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PLASMA LIPOPROTEINS MEDIATE PLATELET ADHESION

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Platelet adhesion to VLDL, LDL, HDL, and to a mixture of purified apolipoproteins was examined. Platelets adhered to all the classes of lipoproteins tested. VLDL and the apolipoprotein mixture promoted the greatest degree of adhesion. Platelet adhesion was inhibited by addition of EDTA, RGD-containing peptides and anti-$GPII_b$-III_a monoclonal antibodies. Platelets from patients with Glanzman's Thrombasthenia which lack the $GPII_b$-III_a receptor adhered to VLDL less than half as well as did normal platelets. These results demonstrate that the major circulating lipoproteins can mediate in vitro platelet adhesion and that this adhesion occurs via platelet integrin receptors. We postulate that lipoprotein mediated platelet adhesion may play an important role in the progression of atherosclerosis.

A wealth of evidence exists linking platelets and their various components to various atherothrombotic processes. Recent studies on platelet-lipoprotein interactions suggested that platelets are important in the initiation and generation of the atherosclerotic plaque (1). The degree of involvement of platelets in atherosclerosis appears to be dependent upon their state of activation. Enhancement of platelet activity has been reported in hyperlipidemic conditions. Patients with primary hypercholesterolemia have been found to have increased platelet adhesion, aggregation and serotonin release. Increase of circulating platelet aggregates have been found in patient blood and patients had reduced bleeding times. Increased aggregation, secretion, and synthesis of arachidonic acid has been observed in platelets enriched with cholesterol (2). On the basis of these observations, a hypothesis can be proposed that abnormal levels of circulating lipids and lipoproteins may produce changes in platelet activation.

The purpose of this study was to investigate the adhesion of platelets to specific plasma lipoproteins, to explore the mechanism of this process, and

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to determine the relationship of platelet-thrombospondin interactions to this process. A further aim was to examine and to identify the platelet adhesion receptor for lipoproteins.

METHODS

Platelet isolation: Human platelets were washed by centrifugation by the method described earlier (3). Briefly, blood from normal volunteers was draw on ACD (6:1 v/v) and centrifuged at room temperature for 20 min at 110xg. Platelet rich plasma was removed from the top of the tube, PGE1 was added to a final concentration of 1 uM. PRP was centrifuged for 20 min at room temperature at 3000xg to sediment platelets. Pellets were gently resuspended in Hepes buffered Tyrode's solution pH 6.5 in the presence of 20 ug/ml apyrase and washed twice in the same buffer by centrifugation for 10 min at room temperature at 3000xg. Platelets were finally resuspended in the Hepes buffered Tyrode's solution, pH 7.4 to the concentration of 5x108/ml in the presence of 100 uM $MnCl_2$ or 5 mM EDTA and used immediately for adhesion assays.

Platelet adhesion assay: Wells of a 96 well microtiter dish (Costar) were incubated for 1 hr with 50 ul of a 40 ug/ml protein solution of either lipoproteins, TSP, or BSA. Proteins were dissolved in Hepes buffered saline (20 mM Hepes, 150 mM NaCl) with 2 mM $CaCl_2$, pH 6.5. After adsorption of the protein, wells were blocked with 200 ul of 1% BSA in the above buffer, for 1 hr, and then washed three times with Hepes buffered saline, pH 7.4. Washed platelets (100 ul) were then added to microtiter plates, and incubated for 30 min. When antibodies are used, platelets were pre-incubated with the respective antibodies for 30 min at room temperature and the adhesion assay performed as described. Non-adherent platelets were removed by aspiration and the wells washed three times with Hepes buffered saline. The total number of adherent platelets was determined by measuring their protein content as described previously (4). Each experiment was carried out in triplicate using platelets from at least five different normal donors and the results presented as the mean plus or minus the standard deviation.

