

Fluid Shear Regulates the Kinetics and Molecular Mechanisms of Activation-Dependent Platelet Binding to Colon Carcinoma Cells

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ABSTRACT This study was undertaken to investigate the kinetics and molecular requirements of platelet binding to tumor cells in bulk suspensions subjected to a uniform linear shear field, using a human colon adenocarcinoma cell line (LS174T) as a model. The effects of shear rate (20–1000 s⁻¹), shear exposure time (30–300 s), shear stress (at constant shear rate by adjusting the viscosity of the medium from 1.3–2.6 cP), cell concentration, and platelet activation on platelet-LS174T heteroaggregation were assessed. The results indicate that hydrodynamic shear-induced collisions augment platelet-LS174T binding, which is further potentiated by thrombin/GPRP-NH₂. Peak adhesion efficiency occurs at low shear and decreases with increasing shear. Intercellular contact duration is the predominant factor limiting heteroaggregation at shear rates up to 200 s⁻¹, whereas these interactions become shear stress-sensitive at ≥400 s⁻¹. Heteroaggregation increases with platelet concentration due to an elevation of the intercellular collision frequency, whereas adhesion efficiency remains nearly constant. Moreover, hydrodynamic shear affects the receptor specificity of activation-dependent platelet binding to LS174T cells, as evidenced by the transition from a P-selectin-independent/Arg-Gly-Asp (RGD)-dependent process at 100 s⁻¹ to a P-selectin/α_{IIb}β₃-dependent interaction at 800 s⁻¹. This study demonstrates that platelet activation and a fluid-mechanical environment representative of the vasculature affect platelet-tumor cell adhesive interactions pertinent to the process of blood-borne metastasis.

INTRODUCTION

Blood-borne metastasis is a highly coordinated, multistep process in which cancerous cells separate from the primary tumor tissue and enter the circulatory system where they interact extensively with various host cells before they lodge in the target organ and form secondary metastatic colonies. Accumulating evidence suggests that the ability of tumor cells to metastasize hematogenously is regulated by their interactions with blood platelets. The most compelling evidence is the inhibition of metastasis by platelet depletion, and the restoration of metastatic potential after platelet repletion (Gasic et al., 1968; Karparkin et al., 1988). Prior work suggests that platelets, by attaching to tumor cells, provide a protective shield against the cytotoxic activity of immune cells (Honn et al., 1992; Nieswandt et al., 1999). Moreover, platelets may assist the escape of tumor cells from the harsh environment of the vasculature by potentiating tumor cell adhesive interactions with the vessel wall (Felding-Habermann et al., 1996). Alternatively, activated platelets may contribute to tumor-induced angiogenesis by secreting potent angiogenic factors (Pinedo et al., 1998). Thus, understanding the molecular interactions between platelets and tumor cells may provide insights ultimately

leading to the development of novel therapeutic strategies to combat metastasis.

Published data indicate that tumor cell recruitment to surface-anchored, activated platelets primarily occurs via a two-step, sequential process of adhesive interactions under dynamic flow conditions (McCarty et al., 2000). In particular, platelet P-selectin is requisite for the efficient tethering and rolling of free-flowing colon carcinoma cells in shear flow, presumably because of its rapid binding kinetics (Smith et al., 1999). Transient P-selectin-mediated binding increases the duration of cell-cell contact, thereby allowing platelet α_{IIb}β₃ integrins to engage and convert these tethering/rolling interactions into firm adhesion. Integrin binding kinetics appears to preclude the formation of adhesive bonds at high shear and corresponding short collision durations in the absence of any selectin contribution due to relatively slow rates of bond formation (Huber et al., 1995; Springer, 1995).

The *in vitro* model in which tumor cells interact with surface-adherent platelets (Karparkin et al., 1988; McCarty et al., 2000; Nierodzik et al., 1995) simulates events that take place at sites of vascular injury, in which platelet deposition to denuded endothelial cell surfaces had occurred. However, tumor cells are more likely to interact with free-flowing platelets throughout the vascular system rather than with isolated sites of platelet deposition. The fundamental physical and molecular requirements of tumor cell binding to either resting or activated platelets in free-cell suspensions as opposed to immobilized, activated platelet substrates remain largely obscure. Although available evidence suggests that in the absence of flow activated

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platelets attach to tumor cells more extensively than do resting platelets (Mannori et al., 1995; Stone and Wagner, 1993), no quantitative comparisons have been reported under well-defined hydrodynamic shear conditions. Nevertheless, platelet-tumor cell binding typically occurs in the presence of shear flow that can critically affect the kinetics and receptor specificity of these heterotypic adhesive interactions. As has been appropriately argued in the literature, data obtained *in vitro* using static binding assays may not be relevant to the fluid flow conditions prevailing in the vasculature (Konstantopoulos et al., 1998a; Springer, 1995). Consequently, this study was undertaken to characterize the molecular interactions of tumor cells and platelets suspended in plasma as a function of the dynamic shear environment using a human colon carcinoma cell model, because colon cancer is among those tumors with a propensity for hematogenous spread. More specifically, the LS174T human colon adenocarcinoma cell line was chosen because it has been extensively characterized and widely used in a number of diverse assays (Capon et al., 1997; Jadhav et al., 2001; Mannori et al., 1995; McCarty et al., 2000).

This study focuses on elucidating the dynamics and molecular constituents of platelet-tumor cell heteroaggregation in a uniform shear field applied by the use of a cone-and-plate rheometer, and analyzed by a dual-color flow cytometric methodology. In particular, we wished to investigate the influence of intercellular contact duration, applied force among colliding cells, collision frequency, and activation-dependent expression of platelet receptors on platelet-LS174T cell heteroaggregation. To this end, the following experimental parameters were systematically varied: shear rate (20–1000 s^{-1}), shear exposure time (30–300 s), shear stress (at constant shear rate by adjusting the viscosity of the medium from 1.3–2.6 cP), cell concentration (25:1–200:1 platelet to LS174T ratio at a constant tumor cell concentration of 10^6 per mL), and platelet activation. Experimental data were analyzed using a mathematical model based on Smoluchowski's two body collision theory that yields numerical estimates of capture efficiency, an index that reflects the binding affinity of interacting cells (Hentzen et al., 2000; Laurenzi and Diamond, 1999).

MATERIALS AND METHODS

Reagents and monoclonal antibodies

The IgG murine monoclonal antibody (mAb) SZ2 (function-blocking anti-CD42b (anti-glycoprotein (GP) Ib)) was purchased from Beckman Coulter (Fullerton, CA). The mAb Beb1 (anti-CD42a conjugated with fluorescein isothiocyanate (FITC); anti-GPIX-FITC), AK4 (anti-CD62P (anti-P-selectin) conjugated with phycoerythrin (PE)) and MOPC-21 (an irrelevant control IgG antibody conjugated with either FITC or PE) were from BD-Pharmingen (San Diego, CA). An anti-P/E-selectin mAb EP5C7 was generously provided by Dr. Cary L. Queen (Protein Design Labs, Fremont, CA). The Fab anti- $\alpha_{\text{IIb}}\beta_3$ mAb c7E3 was from Centocor (Malvern, PA). The antihuman fibrin mAb MH-1 was generously provided by Dr. James McLinden (American Biogenetic Sciences, Inc., Copiague, NY). The non-

peptide small-molecule platelet $\alpha_{\text{IIb}}\beta_3$ antagonist XV454 (Abulencia et al., 2001) was a kind gift of Dr. Shaker A. Mousa (DuPont Pharmaceuticals Co, Wilmington, DE). The synthetic peptide Gly-Arg-Gly-Glu-Ser-Pro (GRGESP) was from Life Technologies (Rockville, MD). The Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) peptide, fibrin polymerization inhibitor Gly-Pro-Arg-Pro-amide (GPRP-NH₂), thrombin, prostaglandin E₁ (PGE₁), trypsin (type III), isotype matched IgG mAbs, and a Fluoro FITC Conjugation Kit, which was used to conjugate c7E3 with FITC, were from Sigma (St. Louis, MO). The CellTracker CMTMR (5-(and-6)-((4-chloromethyl)-benzoyl)amino)tetramethylrhodamine) was purchased from Molecular Probes (Eugene, OR).

Cell line culture and staining

LS174T human colon adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA), and cultured in the recommended medium. Cells were detached from culture flasks by mild trypsinization (0.25% trypsin/EDTA for 2 min at 37°C; Life Technologies) and subsequently incubated at 37°C for 2.5 h to regenerate surface glycoproteins, as previously described (Mannori et al., 1995; McCarty et al., 2000). During this time, the carcinoma cell suspensions (10^7 cells/mL) were incubated with 0.5 μM CMTMR for 60 min at 37°C. LS174T cells were then washed once to remove excess dye, and resuspended in media for an additional 30-min interval to ensure complete modification of the probe. After a second washing step, LS174T cells were resuspended in Dulbecco's phosphate-buffered saline containing $\text{Ca}^{2+}/\text{Mg}^{2+}$, and stored at 4°C for no longer than 4 h before use in aggregation assays or flow cytometry. LS174T cell viability was >97% as assessed by trypan blue exclusion.

