Effects of Aspirin on Clot Structure and Fibrinolysis Using a Novel In Vitro Cellular System


Objectives—The purpose of this study was to investigate the direct effects of aspirin on fibrin structure/function. Methods and Results—Chinese Hamster Ovary cell lines stably transfected with fibrinogen were grown in the absence (0) and presence of increasing concentrations of aspirin. Fibrinogen was purified from the media using affinity chromatography, and clots were made from recombinant protein. Mean final turbidity [OD(±SEM)] was 0.083(±0.03), 0.093(±0.002), 0.101(±0.005), and 0.125(±0.003) in clots made from 0, 1, 10, and 100 mg/L aspirin-treated fibrinogen, respectively (P<0.05). Permeability coefficient (Ks cm²×10⁻⁹) was 1.68(±0.29) and 4.13(±0.33) comparing fibrinogen produced from cells grown with 0 mg/L and 100 mg/L aspirin respectively (P<0.05). Scanning electron microscopy confirmed a looser clot structure and increased fiber thickness of clots made from aspirin-treated fibrinogen, whereas rheometer studies showed a significant 30% reduction in clot rigidity. Fibrinolysis was quicker in clots made from aspirin-treated fibrinogen. Ex vivo studies in 3 normal volunteers given 150 mg aspirin daily for 1 week demonstrated similar changes in clot structure/function. Conclusion—Aspirin directly altered clot structure resulting in the formation of clots with thicker fibers and bigger pores, which are easier to lyse. This study clearly demonstrates an alternative mode of action for aspirin, which should be considered in studies evaluating the biochemical efficacy of this agent. (Arterioscler Thromb Vasc Biol. 2009;29:712-717.)

Key Words: —fibrinogen ■ aspirin ■ fibrin polymerization ■ fibrin structure ■ acetylation ■ fibrinolysis

The use of antiplatelet agents for prevention of atherothrombotic events is now well established, and aspirin is often the first line agent used in individuals with stable disease.¹ Acetylation of platelet cyclo-oxygenase-1 (COX-1) by aspirin results in irreversible inhibition of thromboxane A² (TXA²) production and reduced platelet aggregation potential. This is regarded as the main mechanism for the cardioprotective activity of this agent. However, another mode of action for aspirin, which is not widely considered, is its potential direct effect on clotting factors, including fibrinogen and factor (F) XIII, modulating in the process fibrin clot formation and fibrinolysis.²

A key event in clot formation is the production of thrombin, which mediates the conversion of fibrinogen into a 3-dimensional network of fibrin fibers, and this is further cross-linked and stabilized by thrombin-activated factor (F)XIII.³ Clot structure has a role in atherothrombotic disease; clots with thin fibers, small pores, and compact structure are associated with the development of premature and more severe coronary artery disease, which may be related to slower clot lysis of clots.⁴,⁵

Although the ability of aspirin to acetylate fibrinogen has been known for 4 decades,⁶ little work has been conducted to study the functional effect of fibrinogen-aspirin interactions, with poor characterization of fibrin gel properties. Limited in vitro data of turbidity measures suggest that clots formed from aspirin-treated fibrinogen display a looser clot structure,⁷,⁸ which is easier to lyse. Equally limited in vivo work additionally suggest a direct effect of aspirin on clot structure and lysis.⁹-¹¹ However, these studies have ignored the potential effects of genetic or posttranslational modifications in the fibrinogen molecule on fibrinogen-aspirin interaction.¹²,¹³ Also, the possible intracellular acetylation of fibrinogen has not been taken into account, and protein acetylation has been inadequately studied.¹⁴ Moreover, there is controversy regarding the effects of aspirin dosage, which may reflect the uncertainties created by the study limitations described above.¹⁰,¹¹

To address these issues, we investigated the effects of aspirin on recombinant fibrinogen and clot structure/function using an in vitro cellular system, with acetylation assessed using a monoclonal antibody. The results indicate that aspirin acetylates fibrinogen and directly affects the structure of the resulting clot and its susceptibility to lysis. This alternative mode of action of aspirin needs to be
considered when assessing the clinical effectiveness of this agent, particularly under high-risk conditions such as diabetes in which posttranslational modifications, including oxidation and glycation, might influence the response to aspirin treatment.

