

**THROMBOXANE GENERATION BY HUMAN MONOCYTES
ENHANCES PLATELET FUNCTION**

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Human blood monocytes interact with some plasma proteins involved in hemostasis (1, 2) and promote the activation of the coagulation pathway. This monocyte procoagulant activity (PCA), culminating in local deposition of fibrin, is essential for cell-mediated immunity (3). To characterize better the pathways common to immunity and hemostasis, we investigated whether monocytes, in addition to the clot-promoting activity, also influence the function of platelets. We have found that a specific receptor-ligand interaction mediated by a typical hemostatic protein (fibrinogen) triggers the activation by monocytes of the cyclooxygenase pathway. Furthermore, the monocyte thromboxane (Tx) produced under these conditions has a dramatic proaggregating effect on platelets.

Materials and Methods

Preparation of Cells. Blood obtained after informed consent from drug-free healthy donors was anticoagulated with acid/citrate dextrose solution. Platelets and platelet-free suspensions of human monocytes were prepared and characterized as previously described in detail (2).

Measurement of Platelet Function. Platelet aggregation studies were carried out at 37°C with continuous agitation at 1,000 rpm in an ELVI aggregometer (Milan, Italy). Aliquots (0.25 ml) of washed platelet suspensions (4×10^8 /ml) were stimulated with ADP (1–10 μ M; Sigma Chemical Co., St. Louis, MO) in the presence of purified fibrinogen (4.4×10^{-7} M). Each curve was monitored for 3 min. In some experiments, 10 mM EDTA (Sigma Chemical Co.) or 10 μ g/ml of 10E5 mAb to the glycoprotein IIb/IIIa complex (GP IIb/IIIa), kindly supplied by Dr. B. S. Coller, State University of New York, Stony Brook NY, were preincubated for 3 min with the platelet suspension at 37°C, without agitation, before the addition of the agonists. 125 I-Fibrinogen binding to ADP (10 μ M)-stimulated platelets was carried out basically as described by Kornecki et al. (4). 125 I-Fibrinogen binding to platelets was first studied in time-course experiments with a fixed concentration of labeled fibrinogen (0.2×10^{-7} M). For dose-response studies, increasing concentrations (0.02 – 8.8×10^{-7} M) of 125 I-fibrinogen were incubated at equilibrium with ADP-stimulated platelets.

The effect of autologous monocytes on platelet function was investigated as follows. Mixtures of washed platelets (4×10^8 /ml) and various concentrations of monocytes (0.5 – 2×10^7 /ml) were prepared in the same final volume as control suspensions of platelets without monocytes. Platelet aggregation and 125 I-fibrinogen binding to ADP (1–10 μ M)-stimulated platelet/monocyte mixtures were carried out as described above for suspensions of platelets only.

TxB₂ Production. TxB₂ (stable metabolite of TxA₂) formed during aggregation or 125 I-

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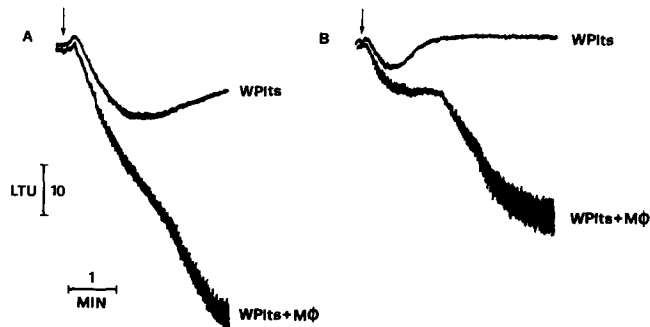


FIGURE 1. Effects of autologous added monocytes ($M\phi$) ($5 \times 10^6/\text{ml}$) on the aggregation of washed platelets ($WPIts$) ($4 \times 10^8/\text{ml}$) stimulated with $10 \mu\text{M}$ (A) or $1 \mu\text{M}$ (B) ADP (arrow) in the presence of 4.4×10^{-7} M fibrinogen. No increase in transmittance was seen when monocytes ($0.25 \text{ ml}; 2 \times 10^7/\text{ml}$) without platelets were challenged with ADP ($1-100 \mu\text{M}$) and fibrinogen (4.4×10^{-7} M), thus excluding that these cells participate in the formation of the aggregates. Finally, the ADP-induced aggregation of platelets, with or without monocytes, was equally blocked by 10 mM EDTA or by 10E5 mAb, confirming that the presence of monocytes potentiated the physiological mechanisms of platelet aggregation. LTU, Light transmission unit.

fibrinogen binding in platelet suspensions or in mixtures of platelets plus monocytes was measured by RIA (5). Specific antibody against TxB_2 was kindly supplied by Dr. J. B. Smith, Cardeza Foundation, Philadelphia, PA. In some experiments, monocytes ($5 \times 10^6/\text{ml}$) were preincubated with $10 \mu\text{M}$ of the cyclooxygenase inhibitor indomethacin (Sigma Chemical Co.) for 45 min at room temperature. Platelet aggregation, ^{125}I -fibrinogen binding and TxB_2 measurement in mixtures of platelets plus indomethacin-treated monocytes were carried out as described above.

