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F11-Receptor (F11R/JAM) Mediates Platelet Adhesion to Endothelial Cells: Role in Inflammatory Thrombosis*

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Keywords

Human platelet F11 receptor (F11R), junctional adhesion molecule (JAM), platelet aggregation, adhesion, F11R peptides, HUVEC

Summary

The F11 receptor (F11R) is a cell adhesion molecule (CAM), member of the immunoglobulin superfamily found on the surface of human platelets, and determined to play a role in platelet aggregation, secretion, adhesion and spreading. The same molecule is present also at tight junctions of endothelial cells (EC) where it is known as JAM and acts as a CAM through homophilic interactions. The role of F11R/JAM in the interaction of platelets with endothelial cells was investigated in the current studies. We report here that washed human platelets adhere specifically to a matrix made of immobilized, recombinant sF11R. Furthermore, platelets adhere to cytokine- (TNF-α, INF-γ) stimulated human umbilical vein endothelial cells (HUVEC), and approximately 40-60% of the adhesive force is exerted by homophilic interactions between the F11R of platelets and EC. This is evidenced by the inhibition of platelet adhesion to endothelial cells by recombinant soluble form of the F11R, and by two F11R peptides with amino acid sequences of the N-terminal region, and in the 1st Ig fold of the F11R, respectively. This study suggests a role for F11R in the adhesion of platelets to cytokine-inflamed endothelial cells and thus in thrombosis and atherosclerosis induced in non-denuded blood vessels by inflammatory processes. Agents that block the F11R-mediated adhesion of platelets to EC may be of therapeutic value in controlling thrombosis and preventing heart attacks and stroke.

Introduction

The vasculature is recognized as a dynamic metabolic organ that exists under normal physiological conditions in an intact, undisturbed state (1). Endothelial cells (EC), which line the exposed (luminal) surface of blood vessels, are normally not thrombogenic, namely, healthy EC do not attract nor bind circulating platelets (2,3). It is well known that the physiological function of the endothelium is to facilitate blood flow by providing a highly thromboresistant surface to flowing blood that inhibits platelet adhesion and clotting (2). However, under inflammatory conditions, the nonthrombotic surface of EC can be transformed to a prothrombotic surface following exposure to cytokines (3, 4), resulting in procoagulant activity and a predisposition to thrombosis (3, 5, 6). Indeed, the adhesion, accumulation and recruitment of NON-stimulated platelets on cytokine-stimulated EC have been reported, with studies implicating the Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1; 7); beta 1 integrin (8), von Willebrand factor (5, 6), and tissue factor (9) in these processes. Thus, under inflammatory conditions, cytokines induce alterations in EC which result in the adhesion of non-stimulated platelets. Recently, a novel adhesion protein of the Ig superfamily has been described with properties indicating a potential triggering role in the pathogenesis of inflammatory thrombosis. This protein was identified first on the surface of human platelets and called the F11 receptor (F11R; 10; 11), and then on the surface of murine endothelial and epithelial cells and called JAM (12).

The human platelet F11 receptor (F11R) is a surface glycoprotein duplex (32 and 35 kDa; core protein: 29 kDa), member of the immunoglobulin superfamily. The F11R was first discovered as the target of a potent stimulatory monoclonal antibody, M.Ab.F11, that induces platelet secretion followed by aggregation (10, 11, 13-19). Signal transduction mechanisms for platelet secretion and aggregation induced by M.Ab.F11 following its initial binding to F11R include: crosslinking of the F11R to the FcγRII (11), activation and translocation of specific PKC isozymes (14), phosphorylation of the F11R through activation of PKC (11, 14), phosphorylation of the F11R following induction of platelet aggregation by the physiological agonists thrombin and collagen and by M.Ab.F11 itself (16-19), and phosphorylation of myosin light chain and pleckstrin, leading to shape change and granular secretion, respectively (10). Following secretion, this signal transduction pathway culminates in the activation of latent fibrinogen receptors and platelet aggregation (10). Partial amino acid sequences representing 30% of the length of purified F11R were reported by us in 1995 (11). Cloning of the full-length cDNA for the platelet F11R has revealed that it is a cell adhesion molecule (CAM), a member of the immunoglobulin superfamily (16-18). As a CAM, the F11R participates in mechanisms underlying adhesion of platelets, endothelial cells, and epithelial cells (12, 18).

The conclusion that in addition to its role as a receptor that triggers signal transduction leading to secretion, the F11R also serves as a CAM involved in platelet adhesion was supported by the high degree of sequence similarity found between the human platelet F11R and an adhesion protein called Junctional Adhesion Molecule (JAM), a protein found in murine endothelial cells (12). Comparison of the murine JAM sequence to the previously-reported sequences of human-platelet F11R (11) revealed over 70% homology of JAM to the N-terminus (23 a.a.)

