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The F11 receptor (F11R/JAM-A) in atherothrombosis: Overexpression of F11R in atherosclerotic plaques

Anna Babinska[1,2], Bani M. Azari[2], Moro O. Salifu[2], Ruijie Liu[1], Xian-Cheng Jiang[1], Malgorzata B. Sobocka[2], Dorothy Boo[3], George Al Khoury[3], Jonathan S. Deitch[3], Jonathan D. Marmur[2], Yigal H. Ehrlich[4], Elizabeth Kornecki[1]

Departments of [1]Anatomy/Cell Biology, [2]Medicine, [3]Surgery, SUNY Downstate Medical Center, Brooklyn, New York, USA and [4]Program in Neuroscience, City University of New York at Staten Island, New York, New York, USA

Summary

F11R is the gene name for an adhesion protein, called the F11-receptor, aka JAM-A, which under normal physiological conditions is expressed constitutively on the surface of platelets and localized within tight junctions of endothelial cells (EC). Previous studies of the interactions between human platelets and EC suggested that F11R/JAM-A plays a crucial role in inflammatory thrombosis and atherosclerosis. The study reported here obtained in-vivo confirmation of this conclusion by investigating F11R/JAM-A protein and mRNA in patients with aortic and peripheral vascular disease and in an animal model of atherosclerosis. Molecular and immunofluorescence determinations revealed very high levels of F11R/JAM-A mRNA and F11R/JAM-A protein in atherosclerotic plaques of cardiovascular patients. Similar results were obtained with 12-week-old atherosclerosis-prone apoE$^{-/-}$ mice, an age in which atherosclerotic plaques are well established. Enhanced expression of the F11R/JAM-A message in cultured EC from human aortic and venous vessels was observed following exposure of the cells to cytokines. Determinations of platelet adhesion to cultured EC inflamed by combined cytokine treatment in the presence of F11R/JAM-A – antagonists provided data indicating that *de novo* expression of F11R/JAM-A on the luminal surface of inflamed EC has an important role in the conversion of EC to a thrombogenic surface. Further studies of these interactions under flow conditions and under in-vivo settings could provide a final proof of a causal role for F11R/JAM-A in the initiation of thrombosis. Based on our in-vitro and in-vivo studies to date, we propose that therapeutic drugs which antagonize the function of F11R/JAM-A should be tested as novel means for the prevention and treatment of atherosclerosis, heart attacks and stroke.

Keywords

F11R, F11 receptor, F11R/JAM-A, inflammatory thrombosis, atherosclerosis, atherothrombosis

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Introduction

F11R is the name of a gene encoding a protein called the F11 receptor, also known by the name junctional adhesion molecule (JAM-A). This protein was identified first as a duplex of proteins constitutively expressed on the surface of human platelets (1). Stimulation of F11R/JAM-A caused platelet activation in a process involving the following events: F11R/JAM-A dimerization (2), phosphorylation by activated PKC associated with transient (reversible) translocation of PKC isoenzymes α and ζ, and lasting translocation of PKC isoenzymes δ, β, n', and θ (3), complex formation of F11R/JAM-A with the integrin GPIIIa (2) and activation of the FcγRII pathway (4). The utilization of specific F11R/JAM-A peptide antagonists and recombinant proteins (5–7) has begun to reveal the role of F11R/JAM-A in the process of platelet adhesion to inflamed endothelial cells and identified F11R/JAM-A as a potentially important molecule in platelet plaque formation leading to inflammatory thrombosis and atherosclerosis, with platelets as critical factors involved in the progression and development of cardiovascular disease (8). Promoter analysis of the human F11R gene has revealed the presence of an NF-kappaB (NF-κB) consensus sequence, thus further implicating F11R's role in inflammatory processes (9).

Enhanced expression and dynamic redistribution of cell adhesion molecules (CAMs) such as intercellular adhesion molecule (ICAM), vascular cell adhesion molecule-1 (VCAM-1),

Correspondence to:
Elizabeth Kornecki, PhD
Department of Anatomy/Cell Biology
SUNY Downstate Medical Center
450 Clarkson Ave
Brooklyn, NY 11203, USA
Tel.: +1 718 982 3932, Fax: +1 718 270 3732
E-mail: ekornecki@downstate.edu

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and platelet endothelial adhesion molecule-1 (PECAM-1/CD31), all members of the immunoglobulin superfamily, occur in the endothelium in response to cytokines (10, 11). Expression and localization of CAMs at the luminal surface of atherosclerotic lesions implicates their participation in the pathology of atherosclerosis (12–15) with ICAM-1, VCAM-1 and PECAM-1 levels modulated within the lesioned vasculature of the atherosclerosis-prone apoE$^{-/-}$ mouse model (15).

Similar to these CAMs, F11R/JAM-A is a cell adhesion molecule and member of the immunoglobulin superfamily (16) whose important role in thrombosis and atherosclerosis is being explored by us and other laboratories. The combined treatment of EC with inflammatory cytokines tumor necrosis factor-alpha (TNFα) and interferon-gamma (INFγ) resulted in a redistribution of F11R/JAM-A from intercellular junctions to the cell surface of the inflamed EC (17). An enhanced expression of F11R/JAM-A was evident already in the early stages of atherosclerosis in the endothelium of apoE-deficient mice fed with high-fat diet (18), and the soluble form of F11R/JAM-A significantly reduced mononuclear cell recruitment to *ex vivo* perfused atherosclerotic carotid arteries (18). The F11R gene is located in a critical region on chromosome 1q21–23 (F11R-USF1), a region that is significantly linked to coronary heart disease and familial-combined hyperlipidemia (19). This finding also suggests that the F11R/JAM-A protein has proatherogenic properties predicted in its involvement in pathophysiological processes underlying cardiovascular diseases (5, 6). Furthermore, the approximate two-fold upregulation of F11R/JAM-A shown to occur in unstable versus stable regions of the atherosclerotic plaque (20), expands the role of F11R/JAM-A to include its involvement in the rupture of the atherosclerotic plaque as well.