Platelet preparation

Human blood was drawn by venipuncture from healthy volunteers, a patient with Glanzmann thrombasthenia (GT) (McCarty et al., 2000), and a patient with Bernard-Soulier syndrome (BSS) (Peng et al., 1991) into sodium citrate (0.38% wt/v) anticoagulant. In selected experiments, citrated blood was treated with either 2 μM PGE₁ or 5 mM EDTA immediately after venipuncture (Konstantopoulos et al., 1998b). Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at $160 \times g$ for 15 min. However, in the case of the BSS patient, PRP was obtained by allowing whole blood to gravity separate for 2 h postvenipuncture. Platelet-poor plasma was obtained by further centrifugation of the blood at $1900 \times g$ for 15 min. The final platelet count of the PRP was adjusted to the desired levels with platelet-poor plasma. Specimens were stored at room temperature (RT) in capped polypropylene tubes and used within 2 h of isolation. For some studies, the PRP viscosity (1.3 cP at 37°C) was adjusted to 2.6 cP by the addition of polymorphonuclear (PMN) leukocyte isolation medium (Robbins Scientific Corporation, Sunnyvale, CA). The latter neither affected the viability of LS174T cells as evidenced by the trypan blue exclusion assay, nor significantly increased the osmolality of the suspension (data not shown). Moreover, it did not alter surface-receptor expression on either platelets (e.g., P-selectin or $\alpha_{\text{IIb}}\beta_3$) or LS174T colon carcinoma cells (data not shown).

Cone-and-plate rheometry assays

Platelet and CMTMR-stained LS174T colon carcinoma cell suspensions were allowed to equilibrate separately to 37°C for 2 min. Thereafter, 50 μL of LS174T cells (1×10^7 cells/mL) along with 450 μL of PRP ($\sim 0.28 \times 10^8$ to $\sim 2.22 \times 10^8$ platelets/mL) were placed onto the stationary platen of a cone-and-plate rheometer (RS150; Haake, Paramus, NJ) to achieve the desired ratio of platelets to LS174T cells (ranging from 25:1–200:1). Shear rates varied from 20 s^{-1} to 1000 s^{-1} (typical of microcirculation) for

prescribed periods of time ranging from 30 to 300 s. Static conditions were achieved by setting the shear rate to 0 s^{-1} . The 0.5° cone and plate of the rheometer were maintained at 37°C during the entire experiment. Upon termination of shear, samples were obtained by pipette and instantly fixed with 1% formaldehyde. Immediately thereafter, specimens were allowed to incubate with a FITC-labeled platelet-specific mAb directed against either platelet $\alpha_{\text{IIb}}\beta_3$ ($5 \text{ } \mu\text{g/mL}$ c7E3-FITC) or GPIX ($0.9 \text{ } \mu\text{g/mL}$ anti-CD42a-FITC) for 30 min in the dark at RT. The labeling reaction was then stopped by further dilution with 1% formaldehyde, and specimens were subsequently analyzed in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

Cell treatment with thrombin, mAbs, and enzymes

To potentiate platelet activation, PRP specimens were incubated for 10 min before shear exposure with thrombin (0.25 or 2 units/mL) in the presence of the fibrin polymerization inhibitor GPRP- NH_2 (2 mM). In selected experiments, platelet suspensions were treated with GPRP- NH_2 alone. The inclusion of GPRP- NH_2 prevented not only fibrin polymerization but also the formation of homotypic platelet aggregates, even after the exposure of specimens to thrombin and/or relatively high shear rates up to 1000 s^{-1} (data not shown).

For inhibition studies, platelets were pretreated for 10 min with EP5C7 ($50 \text{ } \mu\text{g/mL}$), XV454 (100 nM), c7E3 ($20 \text{ } \mu\text{g/mL}$), SZ2 ($20 \text{ } \mu\text{g/mL}$), GRGDSP (20 mM), or Gly-Arg-Gly-Glu-Ser-Pro (20 mM), which were kept present during the aggregation assays. Alternatively, LS174T cells were treated with 0.25% trypsin (type III)/5 mM EDTA for 60 min at 37°C , to remove protease-sensitive glycoproteins. After trypsin treatment, LS174T cells were washed once and used in rheometric assays. In parallel, control experiments were performed in which platelets and tumor cells were treated exactly as stated above but in the absence of any function-blocking mAb or enzyme. Platelet-colon carcinoma cell adhesive interactions in response to hydrodynamic shear were unaltered by the presence or absence of an IgG control mAb (data not shown).

Quantitation of aggregation

The particle distribution and cellular composition of stable aggregates generated in the rheometric assay were determined by a dual-color flow cytometric methodology. CMTMR-stained LS174T cells and FITC-labeled platelets were identified on the basis of their characteristic forward-scatter, side-scatter, and fluorescence profiles in a FACSCalibur flow cytometer. FITC and CMTMR are excited efficiently at 488 nm by the argon laser of a flow cytometer, and their emission spectra are well separated (515 nm for FITC and 570 nm for CMTMR), thereby allowing simultaneous two-color immunofluorescence measurements. Electronic compensation was used to remove spectral overlap between the two fluorescent populations. Acquisition and processing of 3000 CMTMR-stained LS174T cell events was then used to determine 1) the percent of platelet-LS174T cell heteroaggregation and 2) the population distribution of bound platelets to the tumor cell surface (Fig. 1). The FITC-autofluorescence of the LS174T cell population was used to set a threshold (Fig. 1, vertical line) that separates nonadherent single LS174T cells from those bound to platelets. Therefore, the percentage of tumor cells expressing a green fluorescence above this background threshold corresponds to the percentage of LS174T cells binding FITC-labeled platelets.

The following strategy was adapted to determine the number of bound platelets per tumor cell from the flow cytometric measurements (Fig. 1). The mean and standard deviation of single platelet FITC-fluorescence intensity were computed. Six times the calculated standard deviation provides the range of a single platelet fluorescent event with a 99% confidence. This range was then superimposed to the computed threshold value of LS174T cell green fluorescence, thereby characterizing the limiting

values for a single platelet-tumor cell event (P_1T_1). Using this methodology, heterotypic platelet-tumor cell aggregates comprised of a single LS174T cell with one (P_1T_1), two (P_2T_1), three (P_3T_1), or more ($P_{4+}T_1$) adherent platelets were detected and enumerated (Fig. 1). Aggregates consisting of four or more platelets were rare events representing $\leq 1\%$ to 3% of the total LS174T cell population and were therefore grouped into the ($P_{4+}T_1$) category. Using this methodology, differences in the fluorescence intensity of the platelet label between individual experiments did not affect the percentage of platelet-tumor cell aggregates nor the population distribution of heteroaggregates. Under the experimental conditions of this study, homotypic platelet aggregation was negligible with less than 5% of platelets in aggregates even under the most extreme conditions reported here (data not shown).

Determination of adhesion efficiency of platelet binding to LS174T colon carcinoma cells

Platelet-LS174T heteroaggregation in response to hydrodynamic shear exposure is determined by the intercellular collision frequency and the capture efficiency of these collisions. The two-body collision frequency per unit volume, $f_{[(i,j),(m,n)]}$, is a function of the physical parameters of the experimental system, and can be calculated by the Smoluchowski equation:

$$f_{[(i,j),(m,n)]} = \frac{4}{3} (r(i,j) + r(m,n))^3 C(i,j) C(m,n) G \quad (1)$$

in which $r(i,j)$ and $r(m,n)$ are radii of the two colliding particles, one composed of i platelet + j LS174T singlets and other one comprised of m platelet + n LS174T singlets, $C(i,j)$ and $C(m,n)$ are their respective concentrations, and G is the shear rate. The radius of a single LS174T cell was calculated to be $6.0 \text{ } \mu\text{m}$ by image processing, whereas the corresponding one for a platelet singlet was set to $1.34 \text{ } \mu\text{m}$ (Laurenzi and Diamond, 1999; Tandon and Diamond, 1997).

