with 7.5% fetal bovine serum, 2 units/2 μg/ml of penicillin/streptomycin, 2.5 μg/ml of amphotericin B (Fungizone; Squibb) (all from Flow Laboratories, McLean, VA), 0.2 mg/ml of L-glutamine (GIBCO, Grand Island, NY), and 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma Chemicals, St. Louis, MO), pH 7.4, was used continuously from initial seeding. Subculture at confluency was accomplished by treatment with trypsin:EDTA (Flow), with a 1:4 split. Medium was changed 12 to 24 h after subculture, with routine medium changes twice per week. Cells maintained and subcultured as described grew to confluency in 3 to 5 days.

RD cells to be used in aggregation studies were freed from culture plates without proteases by a modification of the method of Bastida *et al.* (5). Cell layers were washed twice with Ca²⁺, Mg²⁺-free HBSS, incubated 15 min with 5 ml of Ca²⁺, Mg²⁺-free HBSS containing 5 mM EGTA (ethyleneguanilylbis(tetraacetic acid), which was poured off and replaced with 1 ml of the same mixture for an additional 15 min. At the end of the incubation time, the cells were completely freed by rapping the flasks sharply on a foam pad. The volume of the cell suspension was increased to 15 ml per flask, after which the suspensions were removed and centrifuged (2000 × g, 10 min, 25°C). The resulting pellets were gently resuspended at 5.5 × 10⁷ cells/ml in Ca²⁺, Mg²⁺-free HBSS containing 1.9% glucose, and suspensions had viabilities of approximately 80% by trypan blue exclusion. Viability for RD cells does decrease with time in suspension, a process particularly noticeable ≥60 min. Cells were therefore used as quickly as possible after isolation.

Aggregometry and Thromboxane Production. Platelet aggregometry studies were performed as previously described (13). Briefly, blood (30 to 50 ml) was obtained from drug-free human volunteers by puncture of an antecubital vein and was anticoagulated with heparin (Upjohn, Kalamazoo, MI; 5 units/ml final) or, in several experiments, with trisodium citrate (3.8%; 1:9, volume:volume). PRPs and PPPs were

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² The abbreviations used are: RD, rhabdosarcoma; HBSS, Hanks' balanced salt solution; PRP, platelet-rich plasma; PPP, platelet-poor plasma; RIA, radioimmunoassay; NGDA, nordihydroguaiaretic acid.

prepared by sequential 25°C centrifugations (150 × *g*, 2000 × *g*, respectively) of whole blood (or whole blood minus PRP). Autologous PPP was used to adjust the PRP platelet count to 300,000 ± 15,000 (Coulter Counter ZB, 70-μm aperture), after which PRP was "rested" 30 to 60 min before use. Aggregation responses of PRP were monitored in a Payton 300 BD aggregation module (1000 rpm, 37°C), with PRP = 0%, PPP = 100%, light transmission. PRP sample size was 100 μl. Each PRP batch was tested for responses to ADP (1 to 10 μM), epinephrine (1 to 10 μM), arachidonic acid (0.5 to 2 mM), and occasionally collagen (1 to 2 μg/ml) to ensure normalcy of responsiveness. Initiators were added in volumes of 1 to 10 μl. Aliquots of tumor cells were added to PRP just as any other initiator.

Thromboxane A₂ production was briefly examined using aggregometry samples. At the end of the response period, indomethacin was added to the cuvetts to prevent further thromboxane production. The samples were then cooled and centrifuged (Eppendorf microfuge), and an aliquot was taken for RIA of the thromboxane A₂ spontaneous, stable metabolite, thromboxane B₂. RIA was performed by standard procedure, using bulk antibody (Upjohn Diagnostics, Kalamazoo, MI) and bulk ³H-thromboxane B₂ (New England Nuclear, Boston, MA). Samples values were determined from a simultaneously done standard curve (log-logit analysis).

