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ADP activation induces bFGF binding to platelets *in vitro*

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ABSTRACT

Basic fibroblast growth factor (bFGF), is a heparin–binding factor with potent angiogenic properties *in vitro* and *in vivo*. bFGF is involved in tumour growth, but it has also been shown to reduce infarct size in experimentally induced acute myocardial infarction. Platelets are also believed to have an important role in both tumour growth and myocardial infarction.

We have studied bFGF binding to platelets by flow cytometry. Platelet activation by ADP induces bFGF binding to platelets. bFGF bound to activated platelets will result in a locally high concentration of bFGF in patients with myocardial infarctions and malignant tumours. Addition of recombinant bFGF to platelet rich plasma reduced the percentage of fibrinogen positive platelets. bFGF may thus have an inhibitory effect on platelet aggregation.

Key words: activation, ADP, bFGF, blood platelets, flow cytometry

INTRODUCTION

Under normal physiological conditions, endothelial cells prevent platelets from adhering to the vascular wall. If the vessel wall is damaged, platelets will adhere to the injured endothelial surfaces and then aggregate at the site of damage to form a thrombus. Depending on the location of the vessel, this may result in unstable angina, myocardial infarction or stroke. The formation of the platelet thrombus is a critical step in the development of ischemic heart disease and the prevention and/or removal of the platelet thrombus are important parts of the treatment of ischemic heart disease. Platelets are also believed to contribute to tumour induced angiogenesis (1) and thrombocytosis is an independent prognostic indicator in patients with cancer (2, 3).

Basic fibroblast growth factor (bFGF) is considered to play an important role in tumour angiogenesis and bFGF has also been shown reduce infarct size in experi-

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mentally induced acute myocardial infarction. The presence of bFGF in megakaryocytes and platelets has previously been shown (4). Platelet activation induces the release of granule proteins from the platelet. Recent studies show that stimulated platelets secrete vascular endothelial growth factor (VEGF) (5). It is likely that bFGF also may be released during platelet activation. Extracellular bFGF is found deposited as a complex with heparan sulphate proteoglycans in the extracellular matrix and on cell surfaces (4). This bFGF provides a reservoir of growth factor, which is capable of mediating biological effects (6). If bFGF is bound to activated platelets this may result in a locally high concentration of bFGF in patients with myocardial infarctions or malignant tumours.

We have used flow cytometry to study the expression of bFGF on the platelet surface after stimulation with ADP *in vitro* to evaluate if platelet activation may influence bFGF binding to platelets. We have also studied the effect of bFGF on platelet activation markers.

MATERIALS AND METHODS

Reagents

Chicken anti–bFGF antibodies were obtained by immunising laying hens with recombinant human bFGF (Fiblast®, Wyeth–Ayerst, Philadelphia, Pennsylvania, USA). The antibodies were purified from egg yolk and FITC-conjugation of the chicken antibodies (IgY) was performed as previously described (7). FITC-conjugated chicken antibodies (IgY) to fibrinogen, p-selectin, whole platelets and von Willebrand factor were from Biopool AB (Umeå, Sweden).

Preparation of samples for flow cytometry

Blood samples were obtained from healthy volunteers who had taken no medication for at least 10 days. All volunteers gave informed consent prior to blood sampling. Blood was obtained from an anticubital vein without tourniquet to avoid platelet activation during blood collection. Blood samples were collected in 5 mL sodium citrate tubes (367704, Becton Dickinson, Rutherford, NJ). Platelet-rich plasma was isolated by centrifugation at low speed and at room temperature (7).

5 µL platelet-rich plasma was added to polystyrene tubes containing 50 µL HEPES-buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L $MgCl_2$, 5.6 mmol/L glucose, 1 g/L bovine serum albumin, and 20 mmol/L HEPES, pH 7.4) (7,8) and 5 or 10 µL FITC labelled chicken antibody. The samples were incubated for 10 min at room temperature and were then diluted and fixed with 1000 µL ice-cold PBS (0.02 mol/L Na_2HPO_4, 0.15 mol/L NaCl, 0.02% NaN_3, pH 7.2), containing 1 % p-formaldehyde. No washing steps were used.

