Fibrinogen mediated activation of platelet aggregation and thromboxane A₂ release: pathological implications in vascular disease

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SUMMARY The effect of a human fibrinogen preparation on in vitro platelet aggregation was assessed. Platelets were obtained from healthy volunteers. Human fibrinogen induced platelet aggregation in 65% of platelet rich plasma samples and enhanced submaximal platelet aggregation induced by heparin or by several conventional agonists in all samples. Aggregation induced by fibrinogen alone was reversed by the in vitro addition of human albumin. Fibrinogen induced aggregation was associated with the release of the vasoconstrictor, thromboxane A₂. Preincubation with indomethacin inhibited both the aggregation and the release of thromboxane A₂. Fibrinogen had no effect on in vitro vascular prostaglandin I₂ synthesis (rat aortic rings) during a 60 minute incubation. The observed effects of fibrinogen on platelet function may be relevant to clinical conditions in which hyperaggregability of platelets is associated with hyperfibrinogenaemia and thrombosis.

The association between increased plasma fibrinogen concentrations and an increased incidence of vascular disease is well documented.¹⁻³ Increased plasma fibrinogen concentrations have been reported in patients with peripheral vascular disease,¹⁻³ diabetes mellitus,¹⁻³ Raynaud's syndrome,¹⁻⁴ nephrotic syndrome,⁵ angina,⁶ myocardial infarction,^{1-3,6,7} stroke,¹⁻³ postsurgical trauma,^{1-3,8} in smokers,¹⁻³ and in women taking oral contraceptives.¹⁻³ The converse also appears to be true: low plasma fibrinogen concentrations are associated with a diminished risk of arterial disease,¹⁻³ and preoperative partial defibrination results in a reduced incidence of postoperative venous thrombosis.^{1,2} Epidemiological studies suggest that fibrinogen may even be a better predictor of coronary death than cholesterol.³

It would, therefore, be of interest to explore, under controlled conditions, the potential mechanisms by which fibrinogen could enhance thrombogenesis. Several mechanisms have been considered, including the effect of fibrinogen on plasma viscosity, coagulation, and fibrinolysis.¹⁻³ Fibrinogen also binds to platelets and is an essential requirement for platelet aggregation induced by cer-

tain agonists.⁹ Platelet hyperactivity has also been reported in most of the conditions in which plasma fibrinogen concentrations are increased.^{1-3,10,11} Clinical trials have reported a highly significant correlation between plasma fibrinogen concentration and circulating platelet aggregates.¹² Furthermore, reduction of the fibrinogen concentration after the administration of anacard resulted in a reduction in the number of circulating platelet aggregates.¹² In vitro studies, however, are conflicting. Some report that fibrinogen enhances platelet adhesion,¹ while others show that fibrinogen inhibits¹ or enhances^{1,13,14} platelet aggregation. To our knowledge, the induction of platelet aggregation in vitro by fibrinogen alone has not been reported. Finally, agents (including thromboxane A₂) that induce aggregation expose platelet fibrinogen receptors, whereas inhibitors of aggregation (including prostacyclin) have the opposite effect.¹⁵⁻¹⁷

The present study investigated certain aspects of the platelet-fibrinogen relation in vitro using a human fibrinogen preparation. Since the vascular endothelium produces prostacyclin (prostaglandin I₂), a potent inhibitor of platelet aggregation,¹⁸ we also investigated the effect of short term incubation with fibrinogen on prostaglandin I₂ synthesis using rat aortic rings as a model.

Material and methods

Human fibrinogen was obtained from Sigma Chemicals (Poole, England). This fibrinogen preparation was 60% protein (over 90% of clottable protein) and contains about 15% sodium citrate and about 25% sodium chloride (information in current Sigma catalogue). Adrenaline, adenosine diphosphate, serotonin, human fraction V albumin, and essentially fatty acid free human albumin were obtained from Sigma Chemical Company. Heparin (Thromboliquine; a conventional high molecular weight fraction sodium heparin of porcine intestinal mucosal origin) was a gift from Organon International BV (Oss, Holland). Collagen was obtained from Hormon-Chemie (Munich, West Germany).

Venous blood was obtained from healthy male and female volunteers who had not ingested any drugs for at least two weeks before sampling. The number and sex of subjects selected in each experiment are described in the appropriate subsections below.