**Methods**

For a detailed Material and Methods section please see the supplemental materials (available online at http://atvb.ahajournals.org).

**Production and Purification of Recombinant Fibrinogen**

Fibrinogen was produced in Chinese Hamster Ovary (CHO) cell as previously described. Aspirin was dissolved in the medium and added to roller bottles at final concentration of 1, 10, and 100 mg l⁻¹. These 3 concentrations of aspirin largely reflect drug plasma levels after the administration of therapeutic doses of this agent. Aspirin was precipitated from culture medium by ammonium sulfate and further purified by affinity chromatography using the IF-1 antibody.

**Western Blotting**

Purity of the proteins was analyzed by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE). For immunoblotting, proteins were transferred to a nitrocellulose membrane. A polyclonal antibody produced in rabbits with a synthetic acetylated lysine-containing peptide (Cell Signaling Technology), which detects acetyl-lysine groups of lysine residues, was used to detect acetylation of the 3 fibrinogen chains.

**Turbidity Measurements**

Turbidity measurements were performed as previously described. Increase in turbidity was continually monitored every 12 seconds at a wave length of 350 nm on a FLX-800 multiwell plate reader (Biotek Instruments Inc) over a period of 60 minutes.

**Scanning Electron Microscopy**

Fibrin clots were as described previously. Clots were observed and photographed digitally using a Field Emission Scanning Electron Microscope (LEO1530 FEGSEM, Leo Electron Microscopy) in 10 different areas at a magnification of 10 000×. Average fiber diameters of 100 fibers were measured from each micrograph using ImageJ 1.29× software (National Institute of Health).

**Fibrinolysis of Recombinant Fibrin Clots**

Macropathic lysis velocity of recombinant fibrin clots was assessed as described previously. Increase and decrease of absorbency were followed as described above for turbidity. Real-time microscopic lysis was determined using laser confocal scanning microscopy. After clot formation, tissue plasminogen activator (tPA) and plasminogen were added to the border of the clot, and fibrinolysis rates were observed as previously detailed.

**Clot Viscoelasticity**

The visco-elastic properties of aspirin- (100 mg l⁻¹) and nonaspirin-treated fibrinogen were studied using an RFS II rheometer (TA Instruments) operated by TA Orchestras software. Strains used were 1%, 2%, 4%, 10%, 20%, 30%, 40%, and 50%. Final data analysis was conducted using the higher strain of 50%. All experiments were repeated 3 times and G’ (dynamic storage modulus), G” (loss modulus), tan δ (loss tangent) were subsequently calculated.

**Effects of Aspirin In Vivo**

Aspirin was given to 3 healthy volunteers, after appropriate ethical approval, at a dose of 150 mg/d for a period of 1 week. Fibrinogen was purified from plasma samples before and after aspirin treatment and clots made as described above. Fibrin polymerization and lysis were assessed by turbidity measurements using identical conditions to those described above. Scanning electron microscopy was used to further study the ultrastructure of clots made from plasma-purified fibrinogen before and after aspirin treatment.

**Results**

**Integrity of Purified Fibrinogen**

Under reducing conditions, SDS-PAGE analysis showed similar Aα, Bβ, and γ chains of recombinant fibrinogen, purified from media in the absence and presence of increasing concentrations of aspirin (supplemental Figure I). Addition of aspirin to culture media had no effect on fibrinogen degradation. Clottability of samples (±SD) was 95% (±0.2), 92% (±1.1), 92% (±1.7), and 94% (±1.2) for clots made from fibrinogen treated with aspirin at 0, 1, 10, and 100 mg l⁻¹, respectively.