Cellular Source and Requirements for TxB_2 Production. Additional experiments were performed to identify the origin of TxB_2 formed in platelet/monocyte mixtures during platelet aggregation and ^{125}I -fibrinogen binding. Aliquots of monocyte suspensions ($5 \times 10^6/\text{ml}$) were incubated without platelets but with $10 \mu\text{M}$ ADP and 1 mM CaCl_2 for 5 min at room temperature in the presence of increasing concentrations of fibrinogen ($0.17-7.3 \times 10^{-7}$ M). Controls were platelets ($4 \times 10^8/\text{ml}$) in the absence of monocytes incubated under the same experimental conditions. At the end of the incubation, cell suspensions were centrifuged at 12,000 g for 2 min at room temperature and the supernatant was assayed for TxB_2 . TxB_2 was measured in suspensions of monocytes ($5 \times 10^6/\text{ml}$) without platelets incubated for 5 min at room temperature in the presence of 1 mM CaCl_2 , either with $10 \mu\text{M}$ ADP only or with 7.3×10^{-7} M fibrinogen only. Finally, aliquots of monocyte suspensions ($5 \times 10^6/\text{ml}$) were preincubated with 10 mM EDTA or 20 $\mu\text{g}/\text{ml}$ of 10E5 mAb for 3 min at room temperature before the addition of ADP and fibrinogen. Monocyte TxB_2 formed under these conditions was measured as described above.

Results

Effects of Autologous Monocytes on Platelet Function. Fig. 1 compares the aggregating response of platelets or platelet/monocyte mixtures with subthreshold concentrations of ADP. Washed platelets stimulated with $10 \mu\text{M}$ ADP (Fig. 1A) had a single reversible wave of aggregation, or only a slight transient increase in transmittance when lower concentrations of ADP ($1 \mu\text{M}$) were used (B). In contrast, monocytes mixed with platelets induced a prompt irreversible wave of aggregation (A) or a biphasic curve with a typical release reaction (Fig. 1B). ^{125}I -fibrinogen binding to ADP-stimulated platelets with or without monocytes gave

data complementary to those of the aggregation studies. In fact, in time-course experiments, the amount of ^{125}I -fibrinogen bound to platelets was four to five times greater in the presence of monocytes than in suspensions of platelets only (Fig. 2A). Dose-response studies showed that for each concentration of ^{125}I -fibrinogen added, mixtures of platelets plus monocytes bound three to four times more labeled protein than platelets only (Fig. 2B).

Mechanisms of Monocyte Enhancement of Platelet Function. We asked ourselves whether a soluble factor released by monocytes under these conditions was responsible for the potentiation of platelet function. We focused our attention on Tx because it is a major product of the monocyte cyclooxygenase pathway (6), and its proaggregating effect on platelets is well known (7). RIA measurements revealed that the enhancement of platelet aggregation seen in platelet/monocyte mixtures was associated with the formation of 65 ± 10 pmol/ml of TxB_2 (means \pm SE, four experiments), while only 5.3 ± 2 pmol/ml of TxB_2 were formed during the aggregation of platelets alone (four experiments). Table I shows that the increased binding of fibrinogen to platelets in platelet/monocyte mixtures was also associated with progressively more synthesis of TxB_2 . In contrast, TxB_2 formed in suspensions of platelets only was insignificant for each concentration of fibrinogen tested (Table I). Furthermore, when monocytes were preincubated with $10 \mu\text{M}$ indomethacin and mixed with platelets, the enhancement of platelet aggregation and ^{125}I -fibrinogen binding was completely suppressed. Control experiments showed that under these conditions the synthesis of TxB_2 in mixtures of platelets plus indomethacin-treated monocytes was abolished (Table I). The requirements for the synthesis of TxB_2 by monocytes were further investigated. Monocytes without platelets but in the presence of CaCl_2 , ADP, and fibrinogen generated the same amounts of TxB_2 as in mixtures of platelets plus monocytes under the same experimental conditions (not shown). This excludes a collaborative participation of platelets in inducing TxB_2 formation by monocytes. Furthermore, effective synthesis of TxB_2 occurred only after monocyte incubation with both ADP and fibrinogen (Table II). Similarly, after cell treatment with EDTA or 10E5 mAb the monocyte generation of TxB_2 was suppressed to basal values (Table II).