Drug Treatments. Drugs used to delineate the mechanism(s) of RD-induced platelet aggregation included cyclooxygenase inhibitors [aspirin, Sigma, 0.5 mM; indomethacin, Sigma, 1 to 5 μg/ml (3 to 14 μM)]; lipoxigenase-cyclooxygenase dual inhibitors [BW 755c, Wellcome Laboratories, UK, 100 ng to 10 μg/ml (0.5 to 50 μM)]; NDGA (Sigma, 1 to 100 μM); specific inhibitors of thromboxane A₂ synthase [dazoxiben (UK 37248), Pfizer, UK, 100 ng to 10 μg/ml (0.46 to 46 μM)]; OKY 046 (Kissei-Ono Pharmaceuticals, Japan; 1 to 100 mM); prostacyclin, a stimulator of platelet adenylyl cyclase [Upjohn, Kalamazoo, MI; ≤10 ng/ml (≤27 nM)] (8); an inhibitor of platelet cyclic nucleotide phosphodiesterase [cilostamide (OPC 3689); Otsuka Pharmaceuticals, Japan; 1 to 70 μM] (14); a specific antagonist of platelet activating factor receptors (triazolam, Upjohn, ≤10 mM) (15); a nonspecific calcium channel blocker (verapamil, Sigma, ≤10 mM); an inhibitor of internal calcium mobilization [TMB-8, 3,4,5-trimethoxybenzoic acid-8-(diethylamino)-octyl ester, ≤10 μg/ml (≤23 μM), Aldrich, Milwaukee, WI (16)]; an antagonist of platelet thrombin responses and binding [hirudin (17), Sigma, 3 units/ml, sufficient to completely antagonize platelet aggregation due to 10U thrombin]; and an ADP scavenging system (apyrase, Sigma, 15 μg/ml; activity, approximately 800 milliunits/mg).

Drugs were dissolved in HBSS or 10% dimethyl sulfoxide in HBSS and were added to platelets 1 min prior to addition of tumor cells with the exception of aspirin, which was added 5 min prior to tumor cells. Apyrase was added to tumor cells 2 to 5 min prior to addition to platelets. In all cases, the effect of drug pretreatment was compared to the effect of vehicle alone (same preincubation procedure). Results were first calculated as the percentage of control, then as the percentage of inhibition (100%–control) for maximum aggregation (light transmission) at 3 min after addition of tumor cells. In cases where complete dose-response data were obtained, results were graphed as the percentage of inhibition *versus* log dose. Linear relationships were defined by linear regression and accompanying correlation coefficient (PC Statistician package, HSD, Reseda, CA).

RESULTS

Rhabdosarcoma cells (10⁶ to 2 × 10⁷ per ml in PRP) were found to dose dependently induce aggregation of platelets (Fig. 1). Variability in the quantity of cells, from the same cell suspension, required to induce aggregation was observed among donors, with occasional donors unresponsive to RD cells but with intact responses to standard aggregating agents. The aggregation patterns were usually monophasic at low concentrations and biphasic at higher concentrations. The patterns resemble those for epinephrine-induced aggregation. Ability to induce aggregation decreased rapidly with time (Fig. 2), with responses decreasing to below 50% of responses immediately after cell

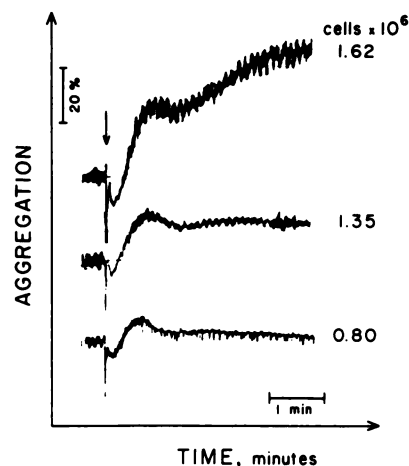


Fig. 1. Dose-dependent human rhabdosarcoma cell-induced aggregation of human platelets in heparinized plasma. Numbers at right, final cell concentration in platelet-rich plasma. Platelet aggregation was monitored via change in light transmission over time after addition of cells (indicated by arrow).