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** M. H. Kedees and H. Athar contributed equally to this work.

of F11R and to two digested products of F11R. In addition, both the human platelet F11R core protein and the murine JAM protein were found to contain a single transmembrane domain and two pairs of cysteine residues in their extracellular domains that allow formation of intramolecular disulfide bridges forming characteristic Ig-like folds. It is now well established that the protein referred to as JAM (12, 20-24) is the murine ortholog of the human F11R (10, 11, 13-19). JAM was localized at intercellular junctions of mouse endothelial and epithelial cells (12).Similarly, the platelet antibody M.Ab.F11 was found to recognize F11R molecules present at intercellular junctions of cultured human umbilical vein endothelial cells (Sobocka et al., manuscript in preparation; 25). A recent study conducted in our laboratory (19) has determined that two domains of F11R are critical for the induction of platelet aggregation by M.AbF11 and the adhesion of platelets to M.Ab.F11. In the present study, we have investigated the role of the F11R in a physiological and pathophysiological process involving the adhesion of platelets to endothelial cells. We now report that the N-terminus and the 1st Ig fold of F11R contain sequences which are critical for the adhesion of platelets to endothelial cells, and that recombinant soluble F11R and F11R-peptides block approximately 60% of the adhesion of platelets to cytokine-treated EC, demonstrating the involvement of the F11R in platelet-endothelial cell interactions, which under pathological conditions result in thrombosis.

Material and Methods

All chemicals and biochemicals were of analytical grade quality.

Preparation of Washed Human Platelets

Whole blood was collected into the anticoagulant ACD (pH 4.6), as detailed (10). Platelets were washed by differential centrifugation in the presence of aggregation inhibitors apyrase, heparin, and PGE_1. The final platelet pellet was resuspended in a Tyrode-albumin (0.35%) solution buffered with 11.9 mM sodium bicarbonate (pH 7.35) (10, 18, 19) in the absence of inhibitors.

Preparation of the Recombinant Soluble F11R (rsF11R) Protein

The plasmid pcDNA3.1/F11R was constructed using the human F11R receptor cDNA as a template (18). PCR amplifications, transfections of plasmids into COS-7 cells, and purification of the secreted F11R protein from COS-7 cell media using M.Ab.F11 affinity chromatography, as well as recombinant DNA methods utilized in this study, were performed using standard molecular biology techniques (26), as previously described (19).

Synthesis of F11R-Peptides

F11R peptides (95% pure) and scrambled peptides were synthesized by New England Peptides, Inc., (Fitchburg, MA) and their mass determined by MALDI-TOF DE mass spectrometry. The sequence of amino acids in these peptides is as follows: F11R peptide 1 ^{1}SVTVHSSEPEVRIPENNPVKLSC^{23}; scrambled peptide 1 ^{1}SPHEVRNPKSESVTVNPIESVLC^{23}; peptide 4 ^{70}KSVTREDTGTYTC^{82}; scrambled peptide 4 ^{70}KTEDTRYTSGTVC^{82} and peptide 5 ^{128}EQDGSPPSEYTWFKD^{142} and peptide 2^{55} SYEDRVTFLPTGITFKSVTRED^{76}.

Platelet Adhesion to an Immobilized Recombinant Soluble F11R (rsF11R) Matrix, and to Human Umbilical Vein Endothelial Cells (HUVEC)

An adhesion assay, based on the determination of cell-derived protein using Bicinchoninic Acid (BCA) protein assay (27), was used for the determination of platelet adhesion to immobilized rsF11R. Wells of a 96-well plate (Nunc-Immuno™ Plate, MaxiSorp™ Surface, flat bottomed) were incubated overnight at 4° C with 150 μl of a 1 μg/ml solution of affinity-purified rsF11R. Wells were aspirated, washed, treated with TBS/1%BSA for 1 h at 37° C, and washed extensively with TBS/0.1 mM $MnCl_2$/0.1 mM $CaCl_2$. Aliquots (100 μl) of washed, isolated platelets, resuspended in Tyrode's buffer (10^8/ml) were applied and plates incubated at 37° C for 90 min. Wells were washed extensively and the number of platelets which adhered to the matrix was measured from a standard curve prepared with known concentrations of platelets. The adhered platelets were dissolved by the addition of 100 μl BCA to each well. Following incubation at 37° C for 2 h, the absorbance at 595 nm was determined (Dynatech Laboratories, Chantilly, VA).

Preparation of Human Umbilical Vein Endothelial Cells (HUVEC)

EC were obtained from human umbilical cords by collagenase digestion following well-established procedures (28, 29) and cultured in complete medium (Medium 199 containing 20% heat-inactivated fetal calf serum (FCS) (GIBCO) and 25 μg/ml EC growth supplement (ECGS) in gelatin-coated tissue culture flasks. At confluence, the cells were detached using 0.125% trypsin and 1 mM EDTA, resuspended in Medium 199 plus 20% FCS and 25 μg/ml ECGS, and plated onto gelatin-coated 96-well plates.