Discussion

In this study, we showed that human monocytes, in addition to PCA, greatly enhance the aggregation of platelets. Monocyte potentiation of platelet aggregation is rapid and is due to an increased binding of fibrinogen to platelets. We investigated the possibility that the monocyte-induced enhancement of platelet aggregation followed the pathways generally involved in the modulation of immune reactions. In fact, the monocytes regulate inflammation through the release of soluble factors, particularly arachidonic acid metabolites. Prostaglandins of the E series modulate the tumoricidal functions (8), leukotrienes are specific inflammatory mediators of allergic reactions (9) and Tx is involved in natural cytotoxic activity (10) and has proaggregating properties (7). Therefore, the role of monocyte Tx in enhancing platelet aggregation was investigated. While virtually no TxB_2 was produced during ADP activation of platelets only, the enhancement of platelet function in mixtures of platelets plus monocytes was

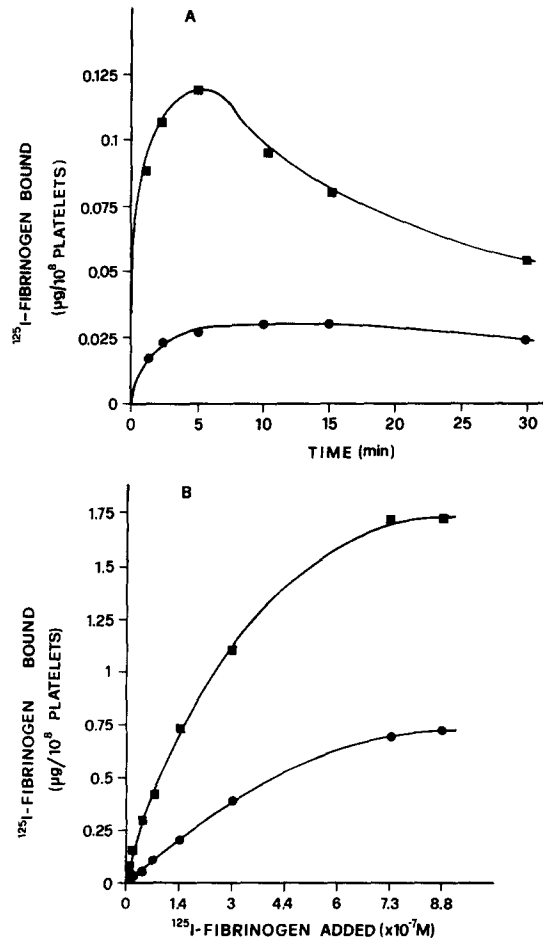


FIGURE 2. Time-course (A) and dose-response curve (B) of ^{125}I -fibrinogen-specific binding to ADP ($10\ \mu\text{M}$)-stimulated platelets ($4 \times 10^8/\text{ml}$) in the presence (filled square) or in the absence (filled circle) of monocytes ($5 \times 10^6/\text{ml}$). Under these conditions both platelets and monocytes bound ^{125}I -fibrinogen. However, control experiments not shown in the figure revealed that the amount of ^{125}I -fibrinogen bound by 5×10^6 monocytes/ml was insignificant as compared with platelet-bound fibrinogen: $0.007\ \mu\text{g}/10^6$ monocytes ($1.4 \times 10^{-7}\text{M}$ ^{125}I -fibrinogen added); $0.013\ \mu\text{g}/10^6$ monocytes ($3 \times 10^{-7}\text{M}$ ^{125}I -fibrinogen added); $0.024\ \mu\text{g}/10^6$ monocytes ($7.3 \times 10^{-7}\text{M}$ ^{125}I -fibrinogen added). Therefore, ^{125}I -fibrinogen bound in platelet-monocyte mixtures was not significantly overestimated because of the amount of specific monocyte-bound fibrinogen. Specific binding data were analyzed by the method of Scatchard. Two classes of receptors were found. The values for binding constants (K_d) were similar with or without monocytes (platelets/platelet-monocyte mixtures: low-affinity $K_d = 8.2/2.4 \times 10^{-7}\text{M}$; high-affinity $K_d = 1.92/2.8 \times 10^{-8}\text{M}$). In contrast, a sevenfold increase in the number of high-affinity fibrinogen receptors was found in the platelet/monocyte mixture ($n = 7,620$) over that in platelets only ($n = 1,160$). Smaller differences were seen in the number of low-affinity receptors (platelets/platelet-monocyte mixtures: $30,120/44,800$). Each point is the mean of three different experiments.