**Fibrin Polymerization**

Clots made from fibrinogen exposed to aspirin in culture media showed a stepwise increase in mean (±SEM) final turbidity of recombinant fibrinogen in the presence of increasing concentrations of aspirin. Mean final turbidity [maximum absorbance (±SEM)] of the fibrin clot was 0.083 (±0.03), 0.093 (±0.002), 0.101 (±0.005), and 0.125 (±0.003) in the presence of 0, 1, 10, and 100 mg l⁻¹ aspirin in culture media, respectively (Figure 1A). These data suggest that aspirin leads to formation of thicker fibrin fibers.

The initiation of clot formation, or lag phase, was prolonged in clots made from fibrinogen treated with high concentration of aspirin (100 mg l⁻¹), as shown in Figure 1B, and this was even more pronounced when lower thrombin concentrations of 0.05 U ml⁻¹ were used (Figure 1C). This suggests an effect of high aspirin concentration on initiation of protofibril formation.

**Permeation**

Clots made from fibrinogen produced in the absence and presence of 100 mg l⁻¹ aspirin had different permeation coefficient (Kₚ [cm²×10⁻⁸]) (±SEM) of 1.88 (±0.29) and 4.13 (±0.33), respectively (P<0.05). Clots formed from fibrinogen not exposed to aspirin had smaller pore radius at 0.72 μm (±0.06) compared with clots formed from aspirin-treated fibrinogen, which had a mean pore radius of 1.14 μm (±0.05; P<0.05).

**Fibrinolysis**

Time to 50% (±3%) lysis in the presence of 0, 1, 10, and 100 mg l⁻¹ of aspirin in culture media was 300, 270, 255, and 240 seconds, respectively (Figure 2A). We also analyzed lysis rate in the linear phase (80% to 20% lysis), and this showed similar results with times of 145, 137, 135, and 122 seconds for clots prepared from 0, 1, 10, and 100 mg
1^-1 aspirin-treated fibrinogen, respectively. Enhanced lysis by aspirin was further confirmed using confocal microscopy, which showed faster lysis of clots made from fibrinogen derived from aspirin-containing media (Figure 2B). The latter experiment was repeated twice with similar results.

**Scanning Electron Microscopy**

Scanning electron microscopy demonstrated a looser 3-dimensional clot structure with increased fiber thickness [147.9 (±4.7) nm] when clots were made from aspirin-treated fibrinogen compared with nonaspirin treated protein [66.3 (±4.2) nm; P<0.05] (Figure 3A and B).

**Clot Viscoelasticity**

Studies using the rheometer, at 50% strain, demonstrated around 30% reduction in stiffness (G') of clots formed from aspirin treated fibrinogen (P<0.05; supplemental Table I). The loss modulus (G'') was also reduced by around 25% in clots made from aspirin treated fibrinogen,
although the difference was only marginally significant (P=0.08). Aspirin had no effect on Tan δ, which was similar in clots prepared from aspirin and nonaspirin treated media (P=0.85).

**Fibrinogen Acetylation**

Using an antibody specific for acetylated lysine residues, a band of \( \sim 68 \) kDa was demonstrated in fibrinogen purified from 100 mg l\(^{-1}\) aspirin treated medium (Figure 4). The size of the band corresponds to the \( \alpha \)-chain of fibrinogen, suggesting that acetylation of lysine residues mainly occur in the \( \alpha \)-chain fibrinogen. A Western blot analysis using an antibody against \( \alpha \)-chain of fibrinogen confirmed that the band detected with the antibody against acetyl-lysine is indeed the \( \alpha \)-chain of fibrinogen (data not shown). We failed to detect acetylation of fibrinogen using lower concentrations of aspirin, probably because of low levels of acetylation below the detection capability of the antibody. It is also possible that low level acetylation occurs in \( \beta \) and \( \gamma \) chains, below the detection sensitivity of the antibody used.