Platelet Adhesion to HUVEC

The number of adhered platelets that bound to HUVEC was determined by the BCA method (27) using a platelet standard curve prepared with various dilutions of washed, isolated platelets. Monolayers of primary HUVECs were plated in 96 well plates in Medium 199 (GibcoBRL) containing 20% FCS plus ECGS (Calbiochem), and heparin (50 μg/ml) (Sigma) in the presence of penicillin-streptomycin-glutamine (PSG) (GibcoBRL). HUVEC were grown in 10% FCS in complete DMEM plus ECGS and heparin in the presence of PSG. At 100% confluency, HUVECs were treated for 48 h at 37° C with either TNF-α (100 units/ml) or INF-γ (200 units/ml) containing 5% FCS in Medium 199. Aliquots of washed platelets (10^8/ml) were applied onto the HUVEC monolayers and incubated at 37° C for 90 min. Following extensive washings of each well, the number of adhered platelets was calculated by the BCA method. The optical densities attributed solely to HUVEC monolayers were subtracted from the total optical densities.

Immunofluorescence Confocal Microscopy

The adhesion of intact, resting platelets (10^8/ml) to immobilized recombinant soluble F11R on glass coverslips was observed using a Biorad Radiance 2000 confocal microscope at 400× magnification. Coverslips were incubated with a solution of 10 μg/ml of rsF11R or an identical concentration of BSA for 1 h at 37° C. Platelet adhesion to BSA was measured as a control. Following extensive washings of the coverslips, the adhered platelets were fixed in paraformaldehyde and labeled with M.Ab.F11 followed by the addition of secondary antibody goat anti-mouse IgG-FITC.

Molecular Modeling

A three-dimensional model of the human soluble recombinant F11R (amino acids 1-208 of the mature protein) was derived from the crystallographic data of the murine junctional adhesion molecule (30), which was used as a template for the human F11R (Brookhaven Protein Database ID:1F97, deposition date: July 7, 2000), as detailed in our previous study (19). The distances between amino acids were calculated using the Kinemage software.

Results

Platelets Adhere to Recombinant sF11R

A soluble, recombinant F11R polypeptide (rsF11R), secreted by COS-7 cells, containing amino acids ser-1 to asn-208 of the extracellular portion of the mature platelet F11R molecule, was purified from COS-7 conditioned media. This recombinant protein was examined for

its ability to act as a platelet adhesion molecule. As shown in Fig. 1, confocal microscopic observations demonstrated adhesion of non-stimulated platelets to an immobilized rsF11R matrix, revealed by immunostaining with M.Ab.F11. A rim-staining pattern of immunofluorescence at the platelet plasma membrane indicated the surface location of F11R in the adhered platelets. Compared to the highly localized staining of platelets by M.Ab.F11, only very faint staining of the rsF11R-immobilized matrix by M.Ab.F11 was seen in areas devoid of platelets. Under the same assay conditions, platelets did not bind to immobilized BSA.

Binding of platelets to rsF11R was also studied by an independent microtiter well-binding assay (Fig. 2). In this assay, purified rsF11R was allowed to adhere to microtiter plates, and then reacted with washed platelets. There was significant binding of nonstimulated platelets to immobilized F11R (see Non-s in Fig. 2). Stimulation by the physiological agonists ADP or collagen, caused a 1.3 and 2 fold increase, respectively, in the adhesion of platelets to immobilized rsF11R. As also shown in Fig. 2, this adhesion was almost *completely* blocked (94.6 ± 2.9%) by adding soluble rsF11R to the medium. In addition, peptide 1 also caused 99.7 ± 0.3% inhibition of the adhesion of nonactivated or activated platelets. At the same concentration, peptide 4 caused approximately 53 ± 16% inhibition in the binding of platelets to immobilized rsF11R. In contrast, the adhesion of platelets was not inhibited by F11R peptides 2 or 5 (data not shown). Peptide 1 is a 23-mer peptide with the sequence of the N-terminal 23 amino acids of the F11R. Peptide 4 represents the sequence of 13 amino acids ^{70}KSVTREDTGTYTC^{82} within the 1st Ig fold (19).