Isolation of plasma lipoproteins: Various human plasma lipoproteins were isolated from fresh plasma of 12-hr fasted normal donors, and then treated with 5 mM N-ethylmaleimide to inhibit lecitin-cholesterol acyltransferase. To prevent proteolytic degradations of apolipoproteins, plasma was treated with 1 uM PPACK, 100 U/ml aprotinin, 0.01% NaN_3, and 0.5 mM PMSF (5). VLDL, LDL, and HDL were isolated by sequential ultra-centrifugation as previously described (6). VLDL was prepared by ultracentrifugation at plasma density (d<1.006 g/ml) for 18 hrs at 160,000xg, 15°C. The supernatant fraction was removed, washed, and dialyzed. LDL (d=1.006-1.063 g/ml) was obtained after adjusting the infranatant density to 1.063 by addition of solid NaBr, and centrifugation at 160,000xg for 20 hrs at 15°C. The preparation of HDL was carried out after removal of the top fraction and adjusting the density to 1.21 by addition of solid NaBr and centrifugation at 180,000 x g for 48 hrs at 15° C. The lipoprotein fractions were dialyzed extensively against Hepes buffered saline with 1 mM EDTA, and 0.02% NaN_3, pH 7.4 and stored at 4° C under nitrogen atmosphere. The purities of the lipoprotein fractions were assessed by agarose gel electrophoresis (7) using precast agarose slides.

Preparation of a mixture of apolipoproteins from HDL. The apolipoproteins obtained from human HDL, in particular apo A-I and apo A-II were isolated in the presence of denaturants using conventional gel filtration chromatography. Total HDL was delipidated with 3:2 (v/v) ethanol/diethyl ether at 0°C (8) and isolated using a modification of the original method (9). The protein was chromatographed on Sephacryl S-200 columns using the elution buffer containing

7M urea, 1 M NaCl, 10 mM Tris-HCl pH 8.6 in the presence of 1 mM EDTA and 0.02% NaN_3. The fractions corresponding to the positions of apo A-I and apo A-II were polled, dialyzed against 140 mM NaCl, 2 mM EDTA, pH 7.4 and stored in lyophilized from. All apolipoproteins or lipoproteins were either dissolved or dialyzed against Hepes-buffered saline before use in the adhesion adhesion assays.

RESULTS AND DISCUSSION

Our prior studies showed that inactivated platelets, treated with PGE_1 adhered to microtiter wells coated with either VLDL, TSP, or FN, but not to wells coated with bovine serum albumin (BSA) or BSA complexed with palmitic acid (10,11). In this study, platelet adhesion to VLDL, LDL, HDL, and to a mixture of apolipoproteins extracted from HDL was compared to that induced by thrombospondin or fibronectin. The results in Fig.1. showed that platelets adhered to all the classes of lipoproteins tested. VLDL and the relative apolipoprotein mixture, promoted the greatest degree of adhesion, compared to those induced by TSP or FN. On the other hand, HDL and LDL promoted adhesion to the extend of 30 - 50 % of that caused by TSP or Fn.

Addition of VLDL to the solution inhibits TSP- and VLDL-mediated platelet adhesion in a dose - dependent fashion, but had little or no effect on FN - mediated platelet adhesion (Fig. 2). Platelet suspensions pretreated for 30 min with various concentrations of VLDL (5-20 ug/ml) were evaluated for adhesion to TSP, VLDL, and FN. The highest concentration of VLDL inhibited in

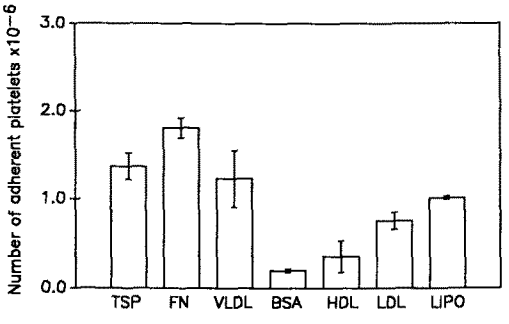

Fig.1. Platelet adhesion to various proteins and lipoproteins. Platelet adhesion was performed as described in Methods. The platelet adhesion proteins are designated by the abbreviations under each bar. TSP=thrombospondin, FN=fibronectin, VLDL=very low density lipoprotein, BSA=bovine serunm albumin, HDL=high density lipoprotein, LDL=low density lipoprotein, LIPO=mixture of apolipoproteins extracted from HDL.
Note: All lipoproteins tested promoted platelet adhesion.