In the present study, we conducted experiments in culture and *in vivo* designed to test the suggestion that the interaction of the newly-expressed F11R/JAM-A sites on the surface of cytokine-inflamed EC with the constitutively-expressed F11R/JAM-A sites on the surface of circulating platelets plays a causal role in the adhesion of platelets to the inflamed endothelium.

Materials and methods

Human endothelial cells

Human aortic (HAEC) and umbilical vein endothelial cells (HUVEC) (10^6) (Cascade Biologics, Inc., Portland, OR, USA), were grown in Medium 200 containing 1% or 2% fetal calf serum (FCS), respectively. At second passage, both HUVEC and HAEC were treated with purified human recombinant TNFα (100 units/ml) (R&D Systems, Inc., Minneapolis, MN, USA), vascular endothelial growth factor (VEGF; 10ng/ml) (R&D Systems) and INFγ (200 units/ml) (Roche Diagnostics, Mannheim, Germany) for 24 hours (h) at 37°C. The procedures involving the adhesion of platelets to endothelial cells were followed as described (6).

Human platelets

Platelets were isolated as described (1). Collagen (native type 1), used as the agonist, was purchased from Chrono-log Corp. (Havertown, PA, USA).

F11R/JAM reagents

Monoclonal antibody F11 (M.Ab.F11; human F11R/JAM antibody), developed in our laboratory against the human platelet F11R (1), was obtained from BD PharMingen (San Diego, CA, USA). JAM-1 polyclonal antibody was purchased from R&D Systems. Human F11R recombinant protein was generated as described (6, 7). The F11R peptides were synthesized and purified (95%) by New England Peptide, Inc. (Gardner, MA, USA). vWF antibody was obtained from Chemicon International (Temecula, CA, USA).

Total RNA isolation

Total RNA was extracted from the isolated aorta of apoE$^{-/-}$ and control C57BL/6J mice utilizing TriZol (Invitrogen, Carlsbad, CA, USA). ApoE knockout mice (apoE$^{-/-}$) with C57BL/6J back-

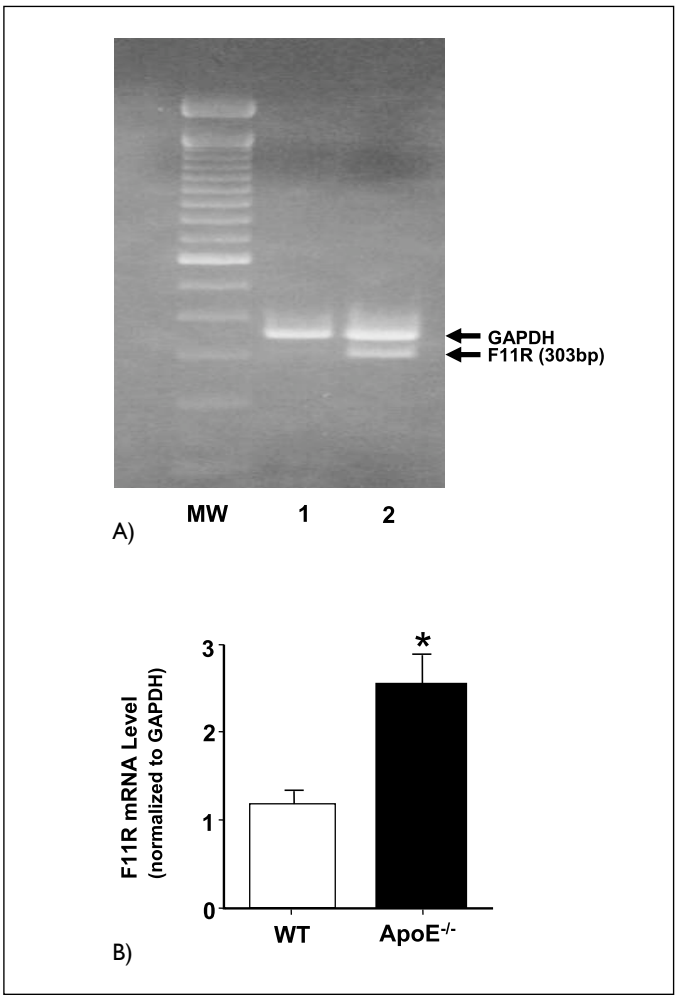

Figure 1: F11R/JAM-A expression in atherosclerotic plaques of apoE$^{-/-}$ mice. A) RT-PCR: F11R mRNA levels obtained from pooled samples of proximal aortae of five apoE$^{-/-}$ and five C57BL/6 wild-type mice (control). Lane 1: control wild-type mice;. Lane 2: apoE$^{-/-}$ mice. Upper arrow, expected size of 350 bp GAPDH; Lower arrow points to the 303 bp F11R fragment. B) Real-time RT-PCR: F11R mRNA levels measure in the proximal aorta of five separate apoE$^{-/-}$ mice (ApoE$^{-/-}$) and five separate C57BL/6 wild-type mice (WT). Measurements were performed in triplicate for each animal for each of the two groups. Values represent the mean ± SEM. *P < 0.05.