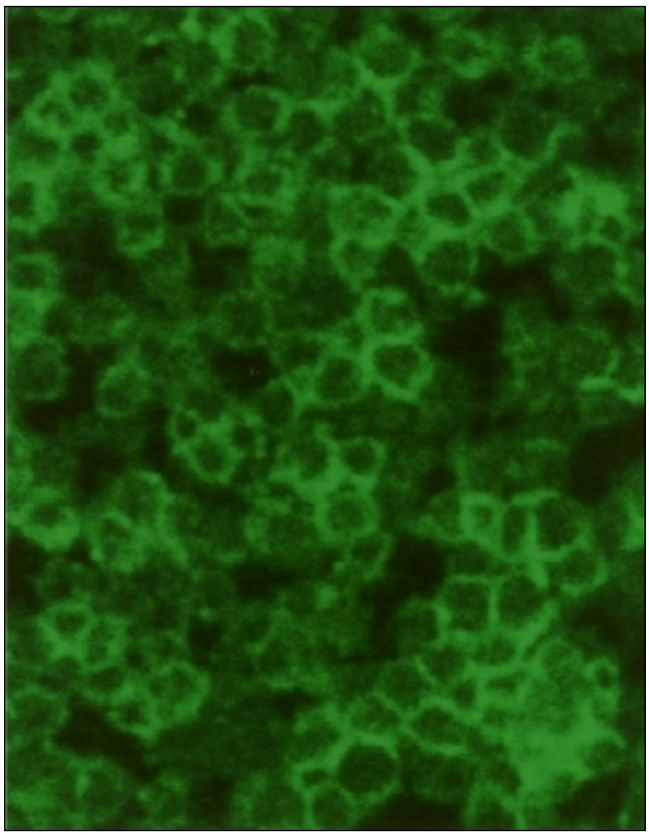

Fig. 1 Adhesion of platelets to recombinant soluble F11R: immunofluorescence staining. The rsF11R (10 μg/ml) was immobilized on a coverglass surface by incubation for 12 h at 4° C. Bovine serum albumin (10 μg/ml) was used as a control. Aliquots of washed platelet suspensions (10^8/ml) were added and the incubation was continued for 90 min at 37° C. The platelets were fixed with 2% paraformaldehyde and incubated at 4° C for 1 h. Following 3 washings, the adherent platelets were immunostained using primary M.Ab.F11 antibody followed by FITC-conjugated 2nd anti-mouse antibody, and viewed by confocal microscopy at 400× magnification. The field of immunostained platelets adhered to the immobilized rsF11R, shown in this figure, is representative of all fields viewed under the microscope. BSA control samples did not exhibit any adherent platelets

Platelets Adhere to Cytokine-Treated Human Umbilical Vein Endothelial Cells (HUVEC)

The adhesion of platelets to an immobilized matrix of rsF11R suggested that F11R can contribute to the adherence of platelets to the endothelium. We have examined this possibility by measuring the adhesion of platelets to monolayers of HUVEC cultured in 96-well plates. As shown in Bar 1 of Fig. 3, and expected from previous reports, nonactivated platelets did not bind to cultured HUVECs, demonstrating that the surface of these cells is nonthrombogenic. Following the exposure of confluent monolayers of HUVECs for 48 h at 37° C to TNF-α (100 units/well), there was significant platelet adhesion to the treated HUVECs (Fig. 3, Bar 2). The contribution of F11R to the binding of intact platelets to TNF-α treated HUVEC was examined by adding to the medium the soluble, recombinant, F11R polypeptide, which represents the extracellular domain of this protein. As shown in Bar 3 of Fig. 3, the presence of rsF11R (150 ng/well) resulted in a 55% inhibition of platelet adhesion to monolayers of HUVEC pretreated with TNF-α. In similar experiments, addition of F11R peptide 1 or peptide 4 inhibited this adhesion by 50-65%. The stimulation of platelets with collagen resulted in a four-fold increase in their adhesion to TNF-α treated HUVECs. The addition of soluble recombinant F11R resulted in 42 ± 1% (mean ± S.E.M) inhibition of the adhesion of collagen-activated platelets to TNF-α treated HUVEC.

Exposure of cultured HUVEC to another cytokine, INF-γ (200 units/well) resulted in platelet adhesion similar to that shown in Fig. 3 with endothelial cells treated with TNF-α. As seen with TNF-α treatment, the activation of platelets with collagen also caused approximately four-fold increase in the adhesion of platelets to INF-γ pretreated EC. Fig. 4 shows the adhesion of collagen-stimulated platelets to cultured HUVEC pretreated with INF-γ. The addition of soluble recombinant F11R to the assay medium caused 40% inhibition of this adhesion, whereas, F11R peptide 1 and 4 caused 54% and 68% inhibition, respectively, of this adhesion.

The specificity of the inhibition by peptides 1 and 4 of platelet adhesion to cytokines (TNF-α or INF-γ)-treated EC was tested by the use of scrambled peptide 1 with the sequence ^{1}SPHEVRNPKSESVTVNPIESVLC^{23} and scrambled peptide 4 with the sequence ^{70}KTEDTRYTSGTVC^{82}. As expected from negative controls, these scrambled peptides had no inhibitory effects in any of the adhesion assays reported here. Another control was F11R-peptide 5 with the sequence ^{128}EQDGSPPSEYTWFKD^{142}, from the 2nd Ig fold of F11R (19). Peptide 5 did not inhibit platelet interaction with M.Ab.F11 (19), and also did not inhibit in the present study platelet adhesion to EC (see Fig. 4).

The two domains of the F11R containing the sequences of peptides 1 and 4 are depicted within the three-dimensional structure of the soluble, human recombinant F11R illustrated in Fig. 5A. The 3D modeling revealed that the N-terminal loops around the 1st Ig fold of the F11R molecule at sequences corresponding to peptides 1 and 4. Furthermore, these sequences were found to reside in space in very close proximity to each other as depicted in Fig. 5B. The close proximity observed