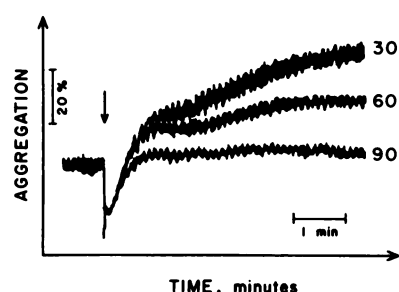


Fig. 2. Time-dependent decrease in aggregation responses of human platelets in heparinized plasma to human rhabdosarcoma cells. Numbers at right are time in minutes from cell isolation to use. Platelet aggregation was monitored via change in light transmission over time after addition of cells (at arrow). Decreases in platelet responses to standard aggregating agents (ADP, epinephrine, etc.) were not seen during this time, indicating tumor cells themselves to be the source of the decreased responses. Tumor cell viability decreases at a slower rate than aggregation-inducing ability.

suspension preparation by 90 min. Importantly, platelet responses to other aggregation stimuli did not change during this same time period, indicating the source of the decreased responsiveness to be the RD cells. Cell viability, which was approximately 80% immediately after suspension preparation, also decreased with time. However, the decrease in viability does not appear to occur as rapidly as the decrease in aggregation induction, with viability decreases ≤10% additional over 90 min.

Pretreatment of either cell suspensions or platelets with apyrase completely inhibited both phases of subsequent aggregation responses to RD cells (Fig. 3, A and B). Supernatant of tumor cell suspensions, added in equivalent volume to cell suspension, evoked a small (≤10% maximal), reversible aggregation response.

Presence of hirudin in platelet suspensions had no effect on platelet responses to RD cells. The amount of hirudin used completely inhibited platelet responses to 10 units (final) of thrombin (Fig. 3, A and C).

Pretreatment of platelets with aspirin, indomethacin, BW 755c, NGDA, dazoxiben, or OKY 046 inhibited the second phase of biphasic aggregation responses to RD cells. A dose-response relationship is shown for BW 755c in Fig. 4. In contrast, pretreatment of RD cells with indomethacin prior to their addition to platelets (indomethacin removed by resuspension of cells) slightly enhanced aggregation responses (data not shown).

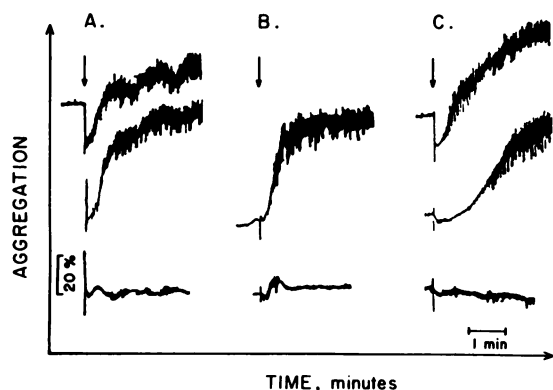


Fig. 3. Effects of apyrase and hirudin on human rhabdosarcoma cell (RD)-induced aggregation of human platelets in heparinized plasma. Aggregation was monitored as change in light transmission over time after addition of stimulus (arrow). A, top, control RD aggregation response; middle, RD aggregation response of hirudin-pretreated (30 units/ml final) platelets, demonstrating no inhibition; bottom, aggregation response induced by RD preincubated with 15 µg/ml (final) of apyrase, demonstrating inhibition. B, top, control ADP (5 µM final) platelet aggregation response; bottom, aggregation response to ADP after apyrase pretreatment of platelets. C, top, control thrombin (10 units/ml final) platelet aggregation response; middle, aggregation response to thrombin after pretreatment of platelets with 10 units/ml (final) of hirudin, demonstrating inhibition (increased lag and decreased slope of response); bottom, aggregation response to thrombin after pretreatment of platelets with 30 units/ml (final) of hirudin, demonstrating complete inhibition.