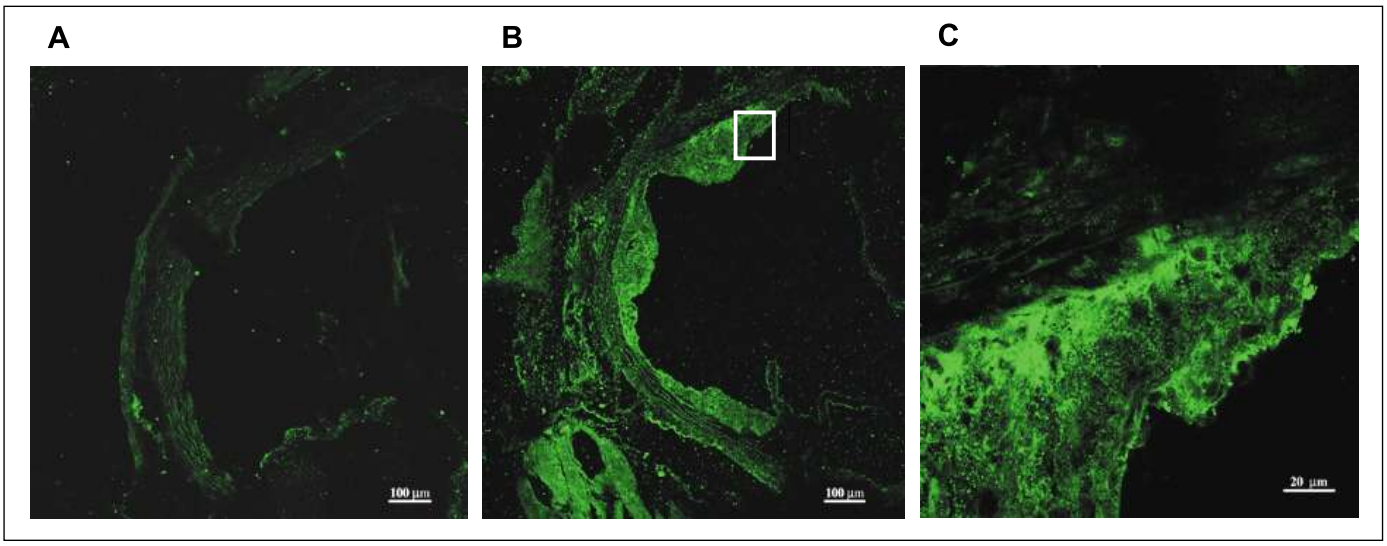

Figure 2: Expression of F11R/JAM-A in atherosclerotic plaques of apoE$^{-/-}$ mice: Immunofluorescence. A) Control, staining of the proximal aorta of wild-type mice by murine F11r/JAM-1 antibody. Scale, 100 µm. B) ApoE$^{-/-}$, staining of proximal aorta of apoE$^{-/-}$ mice by murine F11r/JAM-1 antibody. Scale, 100 µm. Rectangular inset outlines a region of the lesioned area displayed at higher magnification in panel C. C) ApoE$^{-/-}$, inset in panel B, shown at higher magnification, depicts the intense staining of atherosclerotic plaques by the F11r/JAM-1 antibody. Scale, 20 µm.

ground of 12 weeks of age and control age-matched wild-type C57BL/6J mice, obtained from Jackson Labs (Bar Harbor, MA, USA), were fed a regular mouse chow diet. All procedures were conducted in accordance with the SUNY Downstate Institutional Guidelines for Animal Use and Care.

Quantitation of F11R mRNA by reverse transcription (RT)-PCR and real-time PCR

RT-PCR

Reverse transcription (RT) used oligonucleotide primers designed to the N-terminus of the murine F11r, forward primer, GTA CAC TGC TCA ATC TGA CG, reverse primer GAT GGA GGT ACA AGC ACA GT; carried-out at 48ºC, 30 min with AmpliTaq gold activation at 95ºC, 10 minutes (min), PCR denaturation at 95ºC, 15 seconds (sec); and anneal/extend reactions at 60ºC, 1 min, 30 cycles.

Real-time PCR

a) ***Murine:*** F11R mRNA levels in mouse aorta were determined by quantitative real-time PCR with an ABI Prism 7000HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The F11R primers: forward – 825 TCT CCT TGG ACT CTT GAT TTT TGG; reverse–909 ACC CGG TGC AGT CCC TTT; probe-857: TTG CCT ATA GCC GTG GAT ACT TTG AAA GAA CA. The GADPH forward primer –757: TGT GTC CGT CGT GGA TCT GA, and the reverse primer-837: GAT GCC TGC TTC ACC ACC TT; probe-781: CCG CCT GGA GAA ACC TGC CAA GTA TG. Thermal cycles consisted of: 1 cycle at 48ºC for 30 min, 15 sec at 95ºC and 40 cycles for 1 min at 60ºC. Each mRNA level was expressed as a ratio to GAPDH. Normalization to GAPDH mRNA was performed according to the formula $2^{-\Delta\Delta Ct}$ provided by the manufacturer. The results are expressed as the mean ± SEM of the number of observations.

b) ***Collection of human tissue samples:*** Patients: Surgically-excised atherosclerotic specimens were obtained at the time of surgery from the carotid arteries, femoral arteries or aortas of seven patients (6 males, 1 female, 56 to 68 years old). Patients 1, 2, 5, and 7 had severe carotid artery stenosis in excess of 80% and asymptomatic from their coronary artery disease. Patient 3 had significant vascular compromise of the lower extremity as evidenced by femoral artery occlusion and clinical symptomatology. Patients 4 and 6 had atherosclerotic occlusive disease predominantly localized within the aortic wall of the vasculature.

Controls: Specimens of renal artery and aorta were obtained within 12 h of death from young cadaveric kidney transplant donors immediately at the time of organ harvesting for transplantation and used as controls (3 males, 23 to 38 years old). In addition to these cadaveric control specimens, experiments detailed in Figure 3C utilized plaque-free specimens obtained from the aorta of the same patient and were used as internal controls .

All protocols were approved by the SUNY Downstate Institutional Review Board Committee. All patients signed appropriate informed consent forms prior to the surgical excision of the atherosclerotic material.