**Effects of Aspirin In Vivo**

Similarly to our in vitro data, final turbidity increased by 17.9% (±8.2%) after aspirin treatment, whereas time to 50% lysis time was shortened by 9.9% [(±6.5%), Figure 5A]. However, lag time was not affected by aspirin treatment, in contrast to in vitro findings (data not shown). Electron microscopy of clots made from plasma-purified fibrinogen demonstrated an increase in fiber thickness from 56.6 (±2.6) nm at baseline to 139.1 (±5.5) nm after aspirin treatment (P<0.05; Figure 5B and 5C), confirming turbidity data and in agreement with the in vitro results.

**Discussion**

The majority of clinical studies continue to assess the biochemical efficacy of aspirin by only measuring its inhibitory effect on COX-1 activity. However, animal studies have shown alternative antiplatelet actions for aspirin. Furthermore, aspirin has been shown to affect clotting factors including thrombin, fibrinogen, FXIII, and tissue plasminogen activator, thereby potentially having a direct effect on clot formation and lysis. Therefore, focusing only on COX-1 inhibition by aspirin is probably
inadequate and may be misleading when evaluating the biochemical efficacy of this agent.

Previous in vitro work investigating the effects of aspirin on clot structure simply relied on incubating plasma-purified fibrinogen with aspirin, followed by dialysis and functional studies. In the present work, we used recombinant fibrinogen and used a novel cellular system to fully characterize the effects of aspirin on fibrinogen and fibrin clot structure. A strict purification process was undertaken, and therefore the resulting fibrinogen was clear of contamination by proteins or aspirin. A further advantage of this system is the elimination of heterogeneity in plasma-purified fibrinogen secondary to genetic variants and differences in post-translational modification of the protein. Moreover, the use of a mammalian cellular expression system addresses possible intracellular posttranslational modifications of fibrinogen by aspirin treatment. Finally, this work analyzed aspects of clot structure and fibrinolysis that have not been previously investigated. In summary, this is the first study that has extensively investigated the effects of aspirin on clot structure using an in vitro system that mimics, to some extent, the in vivo conditions. Although this study has clear advantages compared with previous in vitro work, it also has limitations when trying to apply the results to the in vivo environment. For example, aspirin may acetylate other clotting factors, which may in turn affect clot structure and fibrinolysis, and this is not addressed in the current work. Moreover, in vivo acetylated fibrinogen may undergo further modification(s), which may impact on clot structure and lysis. Taken together, this work is particularly useful in analyzing the effects of acetylation of fibrinogen by aspirin on clot structure, but it does not take into account the effects of aspirin on other clotting factors and various plasma proteins.

Our findings unequivocally indicate that aspirin has a significant effect on the clotting properties of recombinant fibrinogen. The increased final turbidity and permeation coefficient of clots formed from aspirin-treated fibrinogen suggest that exposure to this agent generates a clot composed of thick fibers and large pores. This loose structure was further confirmed on dried clots using scanning electron microscopy. Formation of a less compact fibrin network is probably responsible for the observed shortening of lysis time in clots formed from aspirin-treated fibrinogen. The lower density of fibrin fibers and larger pores of clots made from aspirin-treated fibrinogen may be important for perfusion of plasminogen and plasminogen activators into the clot. Alternatively, acetylated fibrinogen may affect the rate of conversion of plasminogen to plasmin by tissue plasminogen activator, or it may increase affinity to tPA or plasmin, resulting in increased rate of lysis. There is only one published study that investigated the effects of in vitro fibrinogen-aspirin interaction on clot formation. Turbidity measurement, the sole methodology used in the study, showed an effect on clot structure only with very high aspirin concentrations, more than 7-fold higher than the highest concentration used in the current work. In contrast, we have shown that aspirin has an effect using low and therapeutic concentrations of this agent.

