Effects of age, sex and the oral contraceptive on the platelet membrane fibrinogen binding site (Glycoprotein IIb/IIIa)

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- 1 We have studied the effects of age, sex and the oral contraceptive pill on platelet fibrinogen 'receptor' density ($B_{\max}$) and affinity (K_d).
- 2 [¹²⁵I]-fibrinogen binding to gel-filtered platelets was assessed after stimulation with adenosine 5-diphosphate (ADP) and thrombin in 60 normal subjects; 37 females and 23 males.
- 3 For both agonists there was no significant difference in $B_{\max}$ or K_d between the sexes. A trend towards increasing affinity (reduced K_d) with age was noted in the female group following ADP stimulation. This was not considered to be physiologically relevant. In the group as a whole no significant relationship of $B_{\max}$ and K_d with age was demonstrated.
- 4 A further group of eight women taking low dose oestrogen combined oral contraceptive pill were studied on days 7, 14, 21 and 28 of the treatment cycle and compared with eight women with regular ovulatory cycles. No significant variations in $B_{\max}$ or K_d were detected within or between cycles in these two groups.

Keywords platelets fibrinogen binding site contraceptive pill

Introduction

The role of the platelet is crucial in initiating the haemostatic process. Platelet aggregation is mediated principally by binding of fibrinogen to platelet membrane glycoproteins (GPIIb/IIIa). The exposure of this glycoprotein complex and the binding of fibrinogen is the final common pathway for platelet aggregation however it is induced (Plow & Ginsberg, 1989).

Attention has been recently focused on the role of this platelet fibrinogen binding site or 'receptor' in the thrombo-haemorrhagic diathesis associated with the myeloproliferative disorders (Landolfi *et al.*, 1988; Mazzucato *et al.*, 1989; Mistry *et al.*, 1991). There is however a paucity of data on platelet fibrinogen receptor number and function in other groups who have an increased risk of thrombosis such as the elderly and users of the oral contraceptive pill.

Previous studies have described increased platelet aggregability with age in both sexes (Johnson *et al.*, 1975; Meade *et al.*, 1985). The cause of this is not well understood. Quantitative and/or qualitative changes in fibrinogen binding sites may be responsible.

The risk of coronary and cerebral thrombosis increases

with age. It is lower in pre-menopausal women than in men of the same age but increases markedly post-menopausally. Furthermore there is a well established link between the use of oral contraceptives and thromboembolic disease with a dose-response relationship to oestrogen (Sartwell *et al.*, 1969; Vessey & Doll, 1968). Some studies have looked at the possible relationship between sex and platelet aggregation and found greater aggregation in women than in men (Meade *et al.*, 1985). These observations suggest sensitivity of the coagulation systems to hormonal variation. To elucidate these relationships, a range of haemostatic abnormalities such as alteration in blood flow, activation of platelets, abnormal endothelial surface and decreases in naturally occurring inhibitors have been investigated. In addition, numerous studies have been reported on the effects of oestrogens and progestogens on coagulation and on the fibrinolytic enzyme system with conflicting results (Astedt, 1977; Beller *et al.*, 1972; Hedlin & Monkhouse, 1971; Largelius *et al.*, 1978). However, to our knowledge, no data on the influence of age or hormones on the human

platelet fibrinogen receptor number and function has been published to date.

The aims of this study are (i) to investigate the relationship between age and platelet membrane fibrinogen receptors in healthy males and females over a wide age range and (ii) to investigate the effect of natural and artificial variations in female hormonal levels on this binding site.

Methods

Subjects

Thirty-seven females (mean age 51 years, age range 21–91 years) and 23 males (mean age 49 years, age range 20–83 years) were studied. They consisted of hospital staff, outpatient volunteers and patients admitted for minor surgical procedures. Subjects taking any form of medication were excluded. An additional group of eight women (mean age 27 years, age range 21–33 years) using low dose combined oral contraceptives containing 30–35 µg oestrogen and 0.15–0.50 mg progestogen was compared with eight healthy pre-menopausal control female volunteers (mean age 33 years, age range 21–43 years) with regular cycles between 26–30 days. The ovulatory cycles in the control group were evidenced by measurement of 21-day serum progesterone levels (mean $\pm$ s.e. mean 29.6 ± 4.7 nM). The purpose of the study, which was approved by the hospital Ethics Committee, was explained to all participants and their informed consent obtained.