Unless otherwise stated, venous blood was collected in 3.8% trisodium citrate, as previously described.¹⁹ Platelet rich plasma and platelet poor plasma were prepared by centrifugation at room temperature.¹⁹ Thereafter, all platelet rich plasma was kept at 37°C throughout the experimental procedures: we have previously shown that cooling of platelet rich plasma at temperatures below 37°C affects platelet function.²⁰

STRUCTURE OF EXPERIMENTS

Platelet aggregation and thromboxane A₂ release induced by human fibrinogen preparations

Fibrinogen was dissolved in physiological (0.9%) saline (final fibrinogen concentration 20–40 g/l). Various amounts of this solution (Table 1) were added to platelet rich plasma obtained from 20 healthy volunteers (12 men, eight women).

The concentration of fibrinogen in platelet rich plasma (in the absence of added exogenous fibrinogen) was 1.9 to 2.2 g/l. These concentrations are lower than the expected normal value because of the added citrate, a requirement for the preparation of platelet rich plasma. It follows that, after the addition of 2 and 4 g/l of exogenous fibrinogen, the total concentration of fibrinogen in the platelet rich plasma ranged between 3.9–4.2 and 5.9–6.2 g/l. These small variations in fibrinogen concentration did not influence platelet function in our assay system. It was therefore unnecessary to make minor adjustments to the concentrations of exogenous fibrinogen, especially at the cost of delaying the platelet aggregation experiments, which depend on

Table 1 Effect of human fibrinogen on platelet aggregation (n = 20). Figures are percentage aggregation

Solution	Median	Range
Platelet rich plasma + saline only (control experiment)	4	0–10
Platelet rich plasma + fibrinogen†	37*	0–80
Platelet rich plasma + fibrinogen‡	58*	0–82

*p < 0.01, control platelet rich plasma (PRP) v PRP with fibrinogen added (paired Wilcoxon test).

†Final concentration of added fibrinogen 2 g/l.

‡Final concentration of added fibrinogen 4 g/l.

cell viability and therefore on the freshness of the sample.

As added safeguards, we (a) measured plasma fibrinogen concentrations (while aggregations were being carried out) to ensure that the values did not deviate from the range quoted above. Measurements were carried out using a Cobas Fibro (Roche Instruments, Welwyn Garden City, Herts, England); and (b) compared responses in platelet rich plasma samples with the lowest initial fibrinogen concentration (1.9 g/l) with those samples with the highest initial concentrations (2.2 g/l); there were no differences in the patterns of enhancement.

Aggregation was expressed as the percentage fall in optical density^{19,20} and monitored for up to 15 minutes or until a maximum fall in optical density was sustained for one minute.

The platelet rich plasma from six subjects who reacted (more than 50% aggregation) to human fibrinogen (final concentration of added fibrinogen in the platelet rich plasma 4 g/l) was also assayed for thromboxane A₂ generation, as previously described.²¹ Briefly, when maximal aggregation had occurred the platelet rich plasma (500 µl) was added to 1 ml ethanol (to stop the reaction) and vigorously mixed. The sample was then stored at –70°C until assay using a specific radioimmunoassay²¹ for thromboxane B₂, the stable spontaneous breakdown product of thromboxane A₂. Platelet counts in platelet rich plasma were measured using a Coulter Counter Model D (Coulter Electronics Limited, Luton, Beds, England) to allow the expression of thromboxane B₂ results in terms of platelet count.²¹ Aggregation induced by fibrinogen in these six subjects was also repeated in platelet rich plasma that had been pre-incubated for one minute at 37°C with indomethacin (final concentration 8 mg/l).²²

The aggregation experiments with fibrinogen were repeated in four men and three women, with blood collected in 3.4% citrate instead of 3.8% citrate, in order to establish whether the citrate contained in the human fibrinogen preparation influenced the results.

All experiments were conducted with saline only (same volume as the fibrinogen-saline solution)

added to platelet rich plasma to act as controls. Aggregations were always run in parallel, one aggregometer channel containing platelet rich plasma and fibrinogen and the other channel containing platelet rich plasma and saline (control).

Enhancement of platelet aggregation by fibrinogen

Submaximal platelet aggregation was induced using low concentrations of adenosine diphosphate, adrenaline, serotonin, or collagen. Table 2 shows the final concentrations of these agonists. Eight subjects (five men, three women) were studied with each aggregating agent. The low concentrations of agonists were selected to produce submaximal aggregation and so allow the quantification of any enhancement of platelet aggregation after the addition of fibrinogen.^{23 24}

The potential enhancing effect of fibrinogen was also evaluated after the addition of heparin (Thromboliquine, Organon). The concentration of heparin selected (0.8–3 IU/ml) was always insufficient to cause more than 10% aggregation when added alone to platelet rich plasma.