Platelet-LS174T cell adhesion efficiency (E_{PT}) is defined as the fraction of heterotypic shear-induced collisions that result in stable heteroaggregate formation. This index is a function of the intrinsic biological characteristics of the cells that are pertinent to their aggregation behavior such as number and affinity of receptors and their response to applied shear (Hentzen et al., 2000; Huang and Hellums, 1993). The adhesion efficiency of platelet binding to LS174T cells can be calculated by fitting the aggregation data over the first 60 s after application of shear with a mathematical model based on Smoluchowski's two-body collision theory (Hentzen et al., 2000). This model (Eq. 2) describes the temporal change of the concentrations of aggregates $C(m,n)$ composed of m platelet and n LS174T cell singlets in the most general case (Laurenzi and Diamond 1999):

$$\begin{aligned} \frac{dC(m,n)}{dt} = & \sum_{i=0}^m \sum_{j=0}^n \left[\frac{1}{2} (1 + \delta_{i,m-i} \delta_{j,n-j}) \right. \\ & \times K_{[(i,j),(m-i,n-j)]} C(i,j) C(m-i,n-j) \\ & - \sum_{i=m}^{m_{\text{max}}} \sum_{j=n}^{n_{\text{max}}} [(1 + \delta_{m,i-m} \delta_{n,j-n}) \\ & \times K_{[(m,n),(i-m,j-n)]} C(m,n) C(i-m,j-n) \end{aligned} \quad (2)$$

in which $K_{[(i,j),(m,n)]}$ is the aggregation rate coefficient, $\delta_{i,j}$ is Kronecker delta function, m_{max} and n_{max} represent the maximal number of platelets and tumor cells in an aggregate, respectively, used in the simulation. m_{max} was set equal to four due to the fact that heteroaggregates comprised of a single LS174T cell with more than four adherent platelets were rather rare

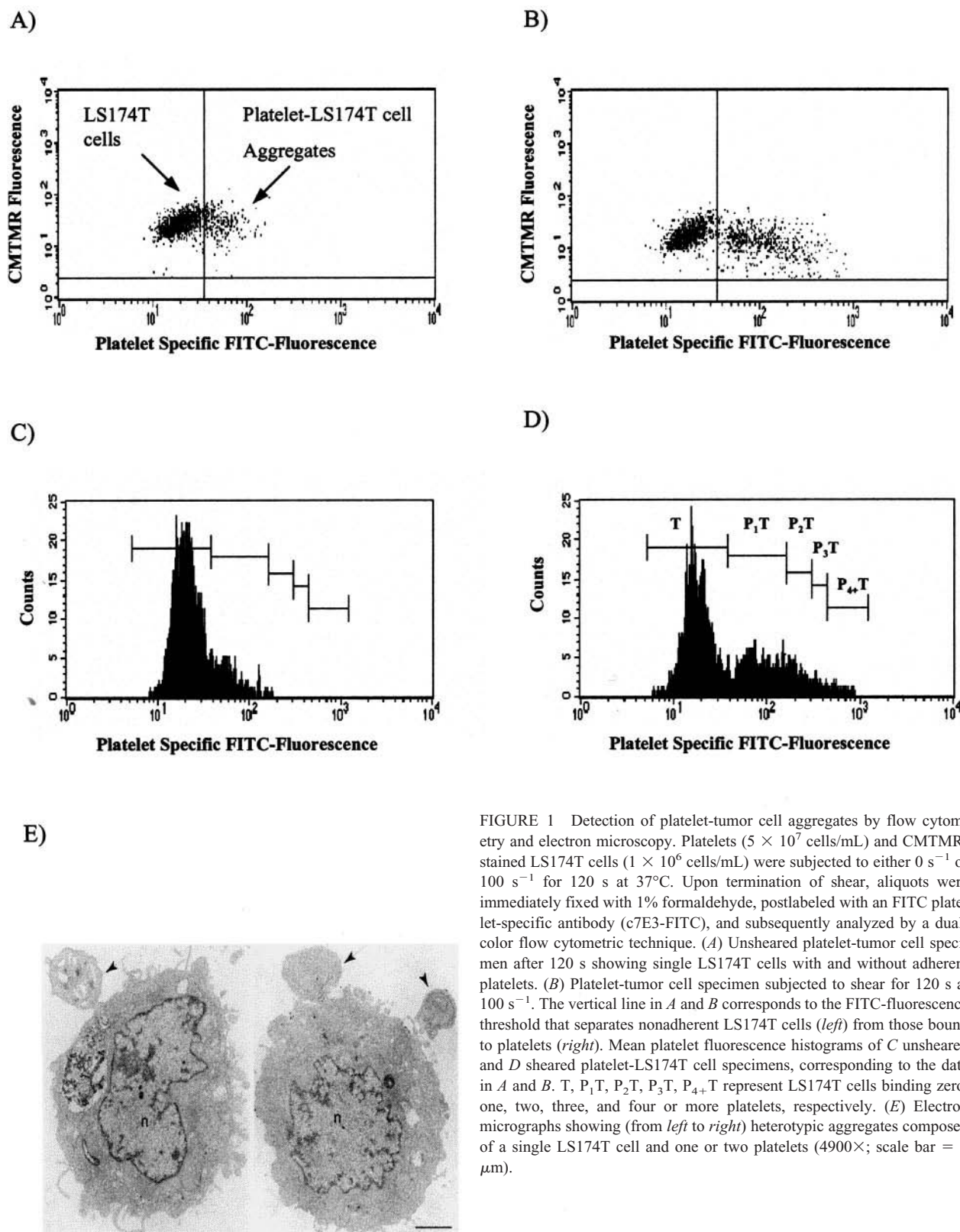


FIGURE 1 Detection of platelet-tumor cell aggregates by flow cytometry and electron microscopy. Platelets (5×10^7 cells/mL) and CMTMR-stained LS174T cells (1×10^6 cells/mL) were subjected to either 0 s^{-1} or 100 s^{-1} for 120 s at 37°C . Upon termination of shear, aliquots were immediately fixed with 1% formaldehyde, postlabeled with an FITC platelet-specific antibody (c7E3-FITC), and subsequently analyzed by a dual-color flow cytometric technique. (A) Unsheared platelet-tumor cell specimen after 120 s showing single LS174T cells with and without adherent platelets. (B) Platelet-tumor cell specimen subjected to shear for 120 s at 100 s^{-1} . The vertical line in A and B corresponds to the FITC-fluorescence threshold that separates nonadherent LS174T cells (left) from those bound to platelets (right). Mean platelet fluorescence histograms of C unsheared and D sheared platelet-LS174T cell specimens, corresponding to the data in A and B. T, P_1T , P_2T , P_3T , $P_{4+}T$ represent LS174T cells binding zero, one, two, three, and four or more platelets, respectively. (E) Electron micrographs showing (from left to right) heterotypic aggregates composed of a single LS174T cell and one or two platelets (4900 \times ; scale bar = 2 μ m).

events (Fig. 1), and thus, grouped into the $P_{4+}T_1$ category. n_{\max} was set to one due to the observation that LS174T cells do not aggregate with each

other under the experimental conditions tested in this study. The first term in the right-hand-side of Eq. 2 accounts for the formation of the combi-

nation from two smaller aggregates, whereas the second term represents the depletion of the combination due to the formation of a higher order aggregate. Because our goal is to measure the initial rate of recruitment of platelets and tumor cells into heterotypic aggregates, the terms describing the fragmentation process have been neglected. Furthermore, heterotypic platelet-LS174T cell aggregates once formed were stable over a wide range of shear stresses for at least 5 min, which corresponds to the longest shear exposure time used in this study.

The set of coupled differential equations represented by Eq. 2 was integrated using the fourth order Runge-Kutta-Gill method and the adhesion efficiency (E_{PT}), defined by Eq. 3, was calculated by using the Golden Section Search Method (Belegundu and Chandrupatla 1999):

$$E_{PT} = \frac{K_{[(1,0),(m,1)]}C(1,0)C(m,1)}{f_{[(1,0),(m,1)]}} \quad (3)$$

in which $K_{[(1,0),(m,1)]}$ is the aggregation rate coefficient that describes the adhesion kinetics when a platelet singlet adheres to a particle comprised of m platelet + 1 LS174T cell singlets. The aforementioned model is based on the following key assumptions: 1) single cells and aggregates have spherical geometries; 2) only single collisions between any two particles are considered at a given time; 3) homotypic platelet and LS174T cell aggregation are absent, and hence, only single platelet binding to either an LS174T cell singlet or an LS174T-platelet aggregate are considered (Fig. 1 *E*).

Quantitation of P-selectin and platelet-bound fibrin expression

PRP specimens, preincubated with thrombin/GPRP-NH₂, GPRP-NH₂ alone, or buffer for 10 min, were subjected to hydrodynamic shear (0, 100, or 800 s⁻¹) for 60 s in the presence or absence of LS174T cells. Upon termination of shear, specimens were fixed with 1% formaldehyde and incubated with a PE-labeled anti-P-selectin mAb, AK4-PE, for 30 min at RT. Thereafter, specimens were diluted with fixative and analyzed in a FACSCalibur flow cytometer. Results were expressed as the percentage of platelets expressing PE fluorescence above the background as previously described (Konstantopoulos et al., 1998b).

To assess the extent of platelet-bound fibrin, PRP specimens pretreated with either thrombin/GPRP-NH₂, GPRP-NH₂ alone, or buffer for 10 min were fixed with 1% formalin (Richard Allan Scientific, Kalamazoo, MI) and washed twice with Dulbecco's phosphate-buffered saline to remove soluble fibrin. After a 15-min incubation with the antihuman fibrin mAb MH-1 (40 μg/mL), platelets were washed once, incubated for additional 15 min with PE-labeled IgG antibody (15 μg/mL), washed again, and analyzed in a FACSCalibur flow cytometer (Konstantopoulos et al., 1997; McCarty et al., 2000). An appropriate isotype-matched IgG mAb was also included for background fluorescence determination.