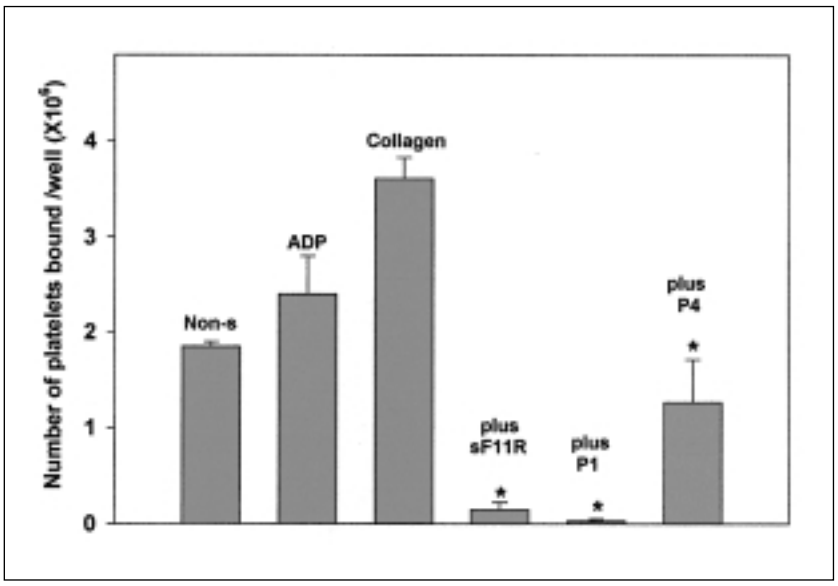

Fig. 2 Adhesion of platelets to immobilized recombinant sF11R (rsF11R): inhibition by soluble rsF11R and F11R-peptides. Purified rsF11R (10 μg/ml) was immobilized onto 96 well plates, as detailed in the Material and Methods section. Platelet suspensions (10^8/ml), intact, nonstimulated or stimulated with ADP or collagen, were applied onto the rsF11R matrix and incubated for 90 min at 37° C, in the absence or presence of soluble recombinant sF11R or F11R peptides 1 or 4. Following extensive washings of each well, the specific binding of platelets to the immobilized recombinant sF11R was quantitated by the BCA method, as detailed in the Material and Methods section. For nonspecific binding, BSA (10 μg/ml) was immobilized in 96 well plates and platelet suspensions were applied onto the BSA matrix. The binding of platelets to BSA was negligible, and the O.D. values of BSA wells were subtracted from the values of recombinant sF11R containing wells. Type of platelets used: Non-s = nonstimulated platelets; ADP = ADP (10 μM)-treated platelets; collagen = collagen (2 μg/ml)-treated platelets; plus sF11R = presence of soluble recombinant F11R (10 μg/ml); plus P1 = presence of F11R peptide 1 (500 μM); plus P4 = presence of F11R peptide 4 (500 μM). The asterisk (*) indicates that significant differences at the $p < 0.05$ level were observed in those wells to which soluble sF11R, peptide 1 or peptide 4 were added

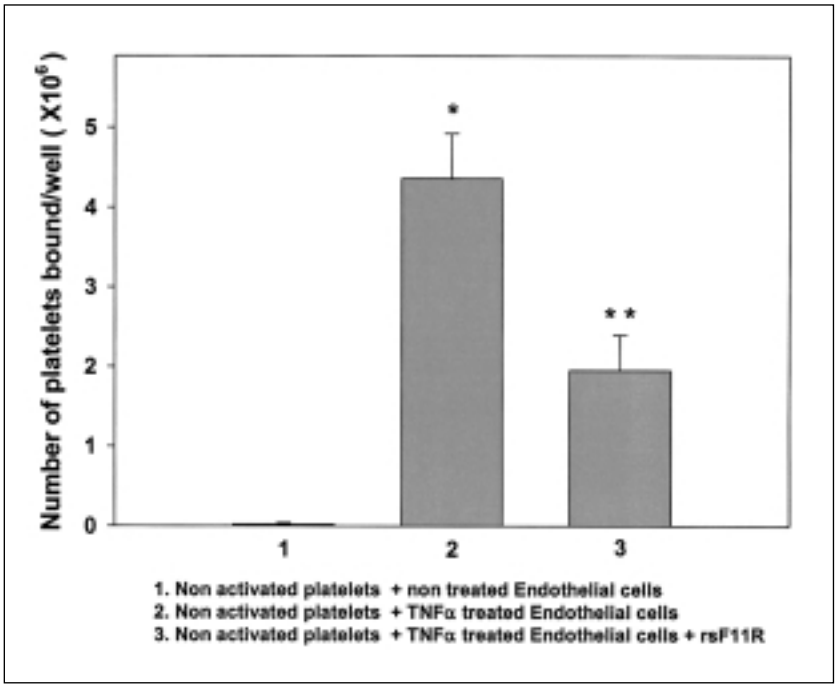

Fig. 3 Adhesion of platelets to TNF-α treated endothelial cells: role of F11R. Confluent monolayers of human umbilical vein endothelial cells (HUVEC) were treated with TNF-α (100 units/ml) for 48 h at 37° C, as detailed in the Material and Methods section. Isolated, washed nonstimulated platelets (10^8/ml) were applied onto the treated HUVECs and incubated for 90 min at 37° C. Wells were washed 3X to remove nonadherent cells, and the adherent platelets were quantitated by the BCA protein method. The O.D. values of HUVEC monolayers alone were subtracted from the total values obtained in the presence of platelets. Bar 1 = the addition of nonstimulated platelets onto monolayers of endothelial cells preincubated without any cytokines. Bar 2 = addition of nonstimulated platelets onto monolayers of TNF-α treated endothelial cells. Bar 3 = the addition of nonstimulated platelets onto monolayers of TNF-α treated endothelial cells conducted in the presence of added soluble recombinant F11R (rsF11R) (10 μg/ml). The asterisk (*) or (**) indicates significant differences at the $p < 0.05$ level, in the absence of presence of rsF11R, respectively