Quantitation of F11R mRNA in human arteries by real-time RT-PCR

Excised segments were placed in RNA Stabilization Reagent, RNA*later* (Ambion, Austin, TX, USA). Total RNA was extracted utilizing RNeasy Protect Mini Kit (Qiagen, Valencia, CA, USA) and analyzed by real-time PCR in triplicate for each of the seven samples. The levels of F11R mRNA were determined by use of an ABI Prism 7000HT Sequence Detection System. The F11R primers consisted of the forward primer – 740: CCG TCC TTG TAA CCC TGA TT, reverse primer – 818: CTC CTT CAC TTC GGG CAC TA and probe –788: TGG CCT CGG CTA TAG GCA AAC C. The GAPDH forward primer – 620: GGA CTC ATG

ACC ACA GTC CA, reverse primer – 738: CCA GTA GAG GCA GGG ATG AT, and the probe – 675: ACG CCA CAG TTT CCC GGA GG. Thermal cycles consisted of: 1 cycle at 48ºC for 30 min, 10 min at 95ºC and 40 cycles for 15 sec at 95ºC, 1 min at 60ºC. Each mRNA level was expressed as a ratio to GAPDH. The mRNA levels were calculated using a standard curve of RNA isolated from cultured HUVEC cells utilizing the ABI Prism 7000 SDS Software (Applied Biosystems).

Statistical analysis

The data were analyzed by Student's t-test and the non-parametric Mann-Whitney U-test. Differences were considered significant at $P<0.05$.

Histology, immunofluorescence and confocal microscopy

Human specimens of atherosclerotic plaque were subjected to standard tissue processing and staining with hematoxylin and eosin (H&E) (21, 22). Pretreated paraffin-embedded human arterial segments were deparaffinized and prepared for immunostaining with primary rabbit polyclonal anti-human von Willebrand Factor (vWF; Chemicon International, Temecula, CA, USA) or murine monoclonal anti-human F11R antibodies, Secondary antibodies consisting of Alexa Fluor 568 conjugated goat anti-rabbit IgG (red color) or FITC-conjugated goat anti-mouse IgG (green color) were mounted with ProLong R Gold anti-fade reagent (Molecular Probes, Eugene, OR, USA) followed by examination by confocal scanning laser microscopy.

Human umbilical vein endothelial cells *(HUVEC),* grown on coverslips in complete growth media (2% FBS), were exposed to cytokines for 24 h at 37°C, incubated for 1.5 h with murine anti-human F11R M.Ab.F11 (25 μg/ml) or goat anti-murine JAM polyclonal antibody (15 μg/ml), fixed in paraformaldehyde (4%), labeled with goat anti-mouse Ig (1:200) or rabbit anti-goat (1:100) FITC conjugated secondary antibodies, and mounted with ProLong R Gold.

Slides of paraffin tissue containing cross-sectional slices of the proximal aorta of mice were processed for deparaffinization, incubated with M.Ab.F11 or goat anti-mouse JAM-1 polyclonal antibody, labeled with secondary FITC conjugated antibodies, and mounted with Prolong Gold anti-fade reagent.

The Biorad MRC 1024ES confocal microscopy system was used to generate fluorescent images. Cultured HUVEC (passages 1 – 3), grown to confluency on glass coverslips, were treated with purified human recombinant TNFα, vascular endothelial growth factor (VEGF) (R&D Systems), and INFγ