Electron microscopy

Samples containing platelet-tumor cell aggregates were prepared for electron microscopy by standard procedures. Briefly, sheared specimens were fixed in 1.5% glutaraldehyde for 1 h at RT and postfixed for 1 h in Palade's 1% osmium tetroxide at 4°C, and subsequently incubated in Kellenberger's uranyl acetate solution overnight. After dehydration with a graded series of ethanol, cells were embedded in Epon. After polymerization, ultrathin sections were obtained on a Leica Ultracut UCT microtome equipped with a diamond knife. Sections were then stained with uranyl acetate and lead citrate before viewing with an electron microscope (Phillips 420 TEM).

Statistics

Data are expressed as the mean ± SE unless otherwise stated. Statistical significance of differences between means was determined by analysis of

variance. If means were shown to be significantly different, multiple comparisons by pairs were performed by the Tukey test. Probability values of $p < 0.05$ were selected to be statistically significant.

RESULTS

Hydrodynamic shear-induced collisions support formation of platelet-LS174T cell aggregates

Previous studies have shown that platelets bind to various carcinoma cells including the colon adenocarcinoma LS174T cell line under static conditions (Karparkin et al., 1988; Mannori et al., 1995; Nierodzik et al., 1995). To investigate how the hydrodynamic shear environment of the circulation modulates these heterotypic adhesive interactions, LS174T cells were combined with platelets suspended in plasma in a cone-and-plate rheometer and subjected to controlled levels of shear for defined periods of time. A ratio of 50 platelets (5×10^7 cells/mL) to one LS174T cell (1×10^6 cells/mL) was maintained in all shearing experiments, unless otherwise stated. Under these conditions, platelet-platelet aggregation was minimal with only 1% to 5% of platelets in homotypic aggregates. In contrast, extensive aggregation and large platelet aggregates, comparable in size with an LS174T cell, were consistently observed when higher platelet concentrations mixed with LS174T cells were subjected to elevated levels of shear (>100 s⁻¹) for relatively long shear exposure times (≥ 60 s) (data not shown). Under the experimental conditions of this study, LS174T cells did not homotypically aggregate.

A baseline level of platelet-LS174T cell binding ($9.3 \pm 1.0\%$ LS174T cells in heteroaggregates, $n = 4$) was detected under static conditions at 30 s (Fig. 2 *A*). Furthermore, the extent of heteroaggregation varied little with time at 0 s⁻¹ ($11.6 \pm 0.9\%$ LS174T cells in heteroaggregates after 120 s of static incubation, $n = 6$) (Figs. 1, *A* and *C*, and 2 *A*). Application of shear in the absence of any exogenously added chemical agonist augmented platelet-LS174T heterotypic aggregation, as determined by the increase in LS174T-bound platelet fluorescence (Fig. 1, *B* and *D*). The extent of platelet recruitment by LS174T cells increased with increasing shear exposure time over a wide range of shear rates (Fig. 2 *A*), plateaued at ~ 120 s, and remained irreversible for at least 300 s. For example, $30.0 \pm 1.9\%$, $28.9 \pm 1.5\%$, and $29.4 \pm 4.0\%$ of LS174T cells had platelets adherent to their surface after 120, 180, and 300 s of shear exposure at 100 s⁻¹, respectively. Similar results were obtained at all other shear rates (data not shown).

Platelet binding to LS174T cells increased with shear rate from baseline levels under static conditions (0 s⁻¹) to a maximum at 100 s⁻¹. However, at shear rates above 100 s⁻¹, the extent of heteroaggregation decreased with increasing shear. The majority ($>85\%$) of platelet-LS174T cell aggregates were present as single tumor cells with one or two platelets bound to their surface (Fig. 1) as confirmed by

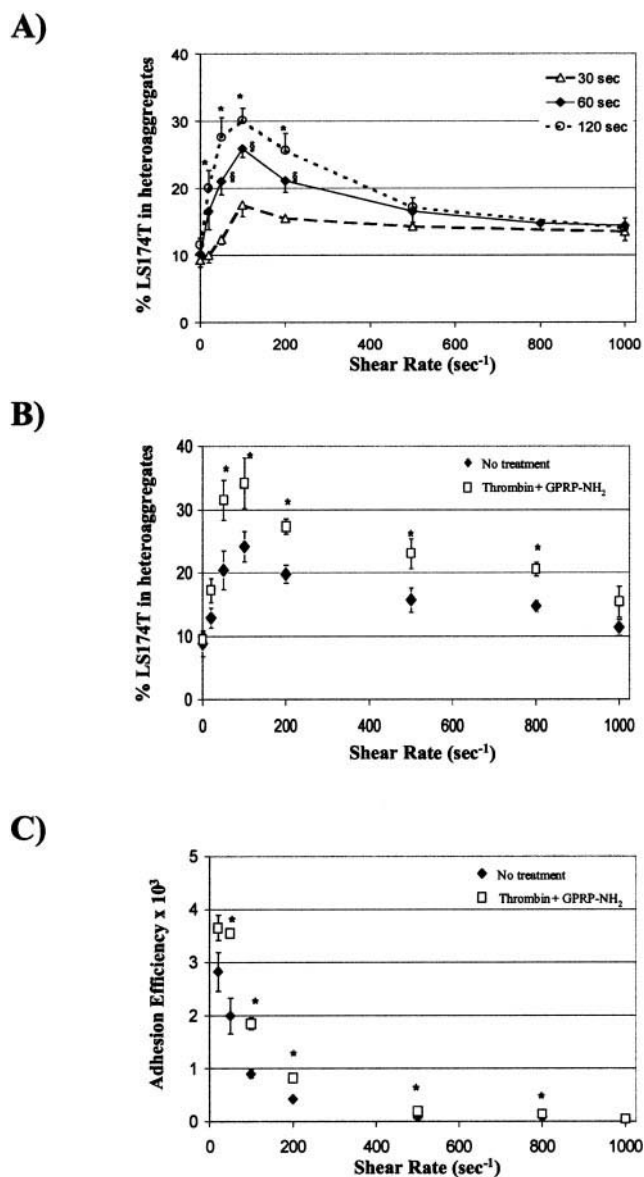


FIGURE 2 Kinetics of platelet-LS174T cell aggregate formation. (A) Effects of hydrodynamic shear on platelet-LS174T cell binding in the absence of any exogenously added chemical agonist. Platelets (5×10^7 cells/mL) were combined with CMTMR-stained LS174T cells (1×10^6 cells/mL) in the cone-and-plate rheometer and subjected to well-defined levels of shear for 30 s (Δ), 60 s (\blacklozenge), or 120 s (\circ). Data represent the percentage of LS174T cells with bound platelets. $*p < 0.05$ with respect to 30 s. $^{\S}p < 0.1$ with respect to 120 and 30 s. Data represent mean \pm SE of 3 to 18 experiments. (B) Effects of hydrodynamic shear on platelet-LS174T cell binding in the presence and absence of thrombin and GPRP-NH₂. Platelets (5×10^7 cells/mL), pretreated for 10 min with (\square) or without (\blacklozenge) fibrin polymerization inhibitor (2 mM GPRP-NH₂) and thrombin (0.25 units/mL), were combined with CMTMR-stained LS174T cells (1×10^6 cells/mL) before the application of shear for 60 s. (C) Adhesion efficiency of platelet binding to LS174T cells as a function of hydrodynamic shear in the presence and absence of thrombin and GPRP-NH₂. $*p < 0.05$ with respect to no-treatment shear. Data represent mean \pm SE of three to nine experiments.

both transmission electron microscopy (Fig. 1 E) and light microscopy (data not shown). Flow cytometric analysis of

sheared specimens reveals that $\leq 3\%$ of LS174T cells had four or more platelets adherent to their surface.

The recruitment of platelets by LS174T cells was significantly potentiated over a wide range of shear rates upon incubation of platelets with thrombin and the fibrin polymerization inhibitor, GPRP-NH₂ (Fig. 2 B). For instance, the percentage of LS174T cells with platelets adherent to their surface increased from $\sim 24\%$ to 36% in the presence of thrombin/GPRP-NH₂ (0.25 units/mL; 2 mM) after 60 s of shear exposure at 100 s^{-1} (Fig. 2 B, Table 1). Treatment of platelets with thrombin/GPRP-NH₂ increased both platelet-bound fibrin and platelet P-selectin expression levels in a concentration-dependent fashion (Table 1). Moreover, thrombin/GPRP-NH₂ stimulation augmented the extent of platelet-LS174T cell binding in a dose-dependent manner only at high (800 s^{-1}) but not low (100 s^{-1}) levels of shear (Table 1). Treating specimens with GPRP-NH₂ alone appeared to slightly increase heteroaggregation only at low shear rates (Table 1), which was more evident at longer shear exposure times ($\geq 120 \text{ s}$) (data not shown).