associated with generation of large amounts of TxB_2 . Similarly, the synthesis of TxB_2 and the enhancement of platelet function were completely suppressed after preincubation of monocytes with indomethacin. These findings confirmed that

TABLE I
¹²⁵I-Fibrinogen (¹²⁵I-FG)-specific Binding and TxB₂ Production in Suspensions of ADP (10 μM)-stimulated platelets (WPlts), mixtures of platelets plus monocytes (WPlts + Mφ), mixtures of platelets plus indomethacin-treated monocytes (WPlts + indo-Mφ)

¹²⁵ I-FG added (×10 ⁻⁷ M)	WPlts		WPlts + Mφ		WPlts + indomethacin-Mφ	
	¹²⁵ I-FG bound (molecules/platelet)	TxB ₂ (pmol/ml)	¹²⁵ I-FG bound (molecules/platelet)	TxB ₂ (pmol/ml)	¹²⁵ I-FG bound (molecules/platelet)	TxB ₂ (pmol/ml)
0.1	586 ± 180	0.5	2,610 ± 1,280	21 ± 8	300 ± 78	0.5
0.3	1,192 ± 310	1.0	5,675 ± 2,641	27 ± 10	ND	ND
0.7	2,176 ± 500	0.9	7,383 ± 4,200	32 ± 14	1,424 ± 12	1.0
1.4	4,129 ± 900	0.2	12,814 ± 6,530	44 ± 4	2,715 ± 25	0.5
3.0	6,390 ± 2,050	0.2	19,600 ± 9,000	50 ± 15	4,552 ± 311	1.5
7.3	12,210 ± 4,677	0.5	30,500 ± 8,000	74 ± 17	ND	ND

¹²⁵I-Fibrinogen binding and TxB₂ measurement were performed as described in Materials and Methods. Results are means ± SE (four experiments).

TABLE II
 Requirements for the Synthesis of TxB₂ by Monocytes

Cell suspension	Number of experiments	TxB ₂ (pmol/ml)
Monocytes + RPMI 1640	4	14 ± 1.4
Monocytes + ADP + fibrinogen	4	115 ± 12.5
Monocytes + ADP	6	20 ± 8.06
Monocytes + fibrinogen	6	14 ± 9.08
EDTA-treated monocytes	6	16.5 ± 3.9
10E5*-treated monocytes	6	18.5 ± 7.1

Experimental procedures and TxB₂ measurement as described in Materials and Methods. Results are means ± SE.

* 10E5, mAb blocking the receptor for fibrinogen on monocytes.

the TxB₂ measured in platelet/monocyte mixtures was of monocyte origin and directly responsible for the effect on platelets.

Previous studies (11) indicate that the common denominator for the monocyte prostaglandin production is a "membrane perturbation" occurring after the binding of a ligand to its specific cell surface receptor. We recently showed (2) that monocytes possess an ADP-inducible receptor for fibrinogen and that the interaction of fibrinogen with monocytes induces rapid expression of PCA by these cells. We now show that only after the ADP-induced interaction of fibrinogen with monocytes is there synthesis of TxB₂ by these cells. Without a stimulus (ADP) or the ligand (fibrinogen), or when Ca²⁺ ions have been removed (cell preincubation with EDTA), fibrinogen does not interact with monocytes, and the synthesis of TxB₂ is abolished. Similarly, when the receptor for fibrinogen on monocytes is blocked by 10E5 mAb there was no synthesis of TxB₂. Therefore, fibrinogen itself might function as a soluble membrane-perturbing agent to induce, like the classical inflammatory stimuli, the synthesis of TxB₂ by monocytes. The biological relevance of this phenomenon is reflected in the dramatic enhancement of platelet response to a stimulus such as ADP, which is relatively ubiquitous in vivo.

In conclusion, the data presented here further emphasize the role of human monocytes as regulatory cells of several biologic systems. Furthermore, plasma

fibrinogen appears to have the same mechanisms as inflammatory stimuli for inducing monocyte prostaglandin production.

Summary

Human monocytes potentiate the ADP-stimulated aggregation of autologous platelets through a fourfold increased binding of ^{125}I -fibrinogen to the platelet surface. The enhancement of platelet function is rapid, relatively transient and is due to thromboxane (Tx) synthesized by monocytes under these conditions. Tx generation by monocytes is triggered by the interaction between fibrinogen and the specific monocyte membrane receptor. These data suggest that the monocyte enhancement of platelet function combined with the clot-promoting activity of these cells might unbalance normal hemostasis.

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