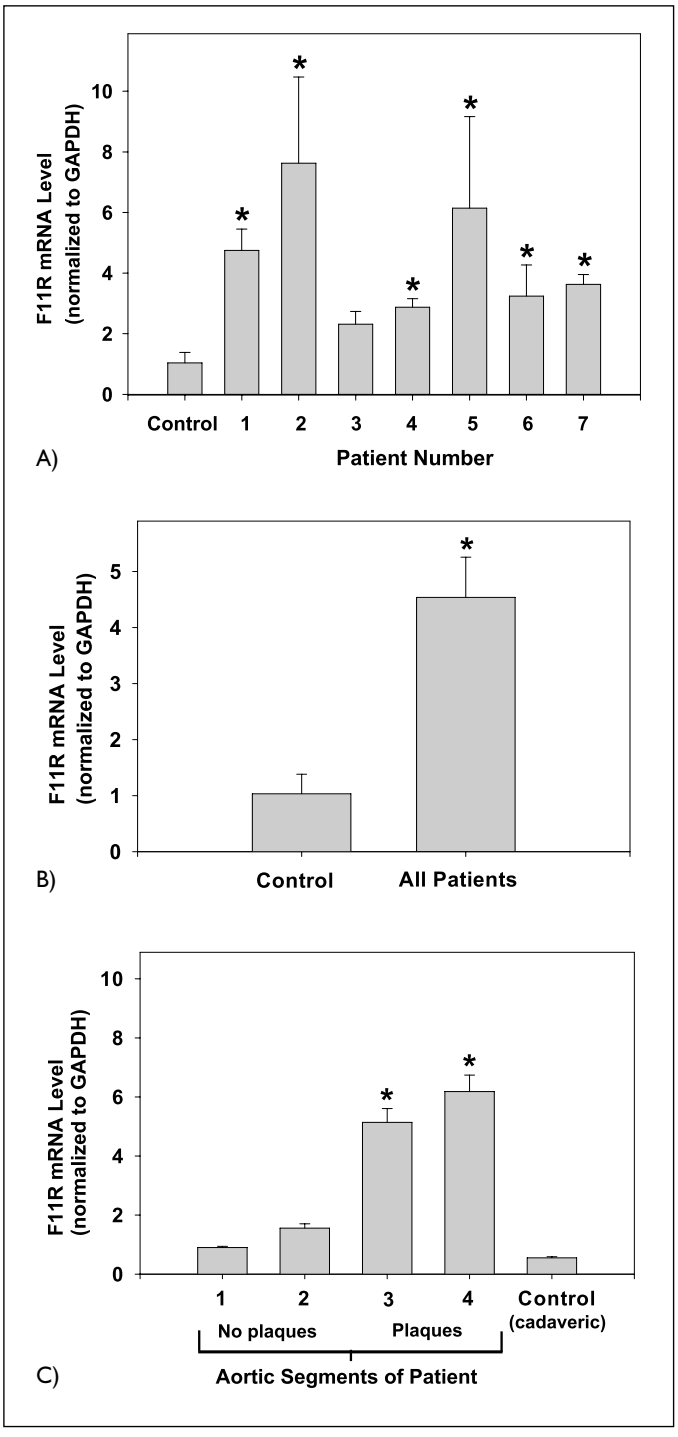

Figure 3: Enhanced expression of F11R/JAM-A mRNA in human atherosclerotic plaques. A) Real-time RT-PCR performed on human arteries. Control, F11R mRNA analyses of separate plaque-free kidney aorta specimens obtained from three cadaveric donors. Real-time RT-PCR was performed three times, in triplicate, for each sample. Patients, F11R mRNA analyses on arteries obtained from seven separate cardiovascular patients (1 thru 7). Values represent the mean ± SEM. *P<0.05. B) Comparison of F11R mRNA expression by real-time RT-PCR: Total values for patients were included for comparison to control values of cadaveric donors. Values represent the mean ± SEM, *P < 0.05. C) Enhanced F11R mRNA expression is associated with regions containing plaques. Total RNA was extracted from four separate excised segments of the same aorta (6 cm) from a representative patient (number 6), and analyzed separately, in triplicate, by real-time RT-PCR. Bar 1, 1st plaque-free segment taken from patient's aorta; Bar 2, Separate adjacent, plaque-free segment of patient's aorta; Bar 3, Aortic segment of patient containing plaques, Bar 4, A separate aortic segment of the patient containing largest amount of plaques. The cadaveric control bar represents the level of F11R mRNA (mean ± SEM) measured in three cadaveric plaque-free aortic and renal artery specimens. Values represent the mean ± SEM. *P<0.05.
Fig. 3C in this reprint has been corrected due to a printing error in the original February 2007 issue. (Erratum will follow.)

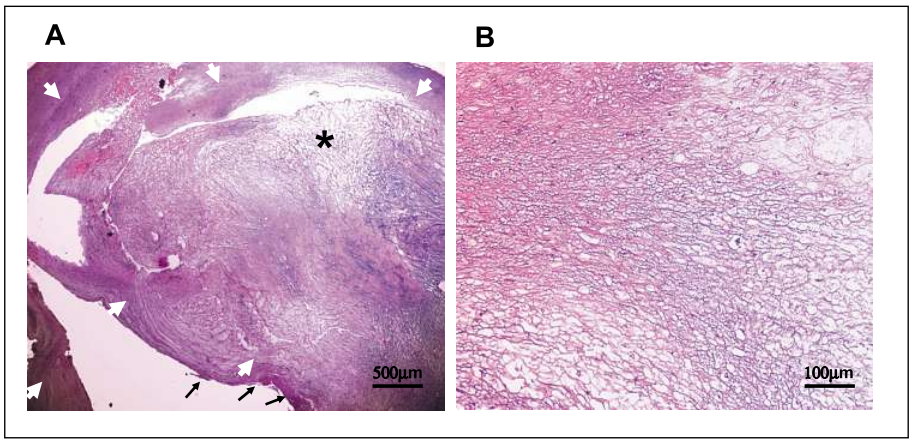

Figure 4: Histological staining of atherosclerotic plaque of carotid arteries. A) Histological staining (hematoxylin-eosin) of a cross-section of the carotid artery of patient 5. The white arrows indicate the boundaries of plaque distribution within layers of the intima and media. The black arrows point to the fibrous cap of the plaque indicating the extension of the plaque into the lumen of the vessel. Scale bar, 500 µm. B) Magnified view (100-fold) of a region (see asterisk in panel A) of the plaque. Scale bar, 100 µm.