The efficiency of platelet capture by LS174T cells provides a measure of the biological properties of the cells that control their aggregation behavior, and was computed by fitting the aggregation data over the first 60 s after application of shear (Hentzen et al., 2000). Fig. 2 C shows the shear rate dependence of adhesion efficiency in the presence and absence of thrombin/GPRP-NH₂ (0.25 units/mL; 2 mM). Maximal efficiency in the absence of any exogenously-added chemical agonist was observed at the lowest shear, at which ~ 2 to 3 of 1000 collisions led to stable platelet-LS174T aggregate formation (Fig. 2 C), and decreased with increasing shear. A similar efficiency versus shear rate pattern was also noted after platelet treatment with thrombin/GPRP-NH₂ (0.25 units/mL; 2 mM), and was significantly enhanced over untreated platelets at shear rates ranging from 50 to 800 s^{-1} (Fig. 2 C). A further increase in adhesion efficiency was observed at high shear (800 s^{-1}) with increasing thrombin concentration (no-treatment: $0.082 \pm 0.01 \times 10^{-3}$ versus 0.25 units/mL thrombin/2 mM GPRP-NH₂: $0.18 \pm 0.01 \times 10^{-3}$ versus 2 units/mL thrombin/2 mM GPRP-NH₂: $0.27 \pm 0.03 \times 10^{-3}$; mean \pm SE, $n = 3-4$). In contrast, an increase in thrombin concentration from 0.25 units/mL to 2 units/mL did not significantly affect the adhesion efficiency at low shear (100 s^{-1}) conditions (data not shown).

Effects of shear rate versus shear stress on platelet-LS174T cell aggregate formation

We next wished to examine the effects of hydrodynamic shear rate not only on the formation of heterotypic aggregates but also on the strength of adhesion by subjecting platelet-LS174T cell suspensions to well-defined levels of

TABLE 1 Effects of thrombin and fibrin polymerization inhibitor GPRP-NH₂

Platelet treatment	Platelet-bound fibrin	P-selectin expression (%)	% of LS174T cell heteroaggregation	
			100 sec ⁻¹	800 sec ⁻¹
—	3.1 ± 0.9	5.5 ± 0.4	23.6 ± 0.2	14.7 ± 0.9
GPRP-NH ₂	3.4 ± 1.4	6.6 ± 0.8	25.9 ± 0.4	14.8 ± 0.5
0.25 units/mL Thr + GPRP-NH ₂	29.4 ± 3.7*	7.7 ± 0.9*	36.5 ± 2.8*	20.5 ± 1.1*
2 units/mL Thr + GPRP-NH ₂	89.8 ± 7.0*§	92.7 ± 3.2*§	34.7 ± 4.0*	33.9 ± 1.7*§

Platelets (5×10^7 cells/mL), either untreated (control) or pretreated for 10 min with fibrin polymerization inhibitor (2 mM GPRP-NH₂) in the absence or presence of thrombin (0.25 or 2 units/mL), were examined for the extent of bound fibrin using an indirect single-color immunofluorescent assay. Values represent the geometric mean fluorescence intensities mean \pm SE ($n = 3-4$) after subtracting background fluorescence measured using an appropriate isotype-matched IgG mAb. P-selectin expression was quantified as percentage of platelets expressing an AK4-PE (anti-P-selectin mAb conjugated with PE) fluorescence above the background. Platelets (5×10^7 cells/mL) were combined with CMTMR-stained LS174T cells (1×10^6 cells/mL) prior to the application of shear for 60 s at 100 and 800 s⁻¹. A dual-color flow cytometric technique was used to quantify the percentage of LS174T cells with bound platelets. * $p < 0.05$ with respect to no-treatment (control) specimens, § $p < 0.05$ with respect to 0.25 units/mL thrombin-treated specimens. Data represent mean \pm SE of three to six experiments.

shear. Application of low shear (100 s⁻¹) for both 60 (Fig. 3 *Ab*) and 120 s (Fig. 3 *Ac*) led to increased platelet-tumor cell binding compared with either static (Fig. 3 *Aa*) or high shear (1000 s⁻¹) conditions (Fig. 3 *Ad, e*). The integrity of the platelet-LS174T aggregate formed under low shear was preserved when exposed to subsequent high shear for 60 s (Fig. 3 *Af*), thus suggesting that heteroaggregates, once formed, are resistant to disaggregation with increasing shear. In contrast, platelets and LS174T cells subjected to high shear for 60 or 120 s did not aggregate. However, they retain the ability to form heterotypic aggregates upon subsequent exposure to low shear for 60 s (Fig. 3 *Ag*). Similar results were obtained in the presence of thrombin/GPRP-NH₂ (data not shown).

Consequently, the increased heteroaggregation observed at low shear rates might be attributed to either the longer intercellular contact time and/or the lower net force acting on the cells. By separately varying the viscosity of the medium and the hydrodynamic shear rate, the contributions of contact duration and shear stress could be differentiated (Hentzen et al., 2000; Rinker et al., 2001). To this end, 20% PMN media was used to increase the viscosity of the suspending medium from 1.3 to 2.6 cP at 37°C, thereby increasing shear stress at a constant shear rate, while maintaining the same contact time. Platelets treated with thrombin/GPRP-NH₂ (0.25 units/mL; 2 mM) before their mixing with LS174T cells and application of shear were used to produce significant heteroaggregation in the absence of platelet-platelet cohesion induced by elevated shear stress in the presence of PMN media. Fig. 3 *B* shows that under low shear (≤ 200 s⁻¹), platelet-LS174T cell binding was essentially independent of the viscosity of the medium. However, at higher shear rates (≥ 400 s⁻¹), the increase in shear stress due to an increase in viscosity significantly decreased the extent of heteroaggregation. In accordance, heterotypic adhesion efficiency decreases with shear stress at higher shear rates (Table 2).

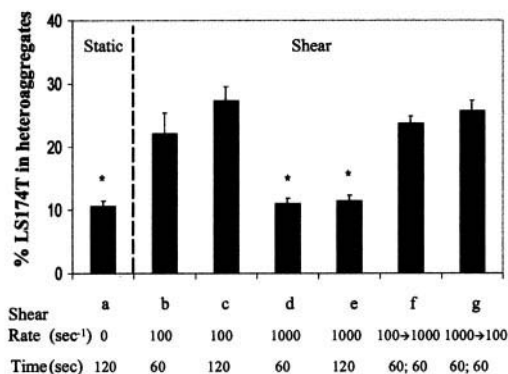
Dependence of platelet-LS174T heteroaggregation on platelet concentration

We next wanted to investigate the effects of platelet concentration on the extent of platelet-LS174T heteroaggregation. To this end, we systematically varied the platelet concentration from 2.5×10^7 to 20×10^7 platelets/mL, whereas the LS174T cell concentration (1×10^6 cells/mL) remained constant, thereby modulating the platelet to LS174T cell ratio from 25:1 to 200:1. To achieve significant heteroaggregation at lower platelet counts and to prevent potential platelet-platelet aggregation at higher platelet concentrations, platelets were treated with thrombin/GPRP-NH₂ (0.25 units/mL; 2 mM) before their mixing with LS174T cells and application of shear. Fig. 4 *A* shows that heterotypic platelet-LS174T cell aggregation increased with increasing platelet concentration under both static and shear (100 s⁻¹) conditions. Moreover, peak heteroaggregation occurred at near physiological platelet concentrations (20×10^7 platelets/mL). However, corresponding adhesion efficiencies varied little over the broad range of platelet concentrations tested in this study. Over an eightfold increase in platelet concentration, the adhesion efficiency reduced moderately by only one-fifth, from 1.37×10^{-3} at 25:1 platelet to LS174T cell ratio to 1.07×10^{-3} at 200:1 ratio.

