Exogenous fibronectin modifies the aggregation of collagen-stimulated human platelets

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Summary. Fibronectins (FN) are adhesive glycoproteins whose role in platelet aggregation is unclear. Addition of 3, 6 and 12 μg/ml of human plasma FN in vitro to isolated human platelets, which had been freed from plasma FN by gel filtration and subsequently stimulated with collagen, inhibited the last stage of platelet aggregation. With 3 and 6 μg/ml of FN a shortening of the lag-time was also observed. These data showed that FN may play a role in platelet-collagen interaction as well as in platelet-platelet interaction.
Key words. Platelet aggregation; fibronectin.

Platelets increased their adhesiveness to collagen or fibrin-covered surfaces following the addition of exogenous fibronectin (FN). Likewise, they showed an increased tendency to express the 'sticking together' and 'spreading' phenomena [1]. In contrast, other authors have reported that addition of FN in vitro to human platelets produces a marked lengthening of the lag time; the period which precedes the irreversible phase of platelet aggregation [2]. On the other hand it was shown that a high concentration of exogenous FN has an inhibitory effect on collagen-induced platelet aggregation [3]. While these observations indicate that FN has a role in platelet spreading and adhesiveness, its activity in normal platelet aggregation remains controversial. In this investigation several concentrations of exogenous human FN were tested on platelets devoid of plasma FN in order to establish whether this glycoprotein modifies platelet aggregation induced by collagen.

Materials and methods

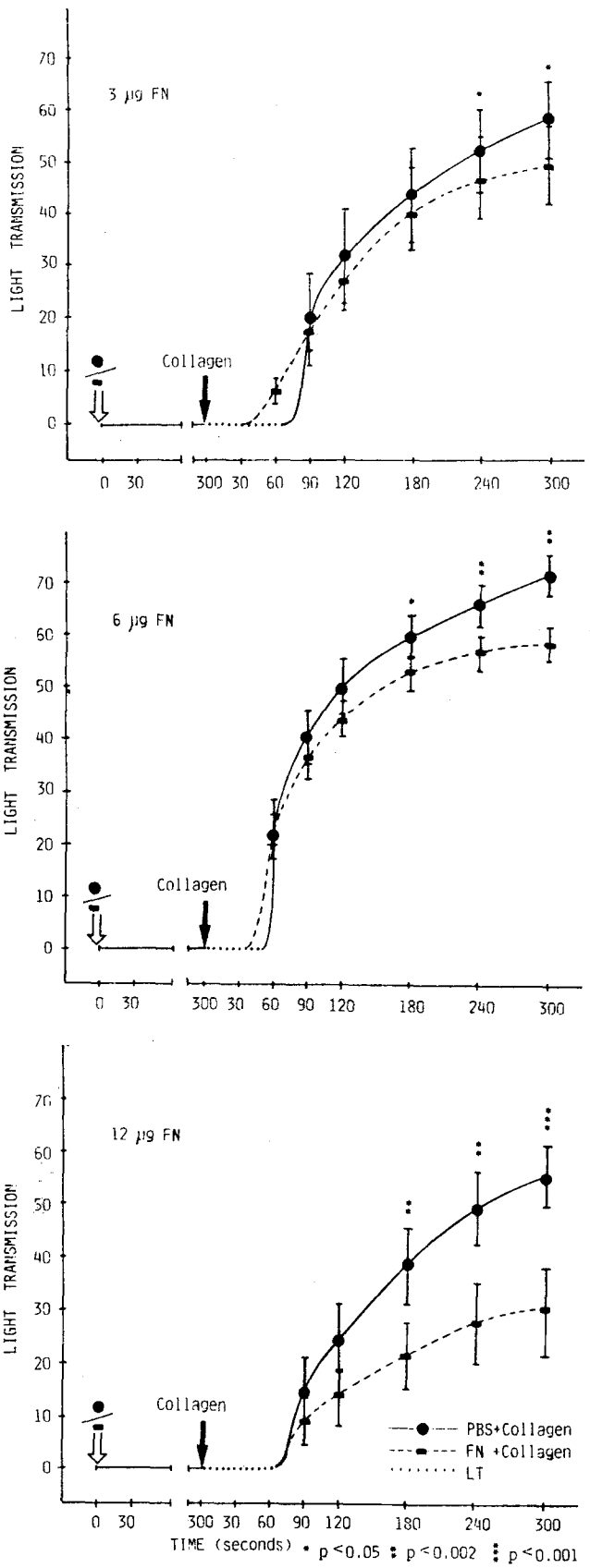
Platelets were obtained from human blood of 19 volunteers of both sexes, 20–40 years old. Blood was collected into 3.8% sodium citrate (9:1, v/v) and centrifuged to obtain platelet rich plasma (PRP) and platelet poor plasma (PPP). To eliminate plasma FN, the plasma of PRP samples was washed off by two centrifugations in phosphate buffered saline (PBS) pH 7.2 [4]. Pelleted washed platelets were then cleared of remnants of plasma proteins by gel-filtration on Sepharose 2-B (Sigma Chemical Co.) [5]. The column was eluted at room temperature with PBS and the first peak at the optical density of 280 nm was distinctive for the platelet-enriched fraction. Maximal concentration ranged from 100.000 to 150.000 platelets/μl. Samples of 500 μl of PRP or gel-filtered platelets (GFP) were pre-incubated for 5 min with FN to a final concentration of 3, 6, 12 or 50 μg/ml and then stimulated with collagen (50 μg/ml, calf skin, Type I, Sigma Chemical Co.). The appropriate collagen concentration was established through preliminary experiments. Fibronectin was obtained from human blood as previously described [6]. Control samples were obtained by incubating PRP or GFP samples with PBS instead of FN and then with collagen. Aggregation studies were performed using a single channel aggregometer (CYBORG Electrónica, Argentina). The following parameters were studied: 1) the length of the lag time (LT) recorded from the addition of the agonist until the onset of the first sharp pen deflection; 2) the maximal transmission (scale deflection), as an indicator of the completion of the aggregation, expressed as per cent of aggregation (PA), and 3) the slope of the aggregation, i.e. the angle with the baseline during the first 30 s, as an indicator of the speed of aggregation (SA) [2]. Results were evaluated by a paired test.