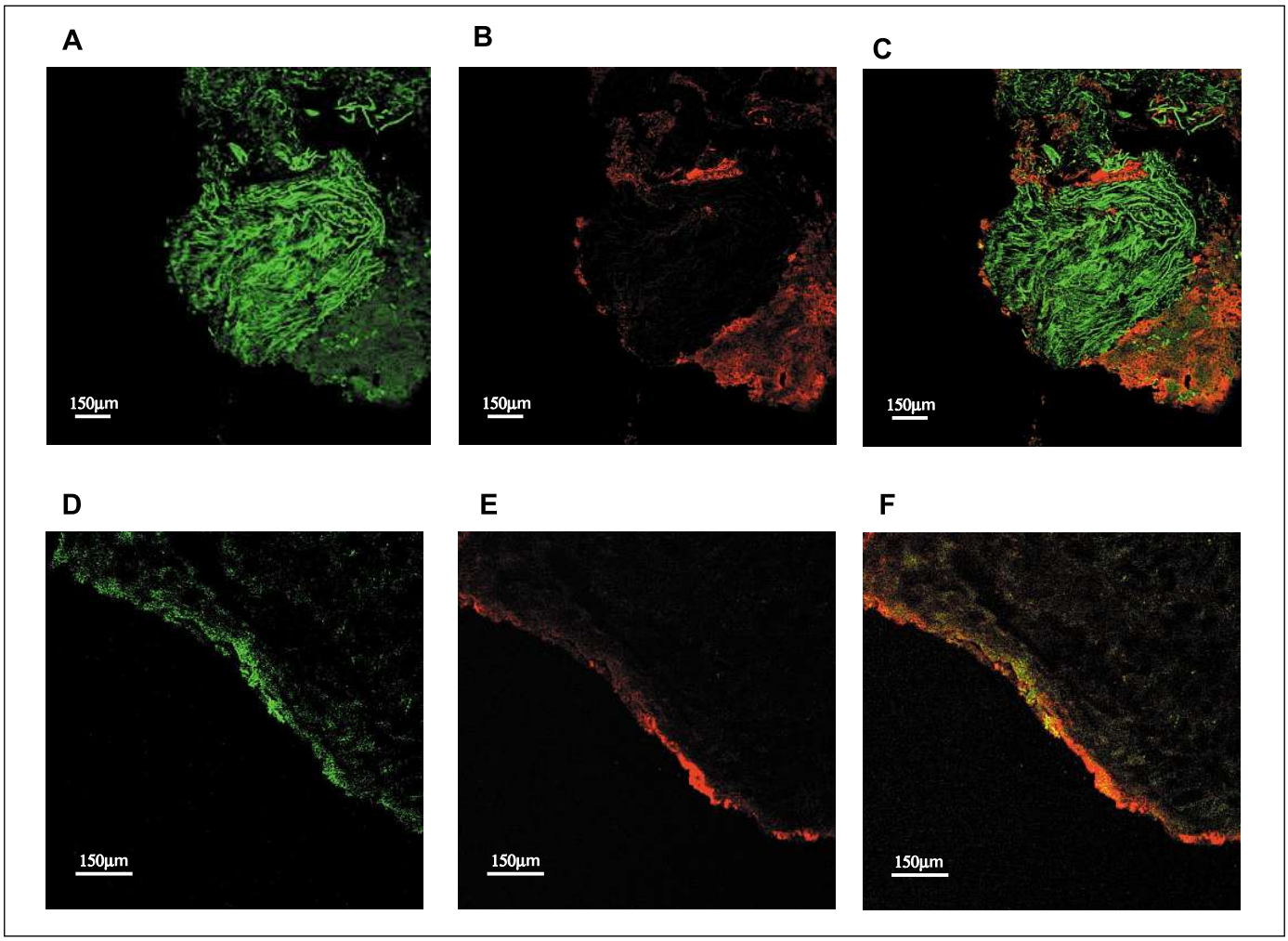

Figure 5: F11R/JAM-A expression in human arteries. A) Immunofluorescence staining for F11R (green) of an atherosclerotic carotid artery specimen (obtained from patient 5). Scale bar, 150 µm. B) Immunofluoresence staining of the endothelial cell marker vWF (red). Scale bar, 150 µm. C) Merged image showing the immunofluorescence staining of F11R and vWF within the atherosclerotic artery. Similar immunofluorescence results for the detection of F11R/JAM-A and vWF were obtained with plaque specimen obtained from three atherosclerotic patients. Scale bar, 150 µm. D) Control immunofluorescence staining for F11R/JAM-A (green color) of the endothelium of aorta obtained from cadaveric kidney transplant donors at the time of organ transplantation. Scale bar, 150 µm. E) Control staining for the endothelium marker, vWF (red color) of the aorta obtained from cadaveric kidney transplant donor at the time of organ harvesting. Scale bar, 150 µm. F) Merged image of panels D and E of the control aorta showing co-localization of F11R/JAM-A and vWF in the endothelium. Similar results were obtained with two additional specimens of renal arteries obtained at time of organ harvesting, from control transplant donors. Scale bar, 150 µm.

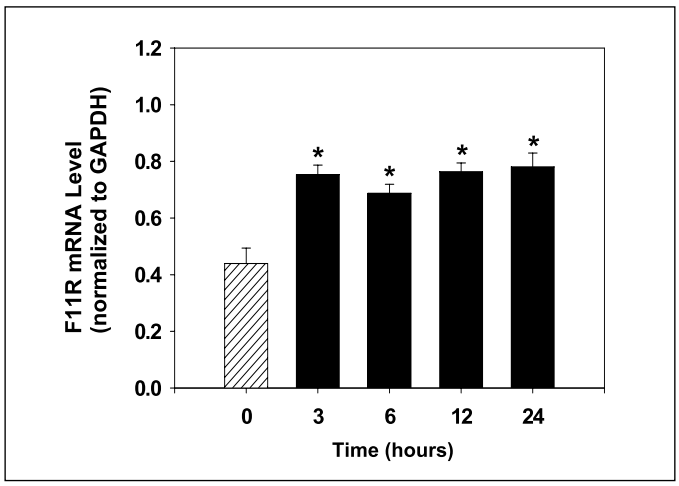

Figure 6: F11R/JAM-A expression in human aortic endothelial cells (HAEC) treated simultaneously with cytokines TNFα and INFγ. Real-time PCR was performed on HAEC treated with combined TNFα (100 u/ml) and INFγ (200u/ml) for 3, 6, 12 and 24 h. Untreated, control, HAEC were maintained at each time point in the absence of cytokines. Real-time PCR was performed three times in triplicate for each time point. Values are the mean ± SEM. * P<0.05.

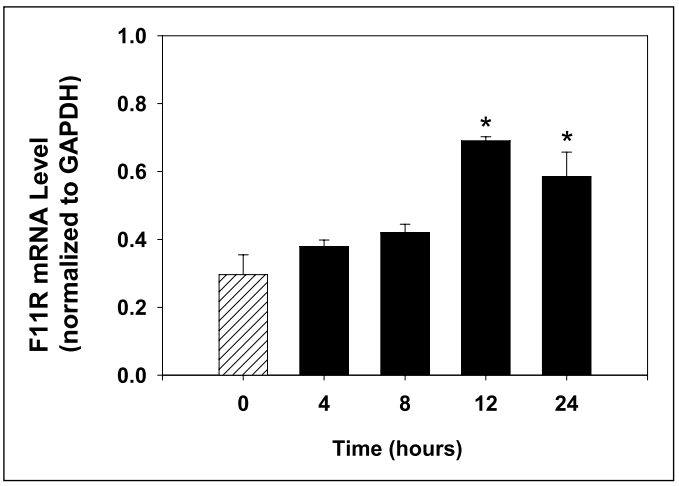

Figure 7: F11R/JAM-A expression in HUVEC treated simultaneously with cytokines TNFα and INFγ. Real-time PCR: F11R mRNA was obtained from HUVEC treated with combined TNFα (100 u/ml) and INFγ (200 u/ml) for 4, 8, 12, and 24 h. Control cells were examined in the absence of cytokines. Real-time PCR was performed two times in triplicate for each time point. Values are the mean ± SEM, *P<0.05.