Roles of platelet P-selectin and $\alpha_{IIb}\beta_3$ -integrins in platelet-LS174T cell heteroaggregation at high and low shear

Ensuing experiments focused on the elucidation of the molecular pathways mediating platelet binding to LS174T cells under dynamic shear conditions. Prior work has shown that both platelet P-selectin and $\alpha_{IIb}\beta_3$ -integrins are required to support optimal LS174T cell adhesive interactions with immobilized, activated platelets under shear (McCarty et al., 2000). Therefore, as a first step, the roles of these platelet receptors were examined in the high shear regime,

A)



B)

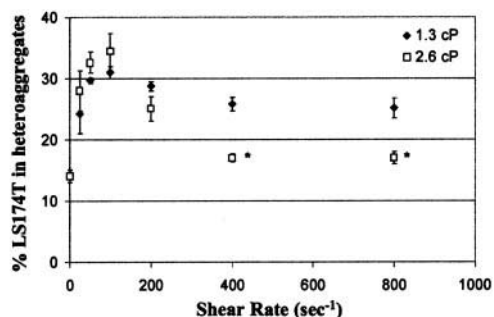


FIGURE 3 Effects of shear rate versus shear stress on platelet-LS174T heteroaggregates. (A) Effects of hydrodynamic shear on platelet-LS174T aggregate formation and disaggregation. Platelets (5×10^7 cells/mL) and CMTMR-stained LS174T cells (1×10^6 cells/mL) were subjected to (a) static conditions for 120 s, (b) 100 s^{-1} for 60 s, (c) 100 s^{-1} for 120 s, (d) 1000 s^{-1} for 60 s, (e) 1000 s^{-1} for 120 s, (f) 100 s^{-1} for 60 s followed by 1000 s^{-1} for 60 s, or (g) 1000 s^{-1} for 60 s followed by 100 s^{-1} for 60 s. Data represent the percentage of LS174T cells in heteroaggregates. * $p < 0.05$ with respect to control shear and time (100 s^{-1} for 120 s). (B) Effects of shear stress on platelet-LS174T cell aggregate formation. Platelets (5×10^7 cells/mL), pretreated for 10 min with fibrin polymerization inhibitor (2 mM GPRP-NH₂), and thrombin (0.25 units/mL) were equilibrated with (2.6 cP, □) or without (1.3 cP, ♦) PMN media at 37°C for 2 min, and then combined with CMTMR-stained LS174T cells (1×10^6 cells/mL) in the cone-and-plate rheometer. Specimens were subjected to well-defined levels of hydrodynamic shear for 60 s. * $p < 0.05$ with respect to control (1.3 cP). Data represent mean \pm SE of three to four experiments.

and in particular at a shear rate of 800 s^{-1} . The results indicate that incubation of platelets (5×10^7 /mL) with either a function blocking anti-P-selectin antibody or $\alpha_{\text{IIb}}\beta_3$ -integrin antagonist inhibited the extent of LS174T cell adhesive interactions with thrombin/GPRP-NH₂-treated platelets (Fig. 5 A). A similar reduction in heteroaggregation was also noted at the physiological platelet concentration of 20×10^7 platelets/mL (data not shown). Regardless of

TABLE 2 Effect of shear stress at a constant shear rate on the adhesion efficiency of platelet-LS174T binding

Shear rate (s ⁻¹)	Adhesion efficiency ($\times 10^3$)	
	Medium viscosity = 1.3 cP	Medium viscosity = 2.6 cP
25	5.52 ± 1.06	6.37 ± 1.06
50	3.63 ± 0.07	4.02 ± 0.29
100	1.82 ± 0.21	1.99 ± 0.16
200	0.84 ± 0.05	0.71 ± 0.10
400	0.32 ± 0.02	$0.19 \pm 0.01^*$
800	0.18 ± 0.01	$0.10 \pm 0.01^*$

Platelets (5×10^7 /mL), pretreated for 10 min with fibrin polymerization inhibitor (2 mM GPRP-NH₂) and thrombin (0.25 units/mL), were equilibrated with (2.6 cP) or without (1.3 cP) PMN media at 37°C for 2 min, and then combined with CMTMR-stained LS174T cells (1×10^6 cells/mL) in the cone-and-plate rheometer. Specimens were subjected to defined levels of shear rate for 60 s, and aggregation was quantified by flow cytometry. The adhesion efficiency of heteroaggregation was calculated from experimental results using a mathematical model based on Smoluchowski's two-body collision theory. * $p < 0.05$ with respect to control (1.3 cP). Data represent mean \pm SE of three to four experiments.

platelet or thrombin concentration, no additive inhibitory effect on platelet-LS174T heteroaggregation was observed with a combination of P-selectin and $\alpha_{\text{IIb}}\beta_3$ -integrin antagonists (Fig. 5 A). Moreover, use of RGD-containing peptides inhibited platelet binding to LS174T cells to a similar degree with that observed with an $\alpha_{\text{IIb}}\beta_3$ specific blocker (data not shown).

We next wished to examine whether these molecular recognition events are required for platelet-LS174T cell interactions in the low shear regime (100 s^{-1}). Our results indicate that blockade of platelet P-selectin and $\alpha_{\text{IIb}}\beta_3$ -integrins, whether alone or in combination, failed to alter the extent of recruitment of thrombin/GPRP-NH₂-treated platelets by LS174T cells, regardless of thrombin concentration (Fig. 5 B).

Characterization of molecular mechanisms mediating platelet-LS174T cell heteroaggregation at low shear

Subsequent experiments aimed to characterize the molecular interactions between LS174T cells and platelets in response to a low level of hydrodynamic shear (100 s^{-1}) in the absence of any exogenously added chemical agonist. As in the case of thrombin/GPRP-NH₂-treated platelets, specific antagonists of platelet P-selectin (EP5C7) and/or $\alpha_{\text{IIb}}\beta_3$ -integrins (c7E3 or XV454) did not inhibit the extent of heterotypic platelet-LS174T cell adhesive interactions (Fig. 6 A).

GP1b is abundantly expressed on the platelet surface (25,000 copies per platelet), and has previously been implicated in platelet-tumor cell interactions under static conditions (Oleksowicz et al., 1995). We therefore, investigated its potential contribution to platelet-LS174T heteroaggrega-

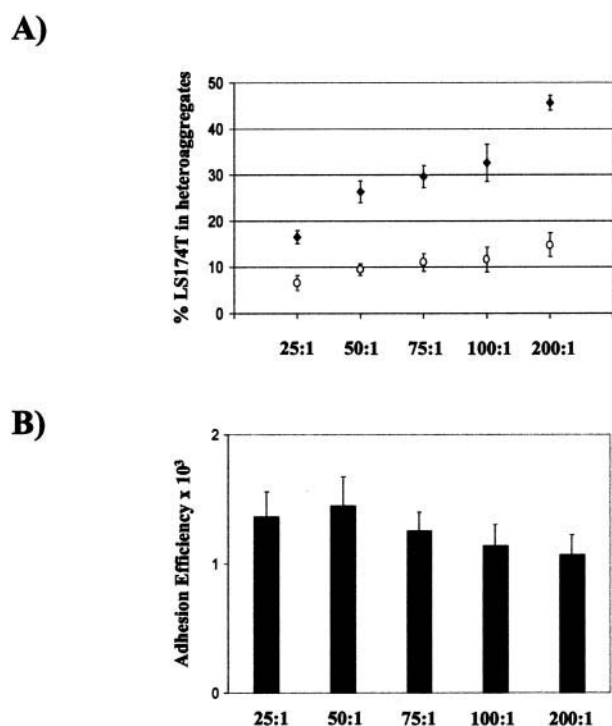


FIGURE 4 Effects of the platelet concentration on platelet-LS174T heteroaggregate formation under well-defined hydrodynamic shear conditions. Platelets ($2.5\text{--}20 \times 10^7$ cells/mL), pretreated for 10 min with fibrin polymerization inhibitor (2 mM GPRP-NH₂), and thrombin (0.25 units/mL), were combined with CMTMR-stained LS174T cells (1×10^6 cells/mL) to achieve the desired tumor cell:platelet ratio (ranging from 25:1–200:1) before the application of shear for 60 s at 100 s^{-1} . (A) Data represent the percent of LS174T cells in heteroaggregates. (B) Adhesion efficiency of heteroaggregation was calculated from experimental data using a mathematical model based on Smoluchowski's two-body collision theory. Data represent mean \pm SE of three to seven experiments.

tion using a function-blocking anti-GPIb specific antibody (SZ2). However, this mAb failed to affect the extent of platelet-LS174T cell binding (Fig. 6 A).

To further validate the aforementioned findings, we used PRP specimens from a patient with GT whose platelets are devoid of $\alpha_{IIb}\beta_3$ integrins, as well as a patient with BSS whose platelets are deficient in the GPIb-IX complex. The results indicate that both GT and BSS platelets adhered to LS174T cells as effectively as platelets from healthy volunteers when subjected to a shear rate of 100 s^{-1} for 120 s (Fig. 6 B). Furthermore, the presence of specific antagonists directed against platelet P-selectin and $\alpha_{IIb}\beta_3$ -integrin did not alter the percentage of LS174T cells with bound BSS platelets (data not shown). Taken together, our data suggest that platelet P-selectin, $\alpha_{IIb}\beta_3$ -integrin, or GPIb/IX are not likely to be involved in platelet-LS174T cell binding at low shear.