Binding studies

Each subject donated 51 ml of blood which was withdrawn from an antecubital vein using a 19-gauge needle into 3 ml of 1 mM aspirin and 6 ml of 0.11 M tri-sodium citrate. Women taking the oral contraceptive pill and the control women had samples taken on days 7, 14, 21 and 28 of either the pill treatment or normal menstrual cycle. Day 1 was defined as the onset of menstruation.

Platelet fibrinogen receptor density and affinity were assessed using [125 I]-fibrinogen as described previously (Mistry *et al.*, 1991). Gel-filtered platelets were prepared using standard methods and finally resuspended in HEPES-buffered modified Tyrodes solution pH 7.5 (HBMT) containing 20 µM CaCl_2 and 2 mM MgCl_2 . These concentrations of divalent ions were shown in preliminary experiments to provide optimum conditions for fibrinogen binding (Mistry *et al.*, 1991). Briefly, the binding of purified human [125 I]-fibrinogen to ADP-stimulated platelets was performed as follows: 500 µl gel-filtered platelets (2.0 – 5.0×10^8 cells ml^{-1}) was incubated with 450 µl of a mixture of labelled/unlabelled fibrinogen (final concentrations ranging from 6.25–200 µg ml^{-1}) and 40 µl of calcium- and magnesium-free HBMT. Binding was initiated by adding 10 µl 1 mM ADP to give a final concentration of 10 µM. Non-specific binding was determined in parallel tubes by substituting 40 µl HBMT with an equal volume of 125 mM EDTA to

produce a final concentration of 5 mM. Quadruplicate samples of 200 µl of reaction mixture were then layered on to 200 µl of phthalate oil in 400 µl microfuge tubes. Incubation was terminated 15 min after the addition of ADP by micro-centrifugation at 13,000 rev min^{-1} for 2 min. The radioactivity associated with the platelet pellet was determined by cutting off the microfuge tube tips and counting them in a gamma counter. Specific binding was determined by subtracting non-specific binding from total binding.

Thrombin-induced fibrinogen binding was measured with a slight difference to avoid proteolysis of fibrinogen by the agonist (Plow & Marguerie, 1980). The gel-filtered platelets were first pre-incubated with 150 mU thrombin for 5 min, followed by the addition of a four-fold excess of hirudin to neutralise the thrombin. The mixture of labelled/unlabelled fibrinogen was then added and the reaction allowed to proceed for 10 min prior to micro-centrifugation. Once again, non-specific binding was measured in the presence of 5 mM EDTA.

Statistics

Data were analysed using the Minitab statistical package. Testing for normality showed data to be predominantly non-parametric, and accordingly Mann-Whitney U-tests were used. To analyse the data for the effects of age, both rank correlation and linear regression analysis were employed. Where regression analysis was used, the residuals were checked for normality. An age-sex interaction was included in the regression model to investigate possible sex differences in the relationship between age and the platelet fibrinogen receptor density and affinity. Simple summary statistics were employed to examine the menstrual cycle data from the pill group of women (Matthews *et al.*, 1990). In all cases, statistical significance was accepted at $P \leq 0.05$.

Results

Overall, maximum density of fibrinogen binding sites and the affinity of radiolabelled fibrinogen was similar in the present study to those we have previously published (Mistry *et al.*, 1991) and values documented by other laboratories using similar techniques (Di Minno *et al.*, 1986; Peerschke, 1984). The coefficient of variation for [125 I]-fibrinogen B_{max} in response to thrombin was 24% as estimated in five male subjects on two separate occasions.

