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IMMUNOCYTOCHEMICAL LOCALIZATION OF FIBRINOGEN DURING THROMBIN-INDUCED AGGREGATION OF HUMAN PLATELETS. H. Suzuki, (1,3), R.L. Kinlough-Rathbone (1), M.A. Packham (2), K. Tanoue (3), H. Yamazaki (3) and J.F. Mustard (1). Dept. of Pathology, McMaster Univ., Hamilton, Canada (1), Dept. of Biochemistry, Univ. of Toronto, Canada (2) and Dept. of Cardiovascular Research, The Tokyo Metropolitan Inst. of Medical Science, Tokyo, Japan (3).

The association of fibrinogen (Fbg) with washed platelets was studied during thrombin-induced aggregation in Tyrode-albumin solution with 2 mM Ca^{2+} or no added Ca^{2+}. Platelets were fixed, embedded in Lowicryl K4M, sectioned, incubated with goat anti-human Fbg, washed, reacted with gold-labelled rabbit anti-goat IgG and prepared for electron microscopy. To ensure that Fbg could be detected with this method, platelets were pretreated with chymotrypsin and aggregated with Fbg; gold particles were apparent on the surface and between adherent platelets and in the alpha granules. In a Ca^{2+}-containing medium in the absence of external Fbg, washed platelets did not have Fbg on their surface although there was extensive gold labelling of the platelet alpha granules. Thrombin (0.05 U/ml) caused platelet aggregation, centralization and apparent fusion of alpha granules. By 60 sec large aggregates had formed, many platelets appeared degranulated but few gold particles were seen between adherent platelets. At 5 min, little Fbg remained in the aggregated platelets. In a few regions gold accumulated in fused granule material, and in occasional clusters between adherent platelets. In the presence of external Fbg (0.4 mg/ml) thrombin caused aggregation, centralization and fusion of granules, and discharge of granule contents. However, numerous gold particles were readily detectable between adherent platelets and on the platelet membrane. Fibrin formed and was abundantly labelled with immuno-gold. Similar findings were obtained in the medium without added Ca^{2+} (20 μM Ca^{2+}). These results agree with observations obtained from measurements of ^{125}I-Fbg binding and show that in the presence of external Fbg thrombin causes Fbg binding to platelets during aggregation. In the absence of added Fbg, thrombin aggregates platelets without extensive binding of released Fbg to the platelets.

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THE SURFACE EXPRESSION OF ALPHA GRANULE PROTEINS FOLLOWING THROMBIN STIMULATION. HR Gralnick, LM Magurder, K Hansmann, M Vail, G. Marti, R McEver*, and S Williams NIH, Bethesda, MD and the U. of Texas, San Antonio, Texas.

We have studied the platelet glycoproteins (GP) GPIb and the GPIIb/IIIa and the expression of alpha granule proteins (AGP) on the platelet (P) surface following thrombin (T) stimulation. The platelets were separated from plasma proteins on a arabinogalactan gradient. The P were stimulated with purified alpha T 0.1u/10^8P. Either monospecific polyclonal or murine monoclonal antibodies were used to detect the P glycoprotein and AGP. The platelets were analyzed on an EPICS V Flow Cytometer. Resting P had small amounts of AGP (2-8%) present on their surface. Within 1-3 min. after T stimulation significantly increased amounts of PF_4 (26%) vWf (8%) I_g (10%) and the 140 kD alpha granule membrane (70%) were present on the P surface. The peak expression of all the AGP occurred within 5 mins. The 140 kD activation protein remained stable over 3-60 mins, in contrast the PF_4 and the vWf expression peaked at 5 mins. and then decreased to near baseline levels. The GPIb and GPIIb/IIIa showed different patterns after activation. The GPIb intensity and number of positive cells decreased over time, while the GPIIb/IIIa increased in flourescent intensity and the number of positive cells. These studies indicate that T stimulation of AGP on the P surface. vWf and P_4 have a transient appearance on the P surface while I_g and the 140 kD activation protein both appear to become stable components of the P plasma membrane. This technique of detecting platelet activation is a specific, sensitive, and rapid method.