Previous work has shown that both homotypic platelet aggregation (Goldsmith et al., 1994) and heterotypic platelet-leukocyte adhesive interactions (Konstantopoulos et al.,

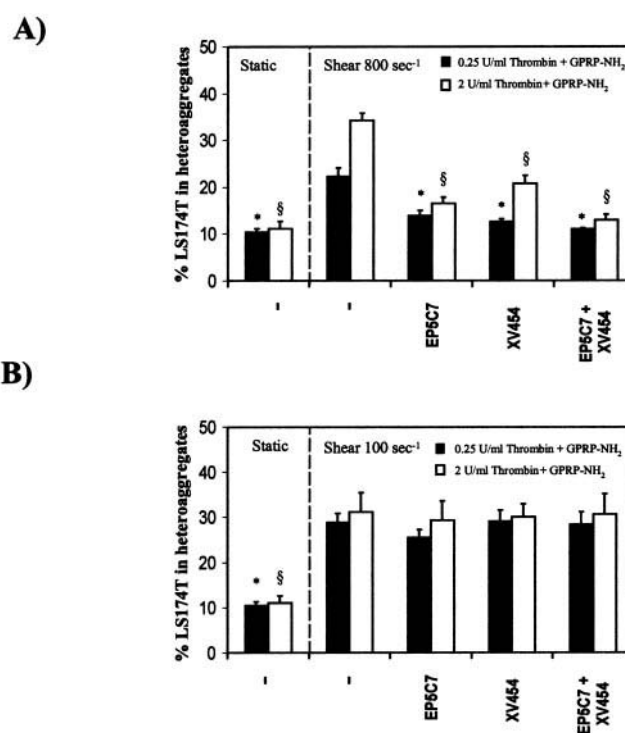


FIGURE 5 Effects of platelet P-selectin and $\alpha_{IIb}\beta_3$ antagonists on platelet-LS174T cell heteroaggregation at (A) high and (B) low shear. Platelets (5×10^7 cells/mL), pretreated for 10 min with fibrin polymerization inhibitor (2 mM GPRP-NH₂), and thrombin (0.25 units/mL or 2 units/mL) were combined with CMTMR-stained LS174T cells (1×10^6 cells/mL) in the cone-and-plate rheometer, and subjected to a shear of (A) 800 s^{-1} or (B) 100 s^{-1} for 60 s. In selected experiments, platelets were treated with agents specific for P-selectin (50 $\mu\text{g/mL}$ EP5C7) and/or $\alpha_{IIb}\beta_3$ -integrin (100 nM XV454) for 10 min before their mixing with untreated CMTMR-stained LS174T cells. Data represent the percentage of LS174T cells in heteroaggregates. * $p < 0.05$ with respect to shear in the absence of antibodies for treatment with 0.25 or 2 units/mL thrombin, respectively. Data represent mean \pm SE of three to five experiments.

1998b) in response to hydrodynamic shear are dependent upon the state of platelet activation. To assess the involvement of platelet activation in the formation of platelet-LS174T heteroaggregates, platelets were treated with PGE₁ immediately after blood collection. This treatment dramatically reduced the ability of platelets to form heterotypic aggregates with LS174T cells (Fig. 6 B), suggesting that platelet activation regulates these adhesive interactions. Consequently, these specific adhesion events are likely to be mediated by the activation of an array of integrins that are present on the platelet surface. Platelet integrins bind principally to Arg-Gly-Asp (RGD)-containing peptide sequences present in adhesive proteins such as fibrin(ogen) and von Willebrand factor (Konstantopoulos et al., 1998a). To assess the potential integrin involvement in platelet-LS174T cell binding induced by hydrodynamic shear, platelets were treated with a GRGDSP peptide before their mixing with tumor cells and application of shear. The re-

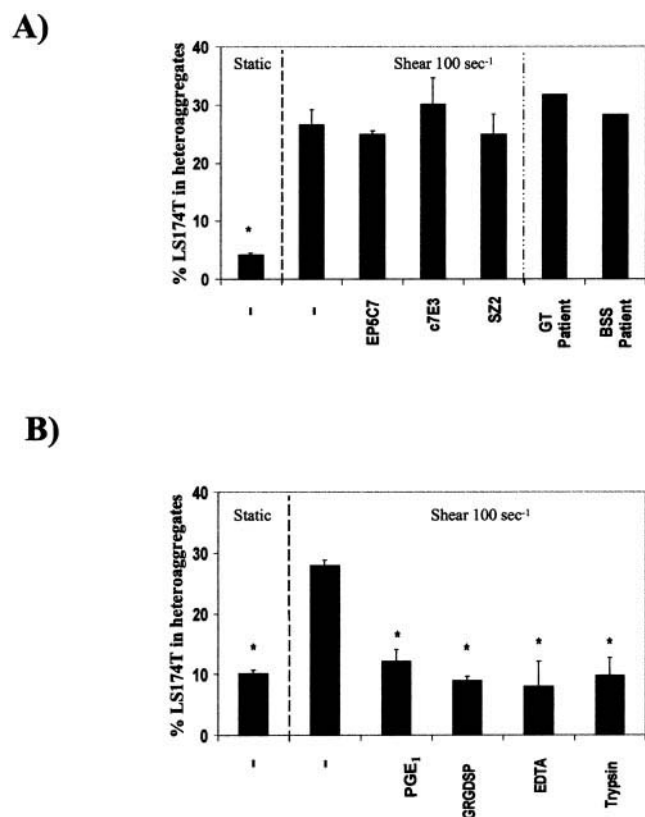


FIGURE 6 Characterization of molecular mechanisms mediating platelet-LS174T cell heteroaggregate formation under hydrodynamic shear conditions. (A) Contributions of platelet P-selectin, $\alpha_{IIb}\beta_3$, and GPIIb on platelet-LS174T cell heteroaggregation at low shear (100 s^{-1}) in the absence of any exogenously added chemical agonist. PRP (5×10^7 cells/mL), isolated either from healthy volunteers, a patient with GT, or a patient with BSS, was combined with CMTMR-stained LS174T cells (1×10^6 cells/mL) before the application of shear for 120 s at 100 s^{-1} . Upon termination of shear, aliquots were immediately fixed with 1% formaldehyde, postlabeled with the appropriate FITC-conjugated platelet-specific antibody (anti-GPIX-FITC or c7E3-FITC), and then analyzed by flow cytometry. In selected experiments, platelets were pretreated with anti-P-selectin ($50 \mu\text{g/mL}$ EP5C7), anti- $\alpha_{IIb}\beta_3$ -integrin ($20 \mu\text{g/mL}$ c7E3), or anti-GPIIb ($20 \mu\text{g/mL}$ SZ2) function-blocking antibodies. $*p < 0.05$ with respect to no-treatment control. Data represent mean \pm SE ($n =$ three to six healthy volunteers; $n = 1$ GT patient performed in triplicate; $n = 1$ BSS patient performed in duplicate). (B) Effects of platelet activation, peptides, and enzymes on platelet-LS174T cell aggregation. Platelets (5×10^7 cells/mL) and CMTMR-stained LS174T cells (1×10^6 cells/mL) were sheared as described in A. In selected experiments, platelets were treated with prostaglandin E_1 ($2 \mu\text{M}$ PGE₁), 20 mM GRGDSP peptide, or 5 mM EDTA before the shearing experiment. Alternatively, CMTMR-stained LS174T cells were treated with trypsin (0.25% type III trypsin). $*p < 0.05$ with respect to no-treatment control. Data represent mean \pm SE of 3 to 47 experiments.

sults indicate that the RGD-containing peptide GRGDSP inhibited the heteroaggregation between platelets and LS174T cells to baseline levels (Fig. 6 B). Similar results were also obtained when thrombin/GPRP-NH₂-treated platelets were sheared with LS174T cells at 100 s^{-1} (data not shown). In distinct contrast, a control Gly-Arg-Gly-Glu-

Ser-Pro peptide failed to affect the extent of platelet-LS174T cell binding (data not shown).

Experiments were also performed in the presence of 5 mM EDTA added to citrate-anticoagulated blood specimens immediately after venipuncture to assess the divalent cation requirements in this process (Konstantopoulos et al., 1998b). Fig. 6 B shows that the presence of EDTA in the suspension essentially abrogates platelet-LS174T cell binding in response to hydrodynamic shear exposure. This inhibitory effect might be ascribed to possible elimination of divalent cation-dependent integrin binding and/or suppression of cation-dependent platelet activation pathways (Konstantopoulos et al., 1998a).

We finally aimed to characterize the LS174T cell ligand(s) mediating heterotypic aggregation with platelets. Enzymatic treatment of the tumor cell surface with trypsin drastically reduced the percentage of LS174T cells with bound platelets. Taken together, our results indicate that platelet-LS174T cell binding in response to hydrodynamic shear is mediated by specific, RGD sequence dependent interactions between the platelet surface, and protease-sensitive glycoproteins on the colon carcinoma cell surface. However, the identity of adhesion receptors mediating platelet-LS174T heteroaggregation under low shear remains unknown.

DISCUSSION

To the best of our knowledge, this is the first study to analyze the kinetics and molecular requirements of platelet-tumor cell binding in bulk suspensions as a function of the dynamic shear environment encountered in the vasculature. The major findings of this work are: 1) hydrodynamic shear-induced collisions augment platelet-LS174T cell binding in the absence of any exogenously-added chemical agonist; 2) the capture efficiency of these heterotypic adhesive interactions is regulated by the state of platelet activation and decreases with increasing shear; 3) a transition in the molecular recognition events mediating heteroaggregation is observed with shear from an P-selectin-independent/RGD-dependent process in the low shear regime to a P-selectin/ $\alpha_{IIb}\beta_3$ -dependent process at high shear.