between these sequences was confirmed by measuring distances between apposed amino acid residues. Distances of 4.436 Å, 4.459 Å, 4.787 Å, 5.076 Å and 5.970 Å were determined between pro-18 and ser-71, pro-18 and lys-70, asn-17 and ser-71, asn-16 and val-72, and asn-17 and val-72, respectively (see Fig. 5B). This steric arrangement produces a small pocket region consisting of six amino acids: the three amino acids asn-16, asn-17, and pro-18 of the N-terminal, and three amino acids lys-70, ser-71 and val-72 of the 1st Ig-fold (see Fig. 5B, encircled region). Distances between other chain atoms outside of this pocket were at least double than 5.970 Å (14 Å or greater). The close proximity of the amino acids within the pocket region suggests that this steric region may form an epitope involved in trans-homophilic interactions between molecules of F11R on the surface of platelets and endothelial cells.

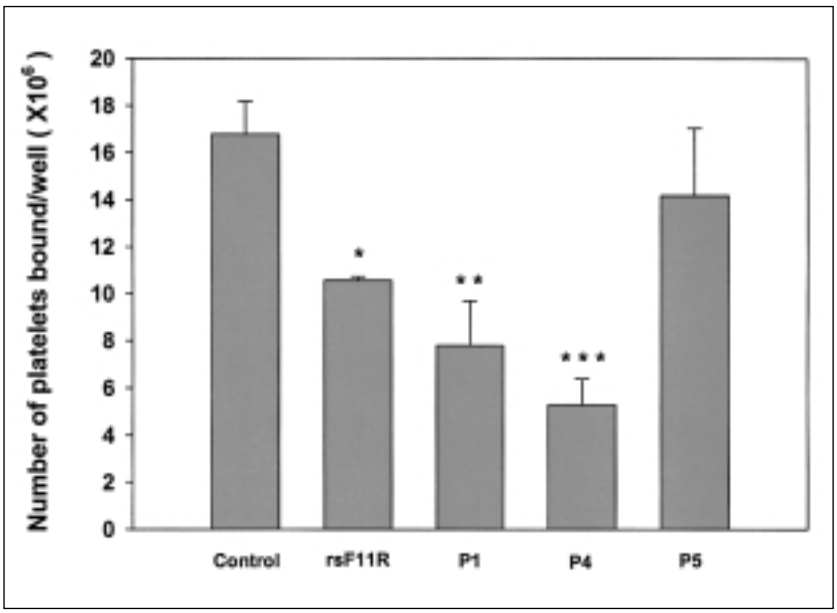

Fig. 4 Adhesion of platelets to INF-γ treated HUVEC: inhibition of platelet adhesion by addition of recombinant rsF11R and F11R peptides 1 and 4. Primary, confluent monolayers of human vein endothelial cells grown in 96 well plates were treated with INF-γ (200 units/ml), and maintained *in culture* for 48 h at 37° C, as detailed in the Material and Methods section. Washed platelet suspensions (10^8/ml) were stimulated with collagen (10 μg/ml), and aliquots (100 μl) were *immediately* applied onto the HUVEC monolayers which contained recombinant soluble F11R protein (rsF11R) (10 μg/ml), F11R peptides 1, 4 or 5 at final concentrations of 500 μM. Following a 90 min incubation period at 37° C, wells were washed and rinsed 3 times, and the number of adhered platelets was quantified using the BCA method. Control = no additions to the medium; rsF11R = addition of 10 μg/ml of soluble recombinant F11R protein; P1 = addition of F11R peptide 1 (500 μM); P4 = addition of F11R peptide 4 (500 μM); P5 = addition of F11R peptide 5 (500 μM). Histograms which represent the addition of rsF11R (*), P1 (**), or P4 (***) were found to be significantly different from the control ($p < 0.05$); but histogram P5 was not significantly different from controls

Discussion

Endothelial cells (EC) lining the luminal surface of blood vessels do not attract nor bind circulating platelets (1-3). The endothelial factors which account for the maintenance of a non-thrombogenic surface include the release of nitric oxide and prostacyclin and expression of an ecto-ADPase (CD39) (33-35). The nonthrombotic surface of EC can be transformed to a prothrombotic surface following exposure to inflammatory agents (3, 4), resulting in procoagulant activity and a predisposition to thrombosis (3, 5, 6). Indeed, the adhesion, accumulation and recruitment of non-stimulated platelets on cytokine-stimulated EC have been reported, with studies implicating P-selectin and P-selectin glycoprotein ligand (PSGL-1) (36-38), Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1) (7, 39, 40); beta1 integrin (6, 8, 41), von Willebrand factor (5, 6), and tissue factor (9) in these adhesive processes. The results of the study reported here confirm previous reports that the surface of endothelial cells is nonthrombogenic, and that the treatment of cultured HUVEC to the inflammatory cytokines TNF-α and/or INF-γ induces the exposure of thrombogenic factors on the cell surface that can cause adhesion of nonstimulated platelets. Furthermore, we demonstrate for the first time that the platelet surface protein called F11R (10) contributes a significant force to this adhesion. Indeed, the main new finding reported here is that 40-60% of the adhesion of non-stimulated human platelets to cytokine-treated, human EC is inhibited by soluble recombinant F11R molecule and specific F11R-peptides. As discussed below, this finding identifies a new, critical factor operating in the formation of platelet plaques on the surface of non-denuded blood vessels, and provides a novel target in the search for clinically useful inhibitors of inflammatory thrombosis.