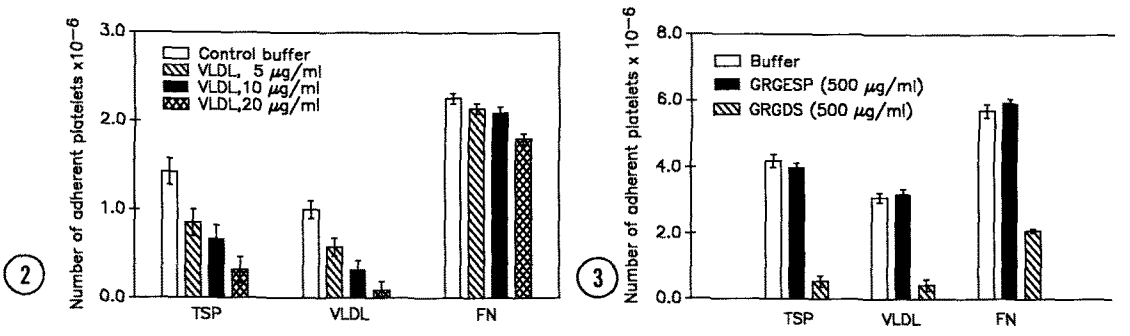

Fig.2. The effect of soluble VLDL on platelet adhesion to immobilized TSP, VLDL, and FN. Platelet adhesion was performed as described in Methods. The proteins adsorbed on the microtiter plate are designated by the abbreviation under each bar. Platelets were incubated with either no VLDL (open bars), 5 ug/ml VLDL (diagonally hatched bars), 10 ug/ml VLDL (solid bars), or 20 ug/ml VLDL (cross-hatched bars). **Note:** VLDL inhibited platelet adhesion to VLDL and TSP but not to FN.

Fig.3. The effect of adhesion peptide GRGDS and the inactive control peptide GRGESP on platelet adhesion to immobilized TSP, VLDL, and FN. Platelet suspensions were incubated prior to adhesion assays in the presence of either buffer (open bars), GRGESP (closed bars), or GRGDS (diagonally hatched bars) for 30 min at room temperature and then adhesion was performed as described in Methods. **Note:** GRGDS inhibits platelet adhesion to TSP, FN, and VLDL by more than 50% whereas GRGDESP has no effect.

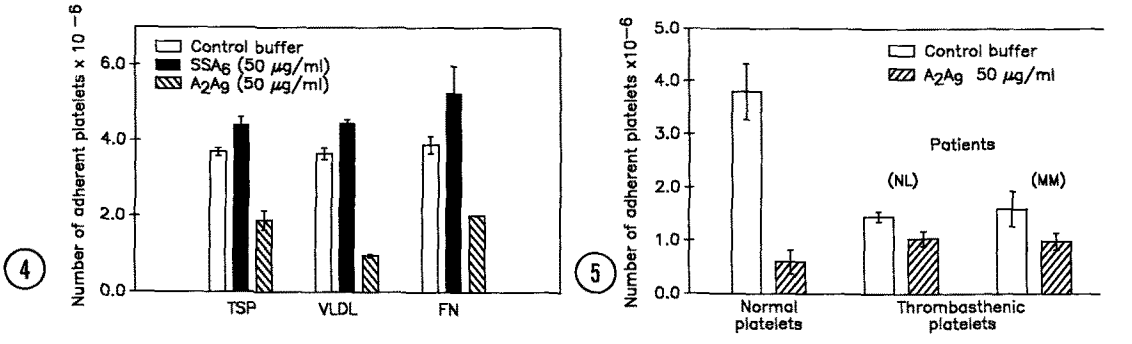

Fig.4. The effect of monoclonal antibodies against GPIIb-IIIa (A2A9) and GPIIIa (SSA6) on platelet adhesion to immobilized TSP, VLDL, and FN. Platelet suspensions were incubated prior to adhesion assays in the presence of either buffer (open bars), anti-GPIIIa (closed bars), or anti-GPIIb-IIIa (diagonally hatched bars) for 30 min at room temperature and then adhesion was performed as described in Methods. **Note:** Anti-GPIIb-IIIa inhibits platelet adhesion to TSP, FN, and VLDL by more than 50% whereas anti-GPIIIa has no effect.