Hydrodynamic shear and platelet activation modulate the kinetics of platelet-LS174T cell heteroaggregation

Our data indicate that platelets readily coaggregated with LS174T cells when subjected to well-defined hydrodynamic shear conditions in the absence of any exogenous chemical stimulation. The efficiency of heteroaggregation varied with both shear rate and shear stress. In particular, peak efficiency was detected at low shear (20 to 50 s^{-1}), at which 2 to 3 of 1000 collisions resulted in stable heteroaggregate

formation. The adhesion efficiency of these interactions reduced with increasing shear rate, reaching a basal value at 1000 s^{-1} . Comparable efficiency values have been reported for the binding of platelets stimulated with low doses of ADP ($<0.5 \text{ } \mu\text{M}$) to fibrinogen-coated polystyrene beads (Bonney et al., 2000).

The adhesion efficiency of platelet-LS174T cell binding increased over a range of shear rates from 50 to 800 s^{-1} upon platelet treatment with thrombin/GPRP-NH₂. Inclusion of exogenous thrombin in the medium can stimulate platelets (by increasing the affinity of platelet receptors (e.g., $\alpha_{\text{IIb}}\beta_3$) for their respective ligands and inducing the release of adhesion molecules from intracellular pools onto the cell surface (e.g., P-selectin)) and degrade plasma and platelet-bound fibrinogen into fibrin. In distinct contrast, no detectable heteroaggregation occurred when platelets were pretreated with PGE₁ before their mixing with LS174T cells in a linear shear field. This compound has been shown to elevate cAMP levels in platelets and to inhibit platelet activation and aggregation (Hardwick et al., 1980). Taken altogether, our data suggest that platelet activation regulates the kinetics of platelet-LS174T cell heteroaggregation.

The efficiency of these adhesive interactions decreases with increasing shear rate, a finding that might be ascribed to either shorter intercellular contact durations and/or higher forces exerted on colliding cells. The influence of contact time and stress on platelet-LS174T cell binding was assessed in thrombin (0.25 units/mL)/GPRP-NH₂ (2 mM)-treated platelets by separately adjusting the shear rate and viscosity of the medium (Hentzen et al., 2000; Rinker et al., 2001). Apparently, the critical parameter (intercellular contact duration versus viscous forces (Chen and Springer 2001; Rinker et al., 2001; Swift et al., 1998)) affecting the rate and extent of platelet recruitment by LS174T cells is regulated by hydrodynamic shear environment. In particular, intercellular contact duration is the predominant factor limiting heteroaggregation at low shear ($\leq 200 \text{ s}^{-1}$), whereas these interactions become shear stress sensitive in the high shear regime ($\geq 400 \text{ s}^{-1}$). Furthermore, heteroaggregates once formed are resistant to breakage when subjected to high shear. This result suggests that the absence of heteroaggregate formation at high shear (1000 s^{-1}) is attributed to the inability of participating receptor-ligand pair(s) to form adhesive bonds at short contact times rather than to the disaggregation of formed aggregates due to their exposure to high shear forces.

The extent of platelet-LS174T cell binding increases with increasing platelet concentration at a constant LS174T cell number density, presumably due to the increase of the intercellular collision frequency. Maximal heteroaggregation was detected at near physiological platelet concentrations (20×10^7 platelets/mL). However, the capture efficiency of these interactions varied little with cell concentration, in agreement with previous work on neutro-

phil-ICAM-1 transfectant interactions (Hentzen et al., 2000).

Hydrodynamic shear regulates the specificity of platelet-LS174T cell interactions

We have recently demonstrated that LS174T cell adhesion to immobilized platelet substrates primarily occurs via a two-step, sequential process of interactions at a wall shear stress of 0.8 dyn/cm^2 (McCarty et al., 2000). Platelet P-selectin is essential for the optimal tethering/rolling of LS174T cells, whereas subsequent involvement of platelet $\alpha_{\text{IIb}}\beta_3$ integrins converts these transient interactions into stable adhesion. This wall shear stress level (0.8 dyn/cm^2) corresponds to a shear rate of $\sim 100 \text{ s}^{-1}$ and an intercellular contact duration of $\sim 1 \text{ ms}$ (Bongrand et al., 1988). However, the rotational streamlines in the cone-and-plate rheometer lead to substantially more frequent intercellular collisions with longer contact times than those occurring in parallel-flow geometry of the perfusion chamber. Previous work has shown that for a fixed shear rate the average contact duration for cell collisions in free-cell suspensions as modeled by the use of a cone-and-plate rheometer is ~ 25 -fold longer than those interactions occurring in a planar geometry (Bartok and Mason, 1957; Bongrand et al., 1988). As a first step, we wished to characterize the molecular recognition events involved in platelet-LS174T cell binding at a shear rate of 800 s^{-1} , and a corresponding intercellular duration of $\sim 3 \text{ ms}$. Treating platelets with thrombin/GPRP-NH₂ was shown to augment the recruitment of platelets to the tumor cell surface. Moreover, the extent of heteroaggregation correlated with platelet P-selectin expression levels in the high shear regime. Blocking P-selectin or $\alpha_{\text{IIb}}\beta_3$ integrin function by the use of mAbs and/or highly specific antagonists also significantly reduced the extent of platelet-LS174T binding. However, no additive effect was observed when P-selectin and $\alpha_{\text{IIb}}\beta_3$ integrin antagonists were used simultaneously. Selectin-ligand bonds have been reported to have high tensile strength and fast molecular association and dissociation rates (Smith et al., 1999). On the other hand, integrin-ligand bonds cannot be formed at high shear and corresponding short contact times in the absence of selectin contribution (Springer, 1995). In light of these observations, our functional data suggest that both P-selectin and $\alpha_{\text{IIb}}\beta_3$ integrins are required to mediate optimal LS174T cell binding in a sequential manner in the high shear regime.

In marked contrast, platelet-LS174T cell heteroaggregation at a shear rate of 100 s^{-1} , with a corresponding contact duration of $\sim 25 \text{ ms}$, is fundamentally different from the aforementioned two-step model. More specifically, blockade of platelet P-selectin and/or $\alpha_{\text{IIb}}\beta_3$ integrins had no effect on these heterotypic adhesive interactions. In accordance with the functional blocking assays, $\alpha_{\text{IIb}}\beta_3$ -deficient platelets isolated from a GT patient coaggregated with

LS174T cells as effectively and extensively as did platelets from normal volunteers. We next examined the potential involvement of platelet GPIb, because this adhesion receptor has previously been shown to mediate platelet-tumor cell interactions under static conditions (Oleksowicz et al., 1995). However, use of a function-blocking anti-GPIb mAb as well as GPIb-deficient platelets did not exhibit altered binding to LS174T cells compared with control platelets from healthy volunteers. Altogether, these data indicate that platelet P-selectin, $\alpha_{IIb}\beta_3$, and GPIb are not likely to be involved in platelet-LS174T cell heteroaggregation at a shear rate of 100 s^{-1} corresponding to an intercellular contact time of $\sim 25\text{ ms}$.

It is noteworthy that the equivalent wall shear rate in a parallel-plate perfusion system that allows collision durations in the order of $\sim 25\text{ ms}$ is $\sim 4\text{ s}^{-1}$, which corresponds to near static conditions. Ample evidence suggests that platelet integrins can effectively bind ligand(s) under static conditions. Moreover, several platelet integrins predominantly bind to RGD-containing peptide sequences present in adhesive proteins such as fibrin(ogen) and von Willebrand factor (Konstantopoulos et al., 1998a). Consequently, we assessed the potential integrin involvement in this adhesion process by incubating platelets with RGD-containing peptides before their mixing with LS174T cells in a linear shear field. Our data indeed indicate that an RGD-, but not a control RGE-, containing peptide essentially eliminated platelet-LS174T cell binding at 100 s^{-1} . Furthermore, the counter-receptor on the LS174T cell surface appears to be a protease-sensitive glycoprotein, as evidenced by abrogation of the heteroaggregation upon tumor cell treatment with trypsin. These observations support a model in which platelet integrins interact with a trypsin-sensitive epitope(s) on the LS174T surface in the low shear regime. It is very likely that fibrin may function as a potential adhesive bridge, as was recently shown for platelet-melanoma cell interactions (Biggerstaff et al., 1999). This is supported by our observations showing that inclusion of thrombin, which increases the extent of platelet-bound fibrin, and the fibrin polymerization inhibitor GPRP-NH₂ in the suspending medium significantly potentiates the extent of platelet binding to LS174T cells. Our data also provide clear evidence for divalent-cation requirements in this adhesion process, as evidenced by the abolition of heteroaggregation upon blood treatment with EDTA. Chelation of divalent-cations may inhibit integrin-ligand binding as well as platelet activation.

Taken altogether, this work clearly shows that the fluid mechanical environment of the circulatory system affects both the kinetics and receptor specificity of activation-dependent platelet binding to LS174T colon carcinoma cells. Elucidation of the detailed physical and molecular basis underlying platelet-colon carcinoma conjugate formation may provide insights for the rational development of novel therapeutic strategies aimed to alter these adhesive interactions.

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