Flow cytometry

The samples were analyzed utilising an Epics Profile XL-MCL cytometer (Coulter Electronics, Hialeah, FL). Data processing from 5,000 platelets was carried out with

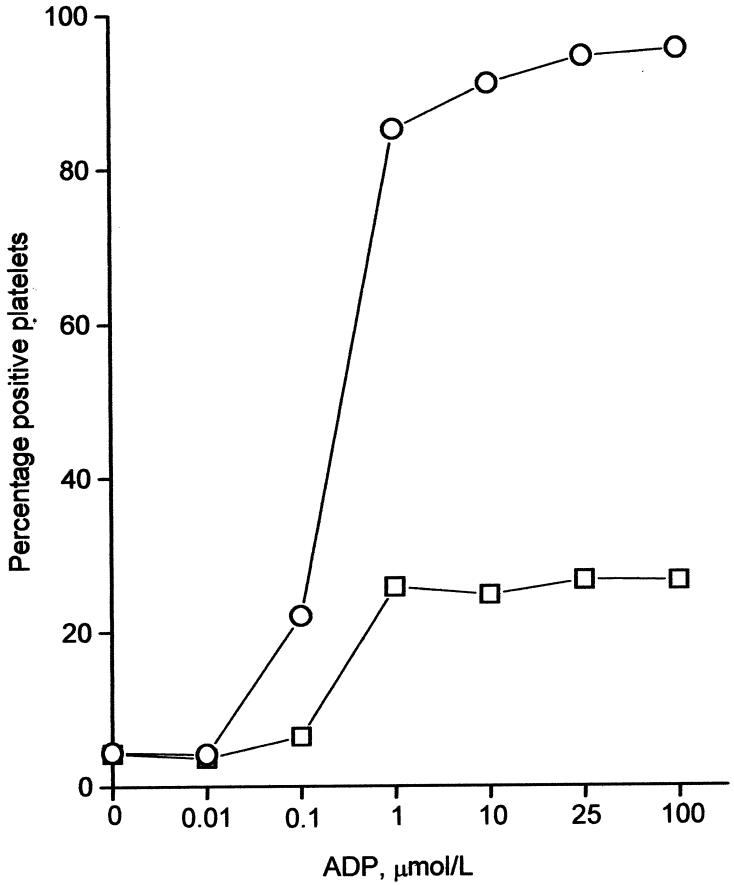

Fig. 1. ADP-induced bFGF binding to platelets measured utilising FITC-labelled chicken anti-bFGF and expressed as percentage bFGF positive platelets. The percentage fibrinogen positive platelets are shown as a comparison. All experiments were run in duplicate.

□ Percentage fibrinogen positive platelets
○ Percentage bFGF positive platelets

the XL software (Coulter Electronics). Based on light scattering properties, each cell is represented by a point in a rectangular co-ordinate system. A discrimination frame is placed around the platelet cluster. The instrument gives percentage of positive cells, mean fluorescence intensity (MFI), complexity (right angle scatter) and the mean cell size (forward angle scatter) of the cell population within the field. Analytical markers were set in the fluorescence channel to divide a negative control sample into two fractions containing 95–99% of the platelets and the 1–5% of the platelets with the highest fluorescent activity. For microvesicle analysis the discrimination frame was set around the platelet cluster using forward angle scatter and FL1 (anti-whole platelet-FITC). Those platelets, with fluorescence lower than the