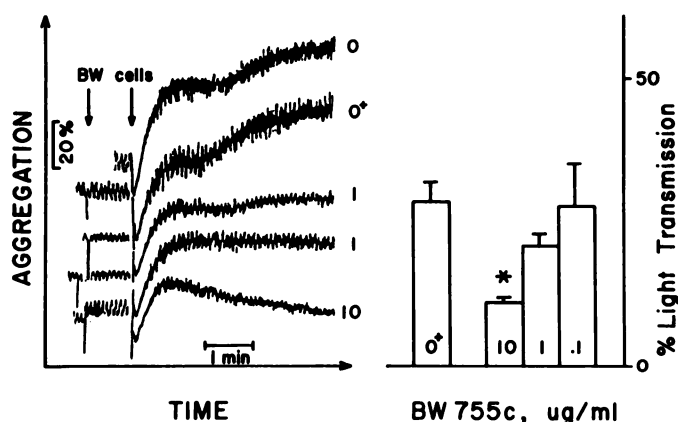


Fig. 4. Dose-dependent inhibition of the second phase of human rhabdosarcoma-induced aggregation of human platelets in heparinized plasma by platelet preincubation with the dual cyclooxygenase-lipoxygenase inhibitor BW 755c ($n = 5$). Numbers to right of aggregation curves and within bars are final BW 755c concentrations (0* is the vehicle control). Aggregation was monitored as change in light transmission over time after pretreatment (first arrow) and addition of cells (second arrow). Similar inhibition of the second phase was obtained with cyclooxygenase inhibitors (aspirin, indomethacin), other dual inhibitors (NGDA), or inhibitors of thromboxane synthase (dazoxiben, OKY 046), indicating an involvement of platelet thromboxane A_2 synthesis and release. *, $P \leq 0.05$, analysis of variance (SAS Institute, IBM 4341 mainframe installation).

Prostacyclin, a potent platelet-inhibitory prostanoid released from vascular endothelium and other tissues (8), dose dependently inhibited RD-induced aggregation. Like apyrase pretreatment of RD cells, prostacyclin pretreatment of platelets inhibited both phases of RD-induced aggregation. Aggregation responses to RD cells were also dose dependently inhibited by pretreatment of platelets with cilostamide. Concentrations of cilostamide up to 70 µM did not alter the initial phase of aggregation, however.

The calcium mobilization antagonist TMB-8 inhibited the second phase of RD-induced aggregation. The initial phase was unaffected at lower TMB-8 concentrations but was slightly attenuated at the highest TMB-8 concentration (10 µg/ml). In contrast, verapamil in the same concentration range did not inhibit any aspect of RD-induced aggregation.

Triazolam had no effect on RD-induced aggregation. The concentration used (10 µM) is sufficient to completely inhibit aggregation induced by pure platelet activating factor.

Platelets did form thromboxane A_2 in response to RD cells: stirred platelets only, 132.4 pg/ml ($n = 2$); RD added to platelets, 412.8 pg/ml ($n = 2$); RD added to indomethacin-treated platelets, 107.5 pg/ml ($n = 2$); and RD alone, below 10 pg/ml (RIA sensitivity limit). For comparison, platelets formed 444.7 ± 72.9 (SEM) pg/ml ($n = 4$) in response to (5 µM) ADP, 694.7 ± 113.6 ($n = 4$) pg/ml with (1 µM) epinephrine, and 677.2 ± 143.6 ($n = 4$) pg/ml with (1 mM) arachidonic acid.