Platelets express constitutively 8,067 ± 1,307 molecules of F11R per cell on their surface membrane (10). Platelet stimulation is not a prerequisite for the binding of a monoclonal antibody to F11R on the platelet surface. Furthermore, the binding of a specific F11R monoclonal antibody to the platelet surface can trigger a signaling pathway that leads to platelet secretion followed by exposure of fibrinogen receptors and platelet aggregation (10). By preparing a recombinant protein consisting of the extracellular domain of the platelet F11R, and using it as an immobilized matrix, we have demonstrated in the present study that F11R exposed on the surface of nonstimulated platelets can participate in an adhesion process in which the counterparts are molecules of F11R. As reported previously for JAM on the surface of EC (21), the inhibition of this adhesion by soluble rsF11R indicated that also adhesion of F11R molecules is mediated by homophilic interactions. This finding, however, does not exclude the possibility that F11R acts also by heterophilic adhesion to another surface protein (42).

The specific domain in F11R responsible for platelet adhesion to HUVEC was investigated by use of specific F11R peptides. We used here peptides that were found previously to specifically inhibit platelet aggregation induced by M.Ab.F11 (19). We observed that the peptides named F11R-1 and F11R-4 blocked the adhesion of platelets to cytokine-treated HUVEC. Thus, the two specific regions of the F11R shown to be responsible for the activation of platelets by the stimulatory antibody M.Ab.F11, operate also in the adhesion of platelets to cytokine-treated HUVEC. In particular, the region which encompasses the N-terminus of the F11R (and contained within the sequence of peptide 1), and the region spanning from the amino acid K-70 to C-82 of the proximal half of the first Ig-like domain (Ig fold) of the mature F11R (and containing the sequence of peptide 4) were found to be within the active site of F11R operating in the adhesion of platelets to endothelial cells.

The involvement of two separate regions of F11R in platelet adhesion suggests that the two sequences involved are located in the vicinity of

one another within the 3 dimensional structure of the molecule, and may produce a single adhesion zone. Modeling of the steric structure of rsF11R revealed that the N-terminal tail of the molecule indeed loops in the direction of the 1st Ig fold, and the sequences included in peptide 1 and peptide 4 produce a single steric pocket for the binding of M.Ab.F11 (19). Fig. 5 demonstrates that such a single pocket in the 3-dimensional structure of the membrane-bound F11R on the surface of platelets and endothelial cells can also provide the critical site for homophilic interactions of F11R that mediate the adhesion of platelets to EC. The sequence and structure of amino acids in this pocket present important information for new drug development (43).

A human homologue of endothelial JAM (20, 21) was sequenced, and found to be *identical* to the sequence of the human platelet F11R (11, 18). The expression of the JAM protein was found to be modulated on the surface of human endothelial cells by the pro-inflammatory cytokines TNF-α and INF-γ (20), suggesting that the human ortholog of JAM, the F11 receptor, plays a role in inflammation. These results suggested to us that F11R molecules exposed on the luminal surface of inflamed EC can provide the trigger for the adhesion of platelets to EC on the inflamed vessel wall. Indeed, the results reported here demonstrate directly that the adhesion of nonstimulated human platelets to human EC treated with inflammatory agents is significantly inhibited by agents that specifically block the action of F11R. Cytokines that are released during inflammatory processes can induce and increase the expression of F11R/JAM molecules on the exposed surface of EC (20). It thus appears that the F11R-mediated adhesion of platelets to cytokine-treated EC may have a causatory role that can trigger the initiation of a thrombotic process under inflammatory conditions. This conclusion has been strengthened by the identification in the regulatory region of the sequenced F11R gene of a unique consensus sequence driven by NF-κB (31), which is known to activate gene transcription under inflammatory conditions. Thus, the increased expression of F11R molecules on the exposed surface of EC that is induced by cytokines is likely mediated by the action of NF-κB. As the regulatory elements for the transcription factor NF-κB were found in the promoter region of the F11R gene (31), these results independently lead to the conclusion that the CAM activity of F11R contributes to the adhesion of platelets to endothelial cells under inflammatory conditions. This conclusion places F11R at the center of interactions