Fibrinogen was added to platelet rich plasma in the aggregometer (final concentration of added fibrinogen 2 g/l). The amount of fibrinogen was selected so as not to cause substantial platelet aggregation (<10%) when added alone to platelet rich plasma from the same subject. After five minutes incubation, at 37°C in the aggregometer cuvette, the submaximal dose of the appropriate aggregating agent (or heparin) was added.

Controls consisted of the appropriate volumes of saline added in place of the fibrinogen-saline solutions. Aggregation was monitored until a maximal fall in optical density was sustained for one minute. Experiments were always run in parallel, one channel recording the platelet rich plasma plus saline plus submaximal dose of agonist (control experiment), the other platelet rich plasma plus fibrinogen plus submaximal dose of agonist.

In addition to the experiments described above, we also investigated whether fibrinogen induced enhancement of aggregation antagonises the

inhibitory action of prostacyclin (prostaglandin I₂). Platelet rich plasma from four subjects was tested in the presence of exogenous fibrinogen (final concentration of added fibrinogen 2 g/l) or of the appropriate volume of the saline vehicle, as described above. After five minutes of incubation of platelet rich plasma with saline or fibrinogen prostaglandin I₂ was added (final concentration 0.5–1 ng/ml), and one minute later aggregation was induced by adding adenosine diphosphate (final concentration 1–2 µmol/l). Percentage aggregation was recorded as the percentage fall in optical density three minutes after adding the adenosine diphosphate. Further details describing the technique of using prostaglandin I₂ as an inhibitor of *in vitro* platelet aggregation are available elsewhere.^{19 20}

“Neutralisation” of human fibrinogen induced platelet aggregation by the addition of albumin preparations

Others have shown that the addition of essentially fatty acid free human albumin to platelet rich plasma can abolish spontaneous platelet aggregation in samples obtained from patients with nephrotic syndrome.¹¹ This condition is associated with both raised plasma fibrinogen concentrations⁸ and enhanced platelet aggregation.^{10 11} We therefore attempted to mimic this “neutralisation” of platelet aggregation in our *in vitro* experiments.

Human fraction V albumin and essentially fatty acid free human albumin were dissolved in physiological (0.9%) saline. Various volumes of these albumin solutions were added to platelet rich plasma (final concentration of added albumin 3.2–6.0 g/l). After two minutes of incubation in the aggregometer the fibrinogen-saline solution was added; the concentration of added fibrinogen (0.8–3.2 g/l) having been previously shown to induce at least 50% aggregation in the same samples of platelet rich plasma (six men, four women) in the absence of added albumin. Aggregation was then monitored for the same period of time as that required to produce maximum fibrinogen induced aggregation in the absence of albumin (time range

Table 2 *Enhancing effect of human fibrinogen on platelet aggregation (n = 8) induced by several aggregating agents. Figures are median and range of percentage aggregation*

Solution	Aggregating agent dose				
	ADP (0.2–1.0 µmol/l)	Adrenaline (0.2–1.0 µmol/l)	Collagen (0.15–0.5 mg/l)	Serotonin (10–50 µmol/l)	Heparin (0.4–3 U/ml)
PRP + saline only (control experiment)	5 (0–10)	8 (0–14)	3 (0–11)	0 (0–17)	3 (0–11)
PRP + saline + fibrinogen*	50 (33–62)	52 (35–56)	37 (27–46)	45 (20–78)	63 (46–68)
p value†	<0.01	<0.01	<0.01	<0.01	<0.01

*Final concentration of added fibrinogen in platelet rich plasma (PRP) 2 g/l.

PRP = platelet rich plasma; ADP = adenosine diphosphate. †Paired Wilcoxon test.

three to 12 minutes). Control experiments consisted of appropriate volumes of saline added instead of the albumin solutions. Experiments were always run in parallel, one channel recording the platelet rich plasma plus albumin preparation plus fibrinogen, the other platelet rich plasma plus saline plus fibrinogen.

The effect of essentially fatty acid free and fraction V albumin, added *in vitro*, on platelet aggregation induced by conventional agents (adenosine diphosphate, adrenaline, collagen) was also assessed. Only the maximum concentrations of essentially fatty acid free albumin and fraction V albumin (6 g/l) used to abolish fibrinogen induced aggregation were evaluated. The concentration of agonists used (adenosine diphosphate 2–3 μmol/l; adrenaline 2–5 μmol/l; collagen 0.2–1 mg/l) were selected so as to induce a percentage platelet aggregation similar to that induced by fibrinogen alone. Nine subjects (five men, four women) were evaluated.