DISCUSSION

Human rhabdosarcoma cells were shown to cause aggregation of human platelets. This is a property shared by many other types of tumor cells (1-3). Following a general scheme suggested by Jamieson *et al.* (3), the aggregation response induced by rhabdosarcoma was shown to involve ADP, with some part of the ADP probably coming from the tumor cells themselves. Very recently (18) ADP release by tumor cells has been shown to be a metabolism-dependent process, rather than a result of damage. The levels of ADP reported in tumor cell medium were 1 to 2 µM, which is very consistent with the reversible aggregation responses observed in this study. Metabolic dependence of ADP release is also consistent with the observed decreased ability of RD to evoke aggregation as a function of time after isolation and could account for the observed loss of effectiveness prior to overt cell death. Not all the ADP involved is released by the tumor cells, however, since equivalent volumes of tumor cell supernatant evoked an almost nonexistent, reversible aggregation response. Tumor cell-induced thrombin formation does not appear to be involved in rhabdosarcoma-induced platelet aggregation, since hirudin did not affect it.

Several drugs that interfere at specific steps in the metabolism of arachidonic acid (8) were examined for effect on rhabdosarcoma-induced aggregation when preincubated with platelets. Aspirin and indomethacin, both of which inhibit the enzyme cyclooxygenase and thus interfere with synthesis of all prostanoids (including thromboxane A_2), NDGA and BW 755c, both of which inhibit both cyclooxygenase and lipoxygenase at the concentrations used, and dazoxiben and OKY 046, both of which specifically inhibit thromboxane synthase, all had a common effect, *i.e.*, they eliminated the second phase of aggregation to RD cells. In contrast, preincubation of RD cells with indomethacin, a cyclooxygenase inhibitor, slightly enhanced aggregation responses. Thus, the common effect of lack of second phase aggregation seen with drugs that limit thromboxane production probably represents an effect of the drugs solely on the platelets. These data provide evidence for an involvement of platelet arachidonic acid metabolism in platelet response to this tumor cell type, consistent with data for other tumor cell types (9). Indeed, thromboxane A_2 synthesis by platelets specifically in response to RD cells was demonstrated by direct measurements of a stable metabolite of thromboxane A_2 . Furthermore, the amount of thromboxane A_2 formed in response to RD was consistent with amounts formed in response to expected amounts of ADP.

Prostacyclin is a potent inhibitor of platelet responses to many stimuli (8), including tumor cells (10). Consistent with previous findings, prostacyclin inhibited RD-induced platelet aggregation. Prostacyclin inhibits platelets by stimulating adenyl cyclase and thus elevating intraplatelet cyclic AMP. Other

drugs that elevate cyclic AMP, *e.g.*, inhibitors of cyclic nucleotide phosphodiesterase(s), would also be expected to inhibit tumor cell-induced platelet activation. The phosphodiesterase inhibitor utilized here, cilostamide, did indeed inhibit rhabdomyosarcoma-induced platelet aggregation. This is consistent with reported *in vivo* effects (reduction in tumor cell adherence, number of metastases, and increased survival) of other phosphodiesterase inhibitors such as dipyridamole (3).

Platelet cyclic AMP levels control the amount of available calcium (19). Basically, the higher the cyclic AMP, the less available is calcium. TMB-8, also an antagonist of internal calcium availability (16), inhibited platelet responses to rhabdomyosarcoma cells. In contrast, verapamil, a nonspecific calcium channel blocker, did not inhibit platelet responses to the tumor cells. Lack of verapamil effect in this *in vitro* system is consistent with the reported lack of *in vivo* effect of verapamil on the process of metastasis (3). Also in support of a lack of requirement for platelet calcium influx for response to RD cells is the observation that RD-induced aggregation occurred in citrated plasma. Thus, rhabdomyosarcoma induction of platelet aggregation appears to require mobilization of intraplatelet calcium. This can be effectively antagonized directly, with TMB-8, or indirectly, by elevation of cyclic AMP levels.

It is concluded that rhabdomyosarcoma cells are capable of activating platelets and that the mechanism by which this activation occurs involves ADP, in part from the tumor cells, platelet calcium mobilization, and the platelet release reaction with formation/release of thromboxane A₂. Further characterization of rhabdomyosarcoma-induced platelet activation may suggest additional therapeutic interventions, as well as add to the basic understanding of tumor-platelet interactions.

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