Similarly to our in vitro findings, we have shown that aspirin administration to healthy volunteers modulate the structure of clots made from plasma-purified fibrinogen. Previous ex vivo work has shown a favorable effect of aspirin on clot structure and lysis, although different doses of this agent have given inconsistent and conflicting results. Using low dose aspirin in healthy volunteers, 2 studies, conducted by 1 group, have shown that higher doses of aspirin are associated with a lesser effect on clot structure. Unfortunately, these studies used plasma samples, rather than purified fibrinogen, and therefore it is unclear whether the described alterations in clot properties were related to a direct aspirin-fibrinogen interaction or an effect on other clotting factors. Also, the baseline starting parameters were different in individuals treated with 75 and 325 mg aspirin, posing a question mark over interpretation of the data. In the study comparing 37.5 mg with 325 mg aspirin, baseline characteristics were well matched but final differences in clot structure were generally too small to draw any definitive conclusions.

A further limitation of these 2 studies is related to their failure to provide a mechanistic explanation for the presumed enhanced efficacy of lower aspirin dose. Interestingly, an earlier study, by the same group, has shown that a dose of 160 mg aspirin is more efficacious than 75 mg at altering fibrin gel porosity in patients with coronary artery disease, and this is in agreement with findings from the current work. Taken together, more studies are warranted to fully evaluate the in vivo optimal dose of aspirin in relation to clot structure and fibrinolysis both in healthy volunteers and in individuals with established cardiovascular disease.

Using a monoclonal antibody, we have shown acetylation of lysine residues on the α-chain of aspirin-treated fibrinogen. This is the first time that such a technique has been used to investigate fibrinogen acetylation. This posttranslational modification in the fibrinogen molecule may lead to changes in charge distribution and possibly conformation, thereby influencing fibrin fiber dimensions and fibrin gel structure. We failed to detect acetylation of β or γ chains, which may be attributable to low levels of acetylation of the lysine groups in these chains, below the detection capability of our antibody. The mechanisms for the decreased acetylation of lysine residues in β and γ chain are unclear but may be related to physical “exposure” of these residues in the α-C-domain, in contrast to β and γ chains, in which lysine residues remain “hidden.” However, this hypothesis does not fit with previous in vitro work, which suggested similar acetylation of the 3 chains of fibrinogen. Therefore, it is possible that amino-acids other than lysine are preferentially acetylated in the β or γ chains of fibrinogen. Alternatively, the observed differences may be related to the different methodology used in our study, possibly secondary to the use of a cellular system that favors α-chain acetylation.

We have also shown, for the first time, reduced rigidity of clots formed from aspirin-treated recombinant fibrinogen. The decrease in elastic modulus (G*) is likely attributable to reduced branch points and the presence of thicker fibers that pack nonuniformly, as shown in the scanning electron micrograph of the aspirin-treated fibrinogen. On the other hand, aspirin also caused a reduction in the loss modulus (G”) such that the ratio of these moduli, tangent delta—the clot’s overall viscoelasticity—remained the same on aspirin treatment. An increase in clot rigidity (G’), however, is known to be associated with enhanced cardiovascular risk, which may be partly attributable to hypofibrinolysis. We have clearly demonstrated a reduction in clot rigidity by aspirin, further
indicating that the protective effects of this agent are not simply attributable to inhibition of platelet function.

In conclusion, the present study has shown that aspirin alters the phenotype of the fibrin clot leading to the formation of fibrin characterized by increased fiber thickness, large pores, reduced rigidity, and enhanced lysis properties. This structural and functional phenotype has been associated in clinical studies with low risk of arterial thrombotic disease, to indicate that these specific aspirin effects are potentially beneficial. These findings may be explained, at least in part, by the observed acetylation of lysine residues in the α-chain of fibrinogen. On the other hand, aspirin may result in other posttranslational modifications in the fibrinogen molecule, by potentially modifying the function of various intracellular enzymes. Therefore, further studies designed to investigate the specific effects of aspirin on the fibrinogen molecule using mass spectrometry are currently underway. In addition to fibrinogen, aspirin may acetylate other clotting factors as detailed earlier, thereby having an even more pronounced effect on clot structure and lysis. This work demonstrates an additional and alternative cardioprotective mode of action for aspirin, which should be taken into consideration in clinical studies assessing the biochemical effectiveness of this agent.