Results

FN exogenously added up to a maximal concentration of 50 μg/ml did not induce aggregation per se in PRP or GFP. Pretreatment of GFP samples with 3, 6 or 12 μg/ml of FN inhibits PA resulting from stimulation with collagen (fig.). Samples of GFP treated with 3 and 6 μg/ml of FN and subsequently stimulated with collagen showed a LT (mean ± SEM) of 47.0 ± 1 and 38.9 ± 5 s respectively, whereas control samples stimulated only with collagen had a LT of 79.3 ± 9 and 52.7 ± 6 respectively ($p < 0.05$). GFP samples pretreated with 12 μg/ml of FN did not have lag times significantly different from those of controls (fig.). No differences were recorded in tracings of SA between samples of GFP pretreated with FN and those post-stimulated with collagen, with respect to their controls. As expected, pretreatment of PRP samples with 3, 6 or 12 μg/ml of FN did not induce modifications in the tracings with respect to the controls when samples were post-stimulated with collagen.

Discussion

The addition of fibronectin to human platelets makes them tend to stick together, and increases spreading on collagen-covered surfaces [7–9]. Furthermore, we observed that pretreatment of platelets with the lowest concentrations of exogenous FN used (3 and 6 μg/ml) re-



Aggregatory response of collagen-stimulated human platelets to which fibronectin had previously been added. Each experiment was run at least in duplicate and the results are expressed as mean ± SEM evaluated by paired test. n = 19.

duced the LT of aggregation in a manner not dependent on FN concentration. Thus, FN adhesiveness may be involved in shortening the LT of platelet aggregation, since platelet activation could be enhanced and potentiated after preincubation with FN. In physiological conditions FN-receptors of resting platelets may be fully saturated by plasma FN[8]. After the addition of low concentrations of FN to suspensions of FN-free platelets the partial and fast occupancy of FN receptors could favor the triggering of the first stage of aggregation, and in turn induce the shortening of LT described at the lower doses in these experiments. Others have shown that FN contained in alpha granules is secreted during the release reaction. Due to its adhesive properties, released platelet FN may enhance the triggering of the irreversible phase of platelet aggregation[10,11]. These data, together with the present results, allow us to suggest that the in vitro pre-treatment of platelets with low concentrations of FN should favor the release of endogenous platelet adhesive glycoproteins, producing in turn the observed short LT. Shortening of the LT was not detected in our experiments when the higher concentration of FN was used. Likewise, it has been reported that exogenous FN addition to human platelets induces a lengthening of LT, due to an early inhibition of irreversible collagen platelet aggregation[2]. However, in these experiments the FN concentrations used were significantly higher than the level used in the present study and higher than that normally present in human plasma[11].

When samples were preincubated with exogenous FN we found a significant inhibition of PA induced by collagen, which was concentration-dependent. These results agree with previous data of Santoro et al., who obtained a maximal inhibition of collagen-induced platelet aggregation by adding FN at concentrations as high as 500 µg/ml[3,12]. These authors showed that the FN-induced inhibition of platelet aggregation was not followed by serotonin release, indicating that such inhibition was an early event during the irreversible phase of platelet aggregation[3,12]. In agreement with that, it has been shown that exogenous FN diminishes the speed of fibrillogenesis of native collagen, which is an important condition for the starting and progression of irreversible platelet aggregation[13,14]. The limited linkage of molecules of exogenous FN when a low concentration of this glycoprotein is available to platelet receptors may increase the bridge function of FN in a fast but limited manner which allows many platelets to remain in suspension[15]. Thus, added FN may play a role in at least two ways: inhibiting platelet-collagen interactions at low concentrations, and altering platelet-platelet interaction through collagen at higher concentrations.