Fig.5. Adhesion of Normal and Thrombasthenic Platelets to Immobilized VLDL. Platelet adhesion was performed as described in Methods. Platelets were incubated with either buffer (open bars), 250 ug/ml GRGDS (closed bars) or 50 ug/ml anti-GPIIb-IIIa antibody (diagonally hatched bars). **Note:** Thrombasthenic platelets adhered 60 % less to VLDL than normal platelets. GRGDS and anti-GPIIb-IIIa inhibited adhesion of normal platelets by more than 80% but had no effect on thrombasthenic platelets.

90% of VLDL -induced adhesion and 80% of TSP -induced adhesion but only 10% of Fn -induced platelets adhesion. Conversely, (data not shown) incubation of platelets with 20 ug/ml TSP for 30 min inhibited 80-90% platelet adhesion to TSP and VLDL, LDL, HDL, and apolipoprotein mixture extracted from HDL, but not to FN or FG. BSA or BSA complexed with palmitic acid did not have any effect on platelet adhesion to either protein or lipoprotein studied. These results suggest that TSP and VLDL share similar platelet receptors.

Lipoprotein - induced platelet adhesion was further studied in the presence of RGD-containing peptides and various monoclonal antibodies against platelet glycoproteins. The presence of peptide GRGDS at a concentration of 500 ug/ml inhibited platelet adhesion to TSP, VLDL, and FN by more than 80% whereas the inactive control peptide, GRGESP at the same concentration had no effect (Fig. 3). Preincubation of platelet suspensions with 50 ug/ml of anti-$GPII_b$-III_a antibody for 30 min at room temperature inhibited platelet adhesion to TSP, VLDL, and FN by more then 50%, while a monoclonal antibody against $GPIII_a$ at the same concentration had no effect (Fig. 4).

We also studied two patients with Glanzman's Thrombasthenia which lack the $GPII_b$-III_a receptor (Fig. 5). Platelets were incubated with either buffer (open bars) or 50 ug/ml of anti-$GPII_b$-III_a antibody (closed bars). Thrombasthenic platelets adhered to VLDL less than 50% as well as normal platelets. Anti-$GPII_b$-III_a antibody inhibited VLDL-mediated normal but not VLDL-mediated thrombasthenic platelet adhesion, suggesting that thrombasthenic platelets adhere to VLDL by receptors other than $GPII_b$-III_a.

These results suggest that platelet adhesion to VLDL is specific and mediated by cell receptors that bind TSP. In addition, at least one of these receptors has been identified as $GPII_b$-III_a, the major fibrinogen binding integrin of platelets.

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REFERENCES

1. Packham, M.A. and Mustard J.F. (1986) Sem. in Hematol. **23**, 8-26.

2. Kramer, R.M., Jakubowski, J.A. et al. (1982) J.Biol.Chem. **257**, 6844-6849.

3. Tuszynski, G.P., Switalska, H.I., and Knudsen K. (1987) Modern Methods in Pharmacology, Vol 4, 267-286.

4. Tuszynski, G.P. and Murphy, A. (1990) Anal. Biochem., **184**, 189-191.

5. Cardin, A.D., Witt, K.R., et al. (1984) J. Biol. Chem. **259**, 8522-8528.

6. Krempler, F., Kostner, G., et al. (1979) Biochim. Biophys. Acta **575**, 63-70.

7. Noble, R.P.: (1968) J. Lip. Res. **9**, 693-700.

8. Scanu, A.M. and Edelstein, C. (1971) Anal. biochem. **44**, 576-588.

9. Scanu, A.M., Toth, J., Edelstein, C., and Stiller, E. (1969) Biochemistry **8**, 3309-3316.

10. Tuszynski, G.P., Kowalska, M.A. et al.(1989) Thromb. Haemost. **62**, 418.

11. Kowalska, M.A., Tuszynski G.P., and Capuzzi, D.M. (1990) Clinical Research **38**, 3A.