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Disclosures

None.

References

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Supplement Material

Full description of methods

Tissue culture

Chinese hamster ovary (CHO) cells, stably transfected with fibrinogen α, β and γ chains and expressing functional fibrinogen, were grown in 850 cm$^2$ roller bottles at 37°C (Corning Incorporated, Acton, MA, USA) containing 200 ml growth medium as previously described$^1$ aspirin was dissolved in the medium and added to roller bottles at final concentration of 1, 10 and 100 mg l$^{-1}$. From this point onwards, 100 ml of media, from aspirin and non-aspirin containing bottles, were removed and replaced every 2-3 days with similar culture media. Harvested media were stored at -40°C until purification. Three concentrations of aspirin were used, which largely reflect drug plasma levels after the administration of therapeutic doses of this agent$^2$-4. Addition of aspirin had no effect on pH of the media, even at higher aspirin concentration, and did not affect cell viability.

Purification of recombinant fibrinogen

Fibrinogen was precipitated from culture medium by ammonium sulphate and further purified by affinity chromatography using the IF-1 antibody$^{1,5}$. Integrity of samples was confirmed by SDS-PAGE analysis. Fibrinogen concentration was determined at 280nm using the extinction coefficient $\varepsilon_{280}=1.506$ and adjusted to a dilution of 1 mg ml$^{-1}$. Clottability was determined as previously described$^1$. 


**SDS PAGE and Western blotting**

Purity of the proteins was analysed by sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE) using a 4-12% Tris-HCl gel (Bio-Rad, Hercules, CA, USA) and GelCode blue stain reagent (Pierce, Rockport, IL, USA). For immunoblotting, proteins were transferred to a nitrocellulose membrane. A polyclonal antibody produced in rabbits with a synthetic acetylated lysine-containing peptide (Cell Signaling Technology, Boston, MA, USA) that detects proteins that have been posttranslationally modified by acetylation on the ε-amine groups of lysine residues was used to detect acetylation of the three fibrinogen chains. The antibody was diluted 1:1000 in TBS-Tween containing 5% non fat dried milk. Blots were developed using an HRP conjugated anti-rabbit antibody (DAKO, Glostrup, Denmark) and chemiluminescence. An identical technique was used to detect α-chain of fibrinogen, using a polyclonal antibody against the fibrinogen α-chain (Accurate Chemicals & Scientific, Westbury, NJ, USA).

**Turbidity measurements**

Turbidity measurements were performed as previously described \(^1\), using fibrinogen at a concentration of 0.45 mg ml\(^{-1}\). Polymerisation was initiated by the addition of thrombin at 0.5 U ml\(^{-1}\) and 2.5 mmol l\(^{-1}\) CaCl\(_2\) in 100 mmol l\(^{-1}\) NaCl, 50 mmol l\(^{-1}\) Tris, pH 7.4 at ambient temperature in a 96 well plate. At least three replicates were performed for each aspirin concentration. Increase in turbidity was continually monitored every 12 seconds at a wave length of 350 nm on a FLX-800 multiwell plate reader (Biotek Instruments Inc, Winooski, VT, USA) over a period of 60 minutes.
**Permeation properties of clots**

Clots formed in open tubes with 0.45 mg ml\(^{-1}\) fibrinogen, 2.5 mmol l\(^{-1}\) CaCl\(_2\) and 0.5 U ml\(^{-1}\) human thrombin, were left for 2 hours at room temperature in a wet chamber. Clot permeability was subsequently measured as previously described\(^1,6\).