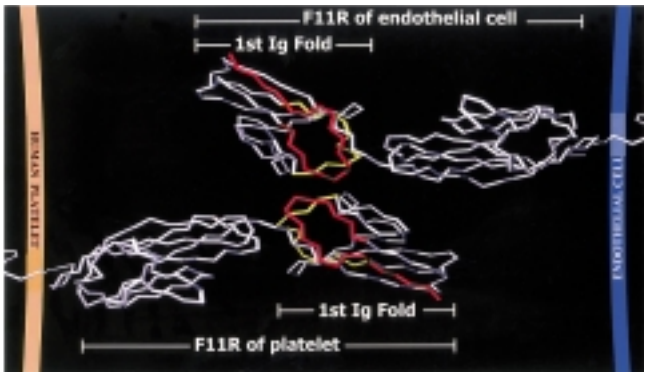

Fig. 5A A schematic drawing demonstrating the adhesion of platelets to endothelial cells through the F11 receptor. The F11R is depicted as a backbone structure in its entirety including the extracellular, soluble domain, consisting of two Ig-folds, a transmembrane domain and a short cytoplasmic portion. The N-terminal 23 amino acid region (^{1}SVTVHSSEPEVRIPENNPVKLSC^{23}) is colored *red*, and was used to synthesize F11R-peptide 1. The 13 amino acid region (^{70}KSVTREDTGTYTC^{82}), found within the 1st Ig fold, is depicted in the color *yellow*; this sequence was used to synthesize F11R-peptide 4. In this model, the F11R of platelets and endothelial cells is shown to align closely in a F11R-mediated trans-homophilic interaction through a pocket formed by these two amino acid regions, as depicted in Fig. 5B and 5C

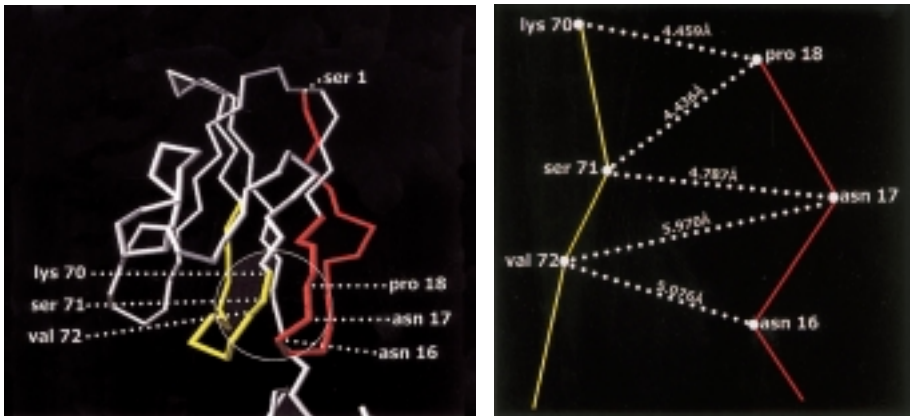

Fig. 5B Magnified View of the peptide 1 and peptide 4 domains of the F11R protein. This picture shows the backbone structure of the 1st Ig fold of the F11R protein (lacking the 2nd Ig fold which was removed for clarity). The red color indicates the amino acid sequence of peptide 1: ^{1}SVTVHSSEPEVRIPENNPVKLSC^{23} with the location of amino acids asparagine$_{16}$ (N), asparagine$_{17}$ (N) and proline$_{18}$ (P). The yellow color indicates the amino acid sequence of peptide 4: ^{70}KSVTREDTGTYTC^{82} with the position of lysine$_{70}$ (K), ser$_{71}$ (S) and valine$_{72}$ (V). The closely-aligned pocket region is encircled

Fig. 5C The distances between each of the amino acids located within the two inhibitory regions were calculated in Angstrom units. The distance between asn$_{16}$, asn$_{17}$ and pro$_{18}$ (which are within the N-terminal 23 amino acid sequence ^{1}SVTVHSSEPEVRIPENNPVKLSC^{23}; colored red) and lys$_{70}$, ser$_{71}$ and val$_{72}$ (which are within the 13 amino acid sequence ^{70}KSVTREDTGTYTC^{82}, colored yellow) of the 1st Ig-fold were calculated to be in closest proximity of each other. Of all of the chain atoms, the smallest distances were found to occur between amino acids ser$_{71}$ and pro$_{18}$ of 4.436 Å, followed by distances between lys$_{70}$ and pro$_{18}$ of 4.459 Å; between ser$_{71}$ and asn$_{17}$ of 4.787 Å and between val$_{72}$ and asn$_{16}$ of 5.076 Å

involved in plaque formation and thrombosis in non-denuded blood vessels. In addition, since we find that platelet activation by collagen results in a 4-fold increase in platelet adhesion to EC, it appears that F11R plays an important role also in thrombus formation participating in wound healing, when a damaged vessel wall exposes collagen to circulating platelets.

In conclusion, the results reported here have the potential to elucidate heretofore unknown mechanisms that can trigger thrombus formation, in particular in non-denuded vessels and under inflammatory conditions. The significant contribution of F11R to the mechanisms of the adhesion process suggests that the sequence of peptide 1 and/or peptide 4 of F11R and the steric structure of the binding-pocket that they produce in F11R may provide the basis for the development of a new generation of therapeutic agents useful for the treatment of inflammatory thrombosis and possibly for the prevention of heart attacks and stroke.

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