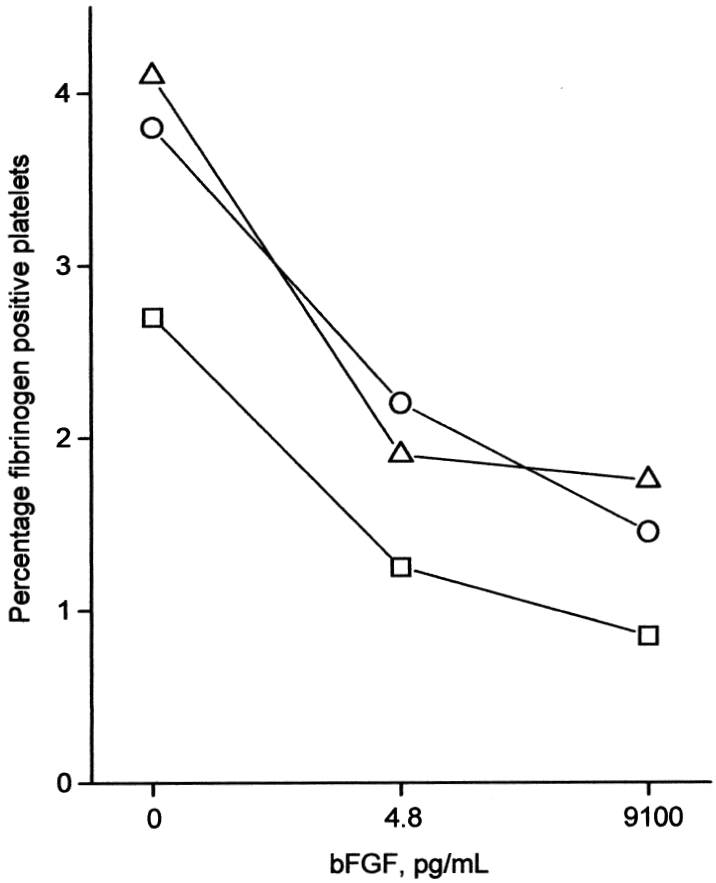

Fig. 2. Effects of addition of bFGF, to platelet rich plasma, on the percentage fibrinogen positive platelets in three healthy volunteers. All experiments were run in duplicate.

marker, were identified as microvesicles. The gates were set prior to the study. All experiments were run in duplicate.

RESULTS

Binding of bFGF to platelet

ADP activation increased the percentage bFGF positive platelets (Fig. 1). The increase in bFGF positive platelets correlated with the increase in fibrinogen positive platelets.

Effects of recombinant bFGF on platelet activation

Addition of bFGF caused a reduction in the percentage of fibrinogen positive platelets (Fig. 2). There was also a decrease in the mean fluorescent intensity. There

was no significant effect of bFGF on microparticle formation or the percentage of p-selectin and von Willebrand factor positive platelets (results not shown).

DISCUSSION

Our experiments show that ADP activation induces bFGF binding to platelets in platelet rich plasma. This bFGF is probably partly platelet derived as platelets contain bFGF and proteins are released from platelets during activation. bFGF is an angiogenic peptide that may have many effects *in vivo*. It stimulates hematopoiesis (9) and it is a pluripotent mitogen of mesodermally derived cells and could theoretically exacerbate neointimal smooth muscle hyperplasia that could cause stenosis (10). bFGF also promotes the formation of new blood vessels. Thus, bFGF could theoretically cause both increased and decreased blood flow. Experimental studies have shown that bFGF increases blood flow and limits the infarct size when used *in vivo* (11, 12). Administration of growth factors is presently emerging as a new therapeutic approach for the enhancement of collateral vessel formation in the ischemic heart (13).

Microspheres injected via a coronary catheter has been used to deliver fibroblast growth factor (FGF). FGF was released from these microspheres and caused proliferation of endothelial cells (14). bFGF bound to platelets resembles this model. Thus, platelet bound bFGF may have biological effects at the site of the platelet thrombus by interacting with endothelial cells. bFGF is released by heparin-like molecules or by plasmin (15). Plasminogen activators (16), heparin and low molecular weight heparin (17) are used to treat patients with myocardial infarctions. bFGF bound to the platelet surface may be released during this treatment. As injection of bFGF has a positive effect on myocardial function it is likely that bFGF released from platelets may have a similar effect.

One producer of ELISA kits for quantification of bFGF (R&D Systems, Minneapolis, MN, USA) states that the mean detectable bFGF concentrations in normal healthy individuals are 2.45 pg/mL in serum and 10.9 pg/mL in EDTA plasma. The binding of bFGF to activated platelets in clotting blood explain why the bFGF concentration is lower in serum than in plasma.

We have used flow cytometry to study interactions between bFGF and platelets. This method provides quantitative information on the expression of specific ligands on the platelet surface. Flow cytometry gives single-cell data, which allows the detection of only a small proportion of cells. We used FITC-labelled chicken antibodies as they have previously been shown to eliminate interference problems encountered with mammalian antibodies (7, 18). Addition of 4.8 pg bFGF/mL to platelet rich plasma caused a decrease in the percentage of fibrinogen positive platelets (Fig. 2). This is a physiological concentration that can be found in plasma from healthy individuals. When increasing the concentration to 9.1 ng/mL there was a further reduction in the percentage of fibrinogen positive platelets. Fibrinogen binding to platelets is essential for platelet aggregation. bFGF is present in platelets

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and is released during platelet activation. bFGF may thus have a negative feedback regulation on platelets similar to platelet-derived growth factor (PDGF) (19). This inhibition of fibrinogen binding to platelets is a possible new role of bFGF in thrombosis and may contribute to the positive effects of bFGF when bFGF is used to enhance collateral vessel formation in the ischemic heart.

The interactions between bFGF and platelets may have clinical implications both in tumour angiogenesis and in thrombosis. Further studies should be carried out to study the interactions between bFGF and platelets *in vivo*.

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