In conclusion, our data indicate that exogenous FN at concentrations as low as 3 µg/ml induces a shortening of LT. On the other hand, FN has an inhibitory effect on the second phase of platelet aggregation, which conforms with findings of other investigators. Present results

should be of interest in pathology, since an antithrombotic action for FN has been postulated, mediated by the limitation of platelet-collagen interaction[2].

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1 Koteliansky, V. E., Leytin, V. L., Sviridov, D. D., Repin, V. S., and Smirnov, V. N. FEBS Lett. *1* (1981) 59.
2 Moon, D. G., Kaplan, J. E., and Mazurkewicz, J. E., Blood *67* (1986) 450.
3 Santoro, S. A., Biochem. biophys. Res. Commun. *116* (1983) 135.
4 Eynard, A. R., Galli, G., Tremoli, E., Maderna, P., Magni, F., and Paoletti, R., J. Lab. clin. Med. *107* (1986) 73.
5 Tangen, O., Berman, H. J., and Marfey, P., Thromb. Diath. Haemorrh. *25* (1971) 268.
6 Rovasio, R. A., Delouvée, A., Yamada, K. M., Rimpl, R., and Thiery, J. P., J. Cell Biol. *96* (1983) 462.
7 Bensusan, H. B., Koh, Th., Henry, K. G., Murray, B. A., and Culp, L. A., Proc. natl Acad. Sci. USA *75* (1978) 5864.
8 Hynes, R. O., and Yamada, K. M., J. Cell Biol. *95* (1982) 369.
9 Ginsberg, M. H., Forsyth, J., Lightsey, A., Chediak, J., and Plow, E. F., J. clin. Invest. *71* (1983) 619.
10 Leung, L., and Nachman, R., A. Rev. Med. *37* (1986) 179.
11 Mosesson, M. W., and Umfleet, R. A., J. biol. Chem. *245* (1970) 5728.
12 Santoro, S. A., and Cunningham, L. W., Proc. natl Acad. Sci. USA *76* (1979) 2644.
13 Kleinman, H. K., Wilkes, C. M., and Martin, G. R., Biochemistry *20* (1981) 2325.
14 Legrand, Y. J., Fauvel, F., Arbeille, B., Leger, D., Mouhli, H., Gutman, N., and Muh, J. P., Lab. Invest. *54* (1966) 566.
15 Yamada, K. M., Hasegawa, T., Hasegawa, E., Kennedy, D. W., Hirano, H., Hayashi, M., Akiyama, S. K., and Olden, K., Prog. clin. Biol. Res. *151* (1984) 1.

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Immunocytochemical demonstration of contractile cells in the human ovarian follicle[1]

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Summary. Actin- and myosin-like immunoreactivity is found in cells located in the theca externa of the follicle wall of the human ovary, and corresponding to previously observed myoid cells. The immunocytochemical observation provides direct structural evidence that non-vascular contractile cells are also present in the follicle wall in humans. As expected, perifollicular blood vessels showed a positive immunoreaction for actin and myosin in their smooth muscle walls.
Key words. Immunoreactivity; actin; myosin; ovary; contractile cells.

The ability of the human follicle wall to contract has been established in pharmacological experiments in vitro[2]. However, the morphological basis for the motor activity has been a matter of dispute[3]. At the ultrastructural level cells have been found in the theca externa layer with several characteristics of smooth muscle cells, i. e. filaments, dense bodies and micropinocytotic vesicles. Since the amount of filaments is somewhat lower than in 'classical' smooth muscle cells, and since transitional forms between fibroblasts and smooth muscle-like cells are present in the follicle wall, it has been questioned whether these ovarian cells are in fact contractile.

With the introduction of immunocytochemical methods for specific demonstration of the smooth muscle proteins, actin and myosin[4,5], it became possible to study whether the follicle wall contains contractile cells. In a study on rat ovaries, contractile proteins were demonstrated in elongated cells forming concentric layers in the theca externa of the Graafian follicle[6]. The present study was performed to answer the question whether the human ovarian follicle also contains cells with actin and myosin.

Preovulatory Graafian follicles were taken from fertile women, who were subjected to hysterectomy and had given their permission for the ovarian biopsy. The tissue was frozen and sectioned in a cryostat at −30 °C. 5-µm-thick sections were cut and placed under a hairdryer for 1–2 h. They were then incubated for 30 min at room temperature with specific γ-globulin enriched rabbit antibodies and their corresponding controls: 1) antiserum (1 mg/ml) raised against purified chicken gizzard smooth muscle myosin, 2) antiserum (1–2 mg/ml) to actin purified from an acetone powder of chicken gizzard smooth muscle, which showed a single band on 10% sodium dodecyl sulfate-acrylamide gel electrophoresis, corresponding to a molecular weight of 42,000, 3) antibody to gizzard smooth muscle myosin previously adsorbed to chicken gizzard myosin, 4) the same antibody adsorbed to striated muscle (pectoralis) myosin and 5) normal non-immune rabbit γ-globulin. After washing, the sections were incubated for 30 min in a solution of fluorescein-labelled immunoglobulin (1 mg/ml) raised in sheep against rabbit. Control sections were incubated with this second antibody alone. The sections were then washed in phos-