(Roche Diagnostics). HUVEC were fixed with 1% paraformaldehyde at 27°C, 10 min, incubated with M.Ab.F11 for 1 h at 37°C, followed by incubation with FITC conjugated secondary antibodies for 1 h. Carbocyanine monomer TO-PRO-3 was used for nuclear staining (Molecular Probes).

Results

Studies of Apo E-deficient mice

At three months of age, apoE$^{-/-}$-deficient mice demonstrate the presence of well-developed plaques along their vasculature. As shown in Figure 1A, the aorta of apoE$^{-/-}$ mice demonstrated a significantly-enhanced expression of F11R mRNA in the proximal aortae (lane 2), as compared to the barely-detectable levels of F11R mRNA measured in controls (lane 1). Quantitative confirmation of these findings was provided by conducting real-time RT-PCR, and as shown in Figure 1B, the level of F11R mRNA in the aorta of the apoE$^{-/-}$ mice was two-fold greater than that observed in wild-type mice. Immunofluorescence of identical specimens utilizing F11R specific antibodies revealed intense staining of the F11R protein in the proximal aorta of apoE$^{-/-}$ mice (Fig. 2B) which followed the exact contours displayed by the atherosclerotic region; low, background staining was observed in the aorta of wild-type mice (Fig. 2A). A magnified view of a por-

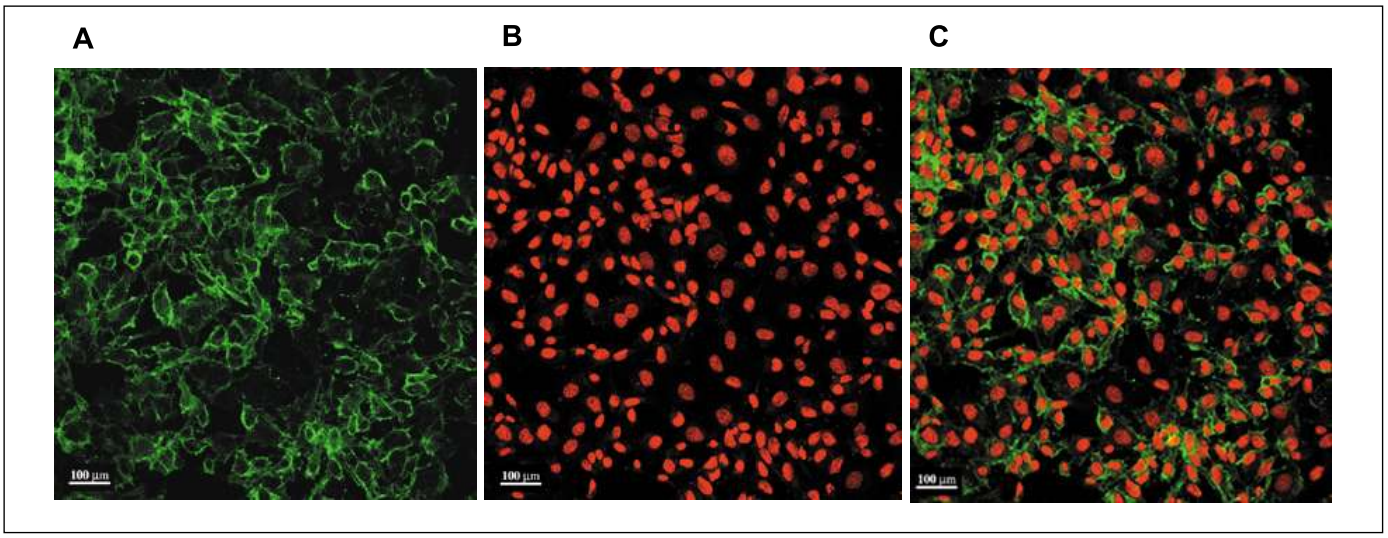

Figure 8: Intercellular localization of F11R/JAM-A in intact, non-treated HUVEC. A) Immunofluorescence staining of F11R in cultured HUVEC using F11R antibody, M.Ab.F11. B) Staining of HUVEC nuclei with To-Pro-3. C) Merged image combining the green immunofluorescence of F11R localized to the plasma membrane with the red-nuclear staining. Scale bar, 100 µm.

tion of the atherosclerotic region from apoE$^{-/-}$ mice (Fig. 2C) demonstrates the highly over-expressed F11R protein found within the plaque of these atherosclerotic-prone animals.

Studies of human subjects

In parallel studies, F11R mRNA levels were measured in endarterectomy specimens of arteries from atherosclerotic patients. Figure 3A shows that F11R levels were found to be significantly higher in the lesioned regions of six of the seven patients examined than in control specimen, obtained from cadaveric kidney donors. A five-fold increase in the overall level of F11R mRNA was demonstrated in the plaques of atherosclerotic arteries as compared to controls (Fig. 3B). In addition, we compared the level of F11R mRNA with plaque content in segments, obtained from the same aorta, that were identified as plaque-free, in comparison to plaque-containing regions. As shown in Figure 3C, regions identified to contain high levels of atherosclerotic plaques (bars 3 and 4) were found to exhibit significantly higher levels of F11R mRNA than two plaque-free segments obtained from the same aorta of this patient (bars 1 and 2). Histological examinations conducted to demonstrate the presence of the atherosclerotic plaque revealed that approximately 80% of the lumen of the carotid of patient 5 was obstructed by the plaque (Fig. 4A), and upon closer inspection (Fig. 4B) consisted of a lipid-laden core with infiltrating cells, connective tissue elements and foam cells. Immunofluoresence staining of the atherosclerotic aorta of the same patient revealed a high level of staining of F11R throughout the entire specimen (Fig. 5A). Staining for vWF in the same specimen demonstrated the presence of the atherosclerotic endothelium (Fig. 5B). The merged image, shown in Figure 5C, depicts the significant distribution of the F11R protein throughout the plaque as it is surrounded by the atherosclerotic endothelium, as shown by the endothelial marker, vWF. In contrast, control specimen of the aorta obtained from cadaveric transplant donors, identified as plaque-free, showed the F11R staining only within the endothelium (Fig. 5D). Staining for vWF demonstrated its presence also within the endothelium of the control aortic specimen (Fig. 5E). The merged immunofluoresence image of the control aorta (Fig. 5F) depicts co-localization of the F11R/JAM-A protein and vWF together within the endothelium of the aorta.