**Scanning electron microscopy**

Scanning electron microscopy was used to analyse the ultrastructure of clots. Fibrin clots were prepared by adding 0.5 U ml\(^{-1}\) thrombin and 2.5 mmol l\(^{-1}\) CaCl\(_2\) 100 mmol l\(^{-1}\) NaCl, 50 mmol l\(^{-1}\) Tris, pH 7.4 to 0.45 mg ml\(^{-1}\) fibrinogen. Samples were prepared for microscopy as described previously\(^1,7\). Clots were observed and photographed digitally using a Field Emission Scanning Electron Microscope (LEO1530 FEGSEM, Leo Electron Microscopy, Cambridge, UK) in 10 different areas at a magnification of 10000x. Average fibre diameters of 100 fibres were measured from each micrograph using ImageJ 1.29x software (National Institute of Health, Bethesda, MD, USA).

**Fibrinolysis of recombinant fibrin clots**

Macroscopic lysis velocity of recombinant fibrin clots was assessed by adding fibrinogen (0.45 mg ml\(^{-1}\)), 10 ng ml\(^{-1}\) tissue plasminogen activator (Technoclone, Vienna, Austria) and 0.1 mg ml\(^{-1}\) Glu-plasminogen (Calbiochem) to the activation mix. Increase and decrease of absorbency were followed as described above for turbidity. All experiments were repeated three times.

Real time microscopic lysis was determined using laser confocal scanning microscopy. Recombinant fibrinogen was used at 0.45 mg ml\(^{-1}\) with calcium at 5
mmol l\(^{-1}\) and thrombin at 1 U ml\(^{-1}\). After clot formation, tissue plasminogen activator (tPA) and plasminogen were added at 10 ng ml\(^{-1}\) and 0.1 mg ml\(^{-1}\) final concentrations respectively to the border of the clot and fibrinolysis rates were observed as previously detailed \(^{1,8}\).

**Clot visco-elasticity**

The visco-elastic properties of aspirin (100 mg l\(^{-1}\)) and non-aspirin treated fibrinogen were studied using an RFS II rheometer (TA Instruments, New Castle, DE, USA) operated by TA Orchestrator software. Recombinant fibrinogen, at a concentration of 0.45 mg ml\(^{-1}\), was incubated with 0.5 U ml\(^{-1}\) thrombin in the presence of 2.5 mmol l\(^{-1}\) CaCl\(_2\) between glass slides attached to the 8 mm parallel plate of the rheometer. The plates were adjusted so that the sides of the clot were perpendicular to the plate, not concave or convex. After 1 hour of incubation, a strain-controlled dynamic time sweep test was performed with a frequency of 5 radians per second with readings every 15 seconds for 1 minute. The first reading of each experiment was discarded and the remaining four were averaged. Strains used were 1%, 2%, 4%, 10%, 20%, 30%, 40%, and 50%. Final data analysis was conducted using the higher strain of 50%. All experiments were repeated three times and \(G'\) (dynamic storage modulus), \(G''\) (loss modulus), tan \(\delta\) (loss tangent) were subsequently calculated.

**Effects of aspirin in vivo**

Aspirin was given to 3 healthy volunteers, after appropriate ethical approval, at a dose of 150 mg/d for a period of one week. Fibrinogen was purified from plasma samples before and after aspirin treatment and clots made as described above. Fibrin polymerisation and lysis were assessed by turbidity measurements using identical
conditions to those described above. Scanning electron microscopy was used to further study the ultrastructure of clots made from plasma-purified fibrinogen before and after aspirin treatment.
Supplementary figure

Fig. I

A

B
Supplementary table

**Table I** Viscoelasticity of clots made from recombinant fibrinogen using rheometer studies. Results are presented as mean $G'$, $G''$ and Tan $\delta$ values of three independent experiments (±SEM), using clots made from fibrinogen purified from aspirin treated media (100 mg l$^{-1}$) and media not treated with this agent (0 mg l$^{-1}$). All clots were prepared using a concentration of 0.45 mg l$^{-1}$ fibrinogen with 2.5 mmol l$^{-1}$ CaCl$_2$ and 1 U ml$^{-1}$ thrombin.