The dissociation constants and the number of fibrinogen binding sites were similar in males and females in response to both ADP and thrombin (Table 1), with no significant differences between median values. Confirming the results of previous studies, thrombin was demonstrated to be a significantly more potent agonist than ADP in its ability to express fibrinogen binding sites ($P < 0.0011$ for both sexes).

Median fibrinogen binding sites and dissociation constants of the total group ($n = 60$) in response to ADP and thrombin are also shown in Table 1. This data was analysed for an association with age. Linear regression

Table 1 Binding of fibrinogen to gel-filtered platelets in response to ADP and thrombin in male and female subjects. Median values are reported. Values in parentheses represent first and third quartiles

		ADP		Thrombin	
Fibrinogen molecules bound/platelet	Males <i>n</i> = 23	12,419 (8,920–23,572)	$P = 0.08$	31,958 (24,026–37,904)	$P = 0.30$
	Females <i>n</i> = 37	10,749 (7,801–17,088)		26,589 (20,926–40,303)	
	Total <i>n</i> = 60	12,016 (8,268–17,548)		29,378 (21,861–38,050)	
Dissociation constant (K_d) (nm)	Males <i>n</i> = 23	83 (58–105)	$P = 0.55$	44 (34–52)	$P = 0.24$
	Females <i>n</i> = 37	78 (66–117)		45 (39–66)	
	Total <i>n</i> = 60	81 (60–112)		45 (36–57)	

analysis did not demonstrate a significant association between age and either number of binding sites (Figure 1) or dissociation constants (figure not shown). An age-sex interaction term introduced into the regression equation suggested that the receptor affinity related to age differed in men and women ($P = 0.02$).

In the 37 female participants, receptor affinity with ADP as agonist was significantly associated with age ($P = 0.01$). However no significant relationships were demonstrated between receptor number and age. This association with affinity was not present when thrombin was used as the agonist ($P = 0.17$). Upon stimulation with either agonist, the male group showed no correlation in either receptor number or affinity with age.

Platelet fibrinogen receptor affinity and density were similar in women taking the oral contraceptive pill and in control women with normal cycles. The sequential data on days 7, 14, 21 and 28 in these groups (Table 2) were analysed by comparison of a summary measure for each group. The mean of each set of sequential measurements was used. The data for binding site density are depicted in Figure 2. No significant differences between the summary measures were detected.

Discussion

Thrombotic disorders are a major cause of mortality in western society today. Platelet aggregates mediated by the activation of GPIIb/IIIa are intrinsic to these pathologies and the exposure of this glycoprotein is the final common pathway for platelet aggregation (Marguerie *et al.*, 1987). Agonists such as ADP and thrombin can cause GPIIb/IIIa exposure in the presence of 'anti-platelet' drugs which block the arachidonic acid or thromboxane A_2 synthesis pathways. Therefore new anti-aggregating drugs such as ticlopidine (Di Minno *et al.*, 1985) and monoclonal antibodies to the fibrinogen binding sites are being investigated for their therapeutic potential in preventing and treating thrombotic episodes (Coller *et al.*, 1989; Fitzgerald, 1989; Haskell *et al.*, 1989). There has been much recent interest in the role of the fibrinogen 'receptor' in

Figure 1 Association between fibrinogen receptor density with age in 60 subjects (23 males and 37 females) in response to a) ADP (●) and b) thrombin (○). Statistical analysis of linear regression with age for the whole group revealed $P = 0.94$ and $P = 0.15$ for ADP- and thrombin-stimulated platelets respectively.

abnormal platelet function (Landolfi *et al.*, 1988; Mazzucato *et al.*, 1989; Mistry *et al.*, 1991), to date however there has been little data on pre-morbid changes in this glycoprotein in normal individuals or in high risk groups. Nor do we know if changes in the

Table 2 Fibrinogen receptor density and affinity in gel-filtered platelets from female subjects taking the oral contraceptive pill and those with normal menstrual cycles (control) in response to ADP and thrombin. Mean $\pm$ s.e mean are reported. For both study groups, $n = 8$