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HIGH MOLECULAR WEIGHT KININOGEN SPECIFICALLY BLOCKS THROMBIN-INDUCED AGGREGATION BY INHIBITING PLATELET CALPAIN. R.N.Puri (1), F.Zhou (1), H.Bradford (1), E.J.Gustafson (1), R.F. Colman (2),and R.W.Colman (1).Thrombosis Research Center, Temple Univ.School of Medicine, Phila. PA, (1), and Dept. of Chemistry, Univ. of Delaware, Newark Delaware, U.S.A.(2)

We have previously shown that platelet-aggregation induced by alpha-thrombin (1.7 nM) involves complete cleavage of the surface membrane polypeptide, M_r= 100 kDa (MP 100) labeled by FSBA in intact platelets. The failure to cleave MP100 in membrane preparations or in platelets treated with metabolic inhibitors or leupeptin, suggested that thrombin was acting by activating platelet calpain. Since high molecular weight kininogen (HMWK) is the most potent plasma inhibitor of calpain(s), we now report that HMWK inhibited thrombin-induced aggregation in a dose-dependent manner over a range of plasma concentrations. HMWK did not inhibit aggregation induced by ADP, collagen, U46619, A23187 and/or PMA. In order to study the action of HMWK in a plasma environment we utilized γ-thrombin. The aggregation induced by γ-thrombin (25 nM) in washed human platelets was also inhibited by HMWK. A much higher concentration of γ-thrombin (200 nM) was required to induce similar aggregation of platelets suspended in normal plasma. In contrast, γ-thrombin (50nM) induced complete aggregation of platelets suspended in plasma completely deficient in total kininogen indicating that a kininogen was predominently responsible for the inhibitory effect of plasma. When platelets were suspended in plasma deficient only in HMWK, aggregation required 75 nM γ-thrombin. When plasma deficient in HMWK was supplemented with a physiological concentration of HMWK (0.67 μM) the aggregation of suspended washed platelets was similar to that in normal plasma. Finally, we found that purified platelet calpain-2 not only exposed fibrinogen binding sites and induced platelet aggregation, but also completely cleaved MP 100 in both intact platelets and membrane preparations. We conclude: a) Thrombin-induced platelet aggregation involves the indirect proteolytic cleavage of MP100 by activating calpain-2, and b) Inhibition of thrombin-induced platelet aggregation by HMWK involves specific inactivation of platelet calpain.

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MONOCLONAL ANTIBODY (ANTI-2B6D4) TO PLASMA FIBRONECTIN INHIBITS COLLAGEN AND THROMBIN INDUCED AGGREGATION OF WASHED PLATELETS. D.A.Kenneally, P.J.Thurlow, J.M.Connellan. Haematology Department, Austin Hospital, Melbourne, Australia.

Fibronectins(Fns) constitute a family of large glycoproteins which are known to bind to a wide range of biological molecules eg.collagen,gelatin,fibrin,heparin and DNA,and to many cells including platelets via discrete structural domains. A murine monoclonal antibody (anti-2B6D4) produced by immunizing BALB/c mice with plasma fn,was used to study the structure and function of fn and its platelet interaction.Anti-2B6D4 reacted specifically with plasma fn was unreactive with FVIII/vWF,BIG,PF4 collagen and fibrinogen nor was it reactive with platelets (unstimulated), human PBLs or a range of tumour cell lines. Immuno-blotting studies(8 % SDS-PAGE) using thermolysin-digested plasma fn with anti-2B6D4 indicated that the 2B6D4 epitope was present on only 2 of the 7 fragments detected by Indian ink. The 2 fragments had an Mr of 145 and 155 K daltons and have been reported to each contain domains which bind cells,DNA and heparin. These fragments were studied further by examining the effect of anti-2B6D4 (Fabs) on the binding of ^{125}I-fn to thrombin-stimulated platelets and demonstrated that anti-2B6D4 binding was inhibited by 50% thus implicating the 2B6D4 epitope as a platelet binding site within the two cell binding domains. Competitive binding analysis of ^{125}I-fn to solid-phase macromolecules i.e.collagen, gelatin, fibrin,heparin,DNA and Con A demonstrated that anti-2B6D4 (Fabs) inhibited the binding of fn to DNA by 50%,but not to the other macromolecules. Therefore, either the DNA and platelet binding sites are shared or the inhibition is due to steric hindrance. However,as Fab fragments of anti-2B6D4 were used,it is more likely that the binding sites are shared. Functional studies were performed to investigate the role of 2B6D4 in platelet-platelet interaction. Anti-2B6D4 totally blocked the aggregation of washed platelets stimulated by low dose collagen (1.6ug/ml) and thrombin (0.05U/ml), partially inhibited arachidonic acid (250ugs/ml) induced platelet aggregation and had no effect on aggregation induced by A23187 (30uM). One other report had demonstrated that fn is a requirement for A23187 and low dose thrombin induced platelet aggregation. We conclude that fn plays an essential role in platelet aggregation induced by low dose collagen and thrombin.