Effect of human fibrinogen on prostaglandin I₂ release by rat aortic rings

Since fibrinogen was found to enhance and even occasionally to initiate platelet aggregation we investigated whether it also exerted any influence on prostaglandin I₂ synthesis using rat aortic rings as a model. Rat aortic rings were prepared as previously described.^{21, 22} They were incubated in phosphate buffered saline (pH 7.4) at 37°C for 60 minutes in the absence and presence of fibrinogen (final concentrations 1, 2, 4, 8, and 16 g/l). The amount of 6-oxo-prostaglandin F_{1α} (the stable spontaneous metabolite of prostacyclin, prostaglandin I₂) released into the buffer during the 60 minutes incubation was quantified using a specific radioimmunoassay.^{21, 22} The amount of citrate in each incubate was kept constant by adding appropriate amounts of citrate to the incubates with the lower fibrinogen content.

Further characterisation of the human fibrinogen preparation

The proportion of clottable protein was confirmed as being 95% for the fibrinogen batch used in the experiments described above. A fibrinogen degradation product assay (in routine use at the Royal Free Hospital) was also performed. Fibrinogen was pre-assayed for proaggregatory potency. Sodium dodecyl sulphate (SDS) gel electrophoresis was also performed.

STATISTICAL ANALYSIS AND EXPRESSION OF RESULTS

Results are expressed as median and range. Statistical comparisons were made using the non-

parametric paired Wilcoxon rank sum test (two tail)²⁵ to compare the observations in the control experiments with those in the test experiments.

Results

PLATELET AGGREGATION AND THROMBOXANE A₂ RELEASE BY HUMAN FIBRINOGEN

Human fibrinogen (final concentration of added fibrinogen in platelet rich plasma 4 g/l) resulted in substantial (>33%) aggregation in 13 (65% of samples) samples of platelet rich plasma obtained from 20 healthy subjects. Table 1 shows the median and range of percentage aggregation. Fibrinogen induced aggregation was more substantial and tended to occur earlier with higher fibrinogen concentrations. Lower and intermediate concentrations of fibrinogen, not shown in Table 1, were tested in a few subjects and confirmed the same trend.

Fibrinogen induced release of thromboxane A₂ in platelet rich plasma obtained from the six subjects who were selected because fibrinogen (final concentration of added fibrinogen in platelet rich plasma 4 g/l) had induced more than 50% aggregation. The median platelet thromboxane A₂ release was 32, and the range was 15–60 ng of thromboxane B₂/ml of platelet rich plasma or 10 (5–18) ng of thromboxane B₂/10⁸ platelets. Samples of platelet rich plasma, with saline only added, did not produce any substantial aggregation or thromboxane A₂ release; in these samples of platelet rich plasma percentage aggregation was 4(0–10)% and thromboxane A₂ release was <2 ng of thromboxane B₂/ml of platelet rich plasma (<1 ng of thromboxane B₂/10⁸ platelets). Preincubation with indomethacin eliminated both the aggregation (to <10% aggregation) and the thromboxane A₂ release (about 95% inhibited).

Collecting blood at lower citrate concentrations (3.4%) did not appreciably alter the results previously observed with fibrinogen and blood collected at the higher citrate concentration (3.8%).

HUMAN FIBRINOGEN INDUCED ENHANCEMENT OF PLATELET AGGREGATION (TABLE 2)

Fibrinogen consistently and appreciably enhanced submaximal platelet aggregation induced by several agonists (adenosine diphosphate, adrenaline, collagen, serotonin, heparin). Heparin alone was unable to induce platelet aggregation, but aggregation was observed when fibrinogen (final concentration 0.8–3.2 g/l) was also present. Exogenous fibrinogen antagonised prostaglandin I₂ mediated inhibition of aggregation, the percentage aggregation always being greater in the samples of platelet rich plasma to which exogenous fibrinogen had been

added than in the samples of platelet rich plasma with saline only.

"NEUTRALISATION" OF FIBRINOGEN INDUCED PLATELET AGGREGATION BY THE ADDITION OF ALBUMIN PREPARATIONS

Both human fraction V albumin and essential fatty acid free human albumin were capable of abolishing the platelet aggregation induced by fibrinogen. Fatty acid free human albumin was more effective than fraction V albumin (Table 3). Both fraction V and essentially fatty acid free albumin, at the maximum final concentrations used in the fibrinogen experiments (Table 3), only minimally inhibited adenosine diphosphate, adrenaline and collagen induced platelet aggregation despite the fact that the percentage aggregation induced by these latter agents was of the same magnitude as that observed with fibrinogen alone. In these latter experiments fraction V albumin had similar effects to those of essentially fatty acid free albumin.