	Day	ADP		Thrombin	
		Pill	Control	Pill	Control
Fibrinogen molecules bound/platelet	7	13,745 $\pm$ 1,374	12,928 $\pm$ 2,202	31,196 $\pm$ 2,036	27,284 $\pm$ 4,316
	14	14,788 $\pm$ 1,795	11,834 $\pm$ 2,255	33,060 $\pm$ 3,295	26,106 $\pm$ 4,618
	21	12,321 $\pm$ 1,949	10,917 $\pm$ 2,189	23,764 $\pm$ 2,993	26,858 $\pm$ 3,829
	28	13,380 $\pm$ 2,239	13,150 $\pm$ 2,946	32,589 $\pm$ 3,360	23,835 $\pm$ 4,541
Dissociation constant (K_d) (nM)	7	89 $\pm$ 12	118 $\pm$ 18	51 $\pm$ 10	46 $\pm$ 4
	14	113 $\pm$ 25	101 $\pm$ 15	42 $\pm$ 8	60 $\pm$ 10
	21	172 $\pm$ 38	92 $\pm$ 9	57 $\pm$ 14	50 $\pm$ 7
	28	102 $\pm$ 21	136 $\pm$ 18	59 $\pm$ 6	63 $\pm$ 15

Figure 2 Fibrinogen receptor density in gel-filtered platelets from female subjects taking the oral contraceptive pill and those with normal menstrual cycles in response to ADP and thrombin. Data shown represents the mean $\pm$ s.e. mean during various stages of the treatment and control cycle. For both study groups, $n = 8$. For ADP-stimulated platelets, analysis of summary means for pill group (13,559 receptors, $\square$) and for control group (12,207 receptors, $\square$), $P = 0.63$. For thrombin-stimulated platelets, summary means analysis of pill group (30,152 receptors, $\blacksquare$) and control group (26,021 receptors, $\blacksquare$) revealed $P = 0.37$.

receptor with ageing contribute to the effects of atherosclerosis on the pathogenesis of ischaemic cerebral and cardiac events. As any such changes would have profound implications for treatment and prevention of thrombotic disorders, the investigation of a large group to determine the influence of age, sex and hormonal status on GPIIb/IIIa is of importance.

Our investigation of 68 subjects (60 on no medication

and eight on the oral contraceptive pill) is the largest study to date in which platelet fibrinogen receptor density and affinity have been examined. Receptor density did not differ between males and females, pre- and post-menopausal women (data not shown) or between women taking the oral contraceptive pill and their normal control group. In addition we found no change in fibrinogen receptor density or affinity with age in the group as a whole over a wide age range (20 to 91 years). However, separate analysis of a subgroup of 37 women not taking the oral contraceptive pill revealed a trend towards increasing affinity of fibrinogen binding with age when ADP but not thrombin was used as the agonist. Thus the median K_d for the first quartile of the age range studied (21–33 years) was 103 nM as compared with that for the upper quartile (66–91 years) which was 76 nM. These numerical differences in K_d are however unlikely to be of clinical significance as they fall within the range of the affinity of fibrinogen for the support of ADP induced *in vitro* platelet aggregation (Mistry *et al.*, 1991).

In those studies which have reported changes in platelet aggregation with age and sex (Johnson *et al.*, 1975; Meade *et al.*, 1985) the mechanisms remain unclear and it is likely that the increased incidence of thrombosis with increasing age and the ingestion of 'pharmacological' doses of oestrogen may be related to vessel wall and plasma factors as well as possible changes in the platelet membrane.

This study suggests that GPIIb/IIIa is a stable entity with little variation occurring with changes in age, between the sexes or changes in hormonal status. The binding site is the final common mediator for platelet aggregation. Its stability in a range of situations where thrombosis is more common encourages the development of novel platelet fibrinogen receptor blocking agents for prevention and treatment of a variety of clinical manifestations of thrombosis.

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