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Figure 1. Turbidity analysis of clots made from recombinant fibrinogen. A. Mean turbidity curves (± SEM) of 3 experiments using fibrinogen purified from culture media without (0 mg l\(^{-1}\)) and with increasing concentrations of aspirin. Clots were made at fibrinogen concentration of 0.45 mg ml\(^{-1}\), 2.5 mmol l\(^{-1}\) CaCl\(_2\) and 0.5 U ml\(^{-1}\) thrombin. Fibrinogen purified from aspirin treated media had increased final turbidity in a dose-dependent manner, suggesting the formation of thicker fibres. B. Lag phase was increased in clots made from aspirin (100 mg ml\(^{-1}\)) compared with non-aspirin treated fibrinogen. C. Increase in lag phase in clots made from aspirin (100 mg l\(^{-1}\)) treated fibrinogen was more pronounced when lower thrombin concentrations of 0.05 u ml\(^{-1}\) were used.

Figure 2. Lysis velocity of fibrin clots formed from recombinant fibrinogen. A. Macroscopic fibrinolysis was assessed directly after the addition of plasminogen and tissue plasminogen activator at 0.1 mg ml\(^{-1}\) and 10 ng ml\(^{-1}\) respectively, to 0.45 mg ml\(^{-1}\) fibrinogen, 2.5 mmol l\(^{-1}\) CaCl\(_2\) and 0.5 U ml\(^{-1}\) thrombin. Clot formation and fibrinolysis were observed every 12 seconds. B. Macroscopic fibrinolysis after correcting data for maximum absorbancy. C. Microscopic fibrinolysis was observed in real time using laser scanning confocal microscopy. Clots were made from untreated and 100 mg l\(^{-1}\) aspirin treated fibrinogen used at a concentration of 0.45 mg ml\(^{-1}\) with calcium at 5 mM and thrombin at 1U ml\(^{-1}\). After clot formation, plasminogen and tissue plasminogen activator were added at 0.1 mg ml\(^{-1}\) and 10 ng ml\(^{-1}\) final concentrations respectively to the border of the clot and fibrinolysis rates were observed every 2 minutes. Scale bars on micrographs indicate 5 μm.

Figure 3. Scanning electron micrographs (EM) of clots made from recombinant fibrinogen. Panels A and B represent EM of clots prepared from recombinant fibrinogen purified from media not containing aspirin (0 mg l\(^{-1}\)) or containing this agent (100 mg l\(^{-1}\)). Recombinant fibrinogen was used at a concentration of 0.45 mg ml\(^{-1}\) in the presence of 2.5 mmol CaCl\(_2\) and 0.5 U/ml thrombin (magnification of ×10000). Scale bars on all micrographs indicate 5 μm.

Figure 4. Detection of fibrinogen acetylation using monoclonal acetyl-lysine antibody. Asp\(^{-}\) and Asp\(^{+}\) represents fibrinogen purified from non-aspirin and aspirin
treated media at (100 mg l$^{-1}$) respectively. A band of ~68 kDa was detected suggesting acetylation of lysine residues in the $\alpha$-chain of fibrinogen.

**Figure 5.** Changes in clot structure using plasma-purified fibrinogen before and after 150 mg/d aspirin treatment. A. Clots were made from plasma-purified fibrinogen of three healthy volunteers at concentrations of 0.45 mg l$^{-1}$ as previously described. Percentage change in final turbidity and time to 50% lysis before and after one week of aspirin treatment are illustrated. B. Electron microscopy of a clot made from plasma-purified fibrinogen of one volunteer before and after one week of aspirin treatment (B and C respectively).

**Figure I.** Recombinant and plasma-purified fibrinogen resolved in 4-12% SDS-Page. A. Recombinant fibrinogen purified from media in the absence (lane 1; 0 mg l$^{-1}$) and presence of increasing concentrations of aspirin (lanes 2-4; 1, 10 and 100 mg l$^{-1}$). B. Plasma purified fibrinogen before (pre) and after (post) 150 mg/d aspirin administration to three healthy volunteers.
References