EFFECT OF HUMAN FIBRINOGEN ON PROSTAGLANDIN I_2 RELEASE FROM RAT AORTIC RINGS

Short term incubation (60 minutes) of rat aortic rings with human fibrinogen did not appreciably alter prostaglandin I_2 production, which was of an order similar to that previously reported by us.^{21, 22}

FURTHER CHARACTERISATION OF THE HUMAN FIBRINOGEN PREPARATION

The fibrinogen degradation product assay (normal range in plasma $<10 \mu\text{g/ml}$) detected $<10 \mu\text{g}$ fibrinogen degradation product/ml of fibrinogen solution (final concentration of fibrinogen preparation 40 g/l). Heat precipitation of fibrinogen showed that the supernatant did not retain the ability to induce platelet aggregation. SDS gel electrophoresis showed the expected bands.

Table 3 *Effect of fraction V human albumin and essentially fatty acid free human albumin on platelet aggregation ($n = 10$) induced by human fibrinogen in vitro. Figures are median and range*

Final concentration (g/l) of albumin preparation added to PRP	Platelet aggregation (%) induced by human fibrinogen [‡]
0 (control)	68 (50-82)
4.0	
Fraction V albumin	48 (4-56)
Fatty acid free albumin	10 (2-47)*
6.0	
Fraction V albumin	25 (1-70)*
Fatty acid free albumin	0 (0-8)**†

* $p = 0.05$; ** $p = 0.01$; controls (no albumin added) v albumin containing samples (paired Wilcoxon rank sum test).

† $p < 0.05$; fraction V containing samples v fatty acid free albumin containing samples.

‡Final concentration of added fibrinogen 4 g/l.
PRP = platelet rich plasma.

Discussion

Our findings clearly show that human fibrinogen cannot only enhance but also induce human platelet aggregation in platelet rich plasma. Other highly purified fibrinogen preparations^{13, 14} also enhance in vitro platelet aggregation induced by other agents under various experimental conditions. To our knowledge, however, platelet aggregation induced by the addition of fibrinogen alone has not been previously reported. In contrast, fibrinogen had no effect on vascular prostaglandin I_2 release, but it did antagonise prostaglandin I_2 mediated inhibition of in vitro platelet aggregation.

The question remains whether our findings with human fibrinogen relate to pathological situations in vivo. Although this question cannot be conclusively answered, there is evidence that our in vitro findings are in accord with in vivo observations. The behaviour of human fibrinogen in the present study would go some way to explaining why plasma fibrinogen concentrations correlate with platelet aggregation²⁶ and increased fibrinogen concentrations are associated with platelet aggregation and an increased incidence of vascular disease.²⁷ Our results are also very similar to those showing that albumin, added in vitro, abolished spontaneous aggregation in samples obtained from patients with nephrotic syndrome.¹¹ It is of interest that the concentrations of essentially fatty acid free human albumin required to neutralise aggregation in our experiments and the nephrotic syndrome study¹¹ are of a similar order. The mechanism involved in the abolition of aggregation by albumin is unclear but may relate, as previously suggested, to the ability of albumin to bind arachidonic acid, thromboxane A_2 , or endoperoxides.¹¹ Such a view is compatible with the greater inhibitory potency of fatty acid free albumin than of fraction V albumin since the latter is rich in fatty acids.²⁸ Our experimental observations would therefore predict that in the nephrotic syndrome, a condition where a low plasma albumin¹¹ and an increased plasma fibrinogen⁵ concentration are usually found, platelet hyperaggregability should occur. This is indeed the case; platelets have been shown to be hyperaggregable and to generate appreciably greater quantities of thromboxane A_2 .^{10, 11} An increased incidence of thrombotic events has also been reported in this condition.^{10, 11}

We also investigated the fibrinogen-heparin interaction since there is increasing evidence that, in certain situations, heparin can directly activate platelets in vivo and in vitro.²⁹⁻³¹ Since fibrinogen and heparin potentiated each other our findings predict that heparin administration would be less

beneficial in conditions in which plasma fibrinogen concentrations are increased. Thus in severe peripheral vascular disease prophylactic heparin administration has been shown to be less beneficial than in other surgical patients.³² Furthermore, vascular surgeons have noted an association between heparin administration and serious vascular occlusion with platelet thrombi (the white clot syndrome).³³ It is of interest that both heparin²⁹ and fibrinogen have been shown to antagonise prostaglandin I₂ mediated inhibition of platelet aggregation.

In conclusion, the proaggregatory effect of fibrinogen may contribute, at least in part, to prothrombotic and atherogenic effects. This area needs further elucidation.

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