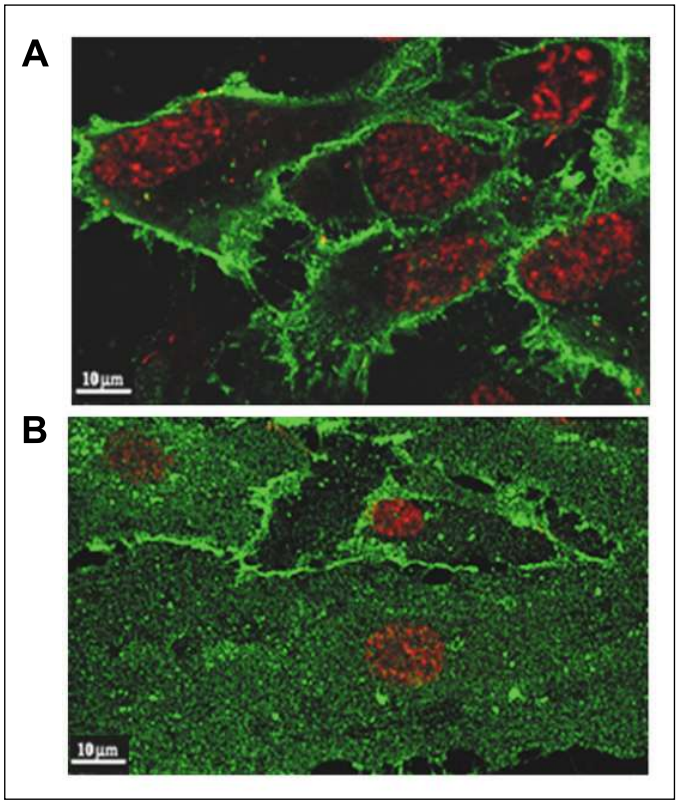

Figure 9: Localization of F11R/JAM-A following the combined treatment of cultured HUVEC with TNFα plus INFγ. A) Intact, non-treated HUVEC: Merged image showing the immunofluorescent staining of non-treated HUVEC with F11R antibody, M.Ab.F11, showing intercellular staining of F11R at tight junctions with co-staining of nuclei by To-Pro-3. Scale bar, 10 µm. B) Cytokine-treated HUVEC: Merged image showing the diffuse immunofluorescent staining pattern of F11R throughout the entire cell following the treatment of HUVEC for 24 h with a combination of cytokines TNFα (100 u/ml) and INFγ (200 u/ml). Nuclei stained by To-Pro-3. Scale bar, 10 µm.

Investigations of human endothelial cells in culture

In studies designed to determine the potential causes of the increased levels of F11R in atherosclerotic plaques observed *in vivo*, we examined the effects of the treatment of EC with inflammatory cytokines on the interactions between platelets and EC. In previous studies we exposed human EC to only a single type of cytokine. However, a combined cytokine treatment is known to cause an even greater redistribution of the F11R from tight junctions to the luminal surface of EC. Therefore, in the present study, our experiments specifically focused on the exposure of EC to a combination treatment using the cytokines TNFα and INFγ. As shown in Figure 6, the simultaneous addition of both cytokines to cultured human aortic EC (HAEC) resulted in significant upregulation of F11R mRNA that remained significantly elevated for 24 h following the initial application of the cytokines. Similar to aortic cells, HUVEC also demonstrated significantly increased levels of F11R mRNA with time following the simultaneous addition of both cytokines (Fig. 7), although differences in sensitivity to the cytokines between these two cell types were observed. Differences in sensitivity to cytokine stimulation between aortic endothelial cells and HUVEC could not be explained by different passages, as the same cell passage was utilized for both cell types in these experiments.

Immunofluoresence staining of non-treated HUVEC revealed the presence of F11R within the plasma membranes of these cells (Fig. 8A); counterstaining with a nuclear stain revealed the HUVEC nuclei (Fig. 8B). The merged image shown in Figure 8C demonstrates the association of F11R only with plasma membranes; the clear regions of the HUVEC cytoplasm depict the lack of staining for F11R under these control conditions. Such merged images were utilized to analyze changes in F11R immunofluorescence caused by the treatment of confluent HUVEC cultures with the combination of cytokines. As shown in Figure 9, such simultaneous cytokine treatment caused substantial changes in the localization of F11R. In comparison to the non-treated HUVEC (Fig. 9A), the treatment of HUVEC with the cytokines TNFα plus INFγ for 24 h resulted in a decreased intensity for F11R staining at membrane intercellular junctions. As shown

in Figure 9B, following the combined cytokine treatment of HUVEC, the F11R protein became evenly distributed throughout the entire cell body of the HUVEC rather than concentrated within tight junctions.

Investigations of platelet adhesion to EC in culture

To examine the contribution of F11R to the force of adhesion operating between platelets and inflamed endothelial cells, we examined the effects of specific F11R-inhibitors on the adhesion of platelets to HUVEC treated simultaneously with cytokines TNFα plus INFγ. We have previously reported that peptides designated F11R-peptide-1 (amino acid sequence ^{28}SVTVHSSEPEVRIPENNPVKLSC^{50} present in the N-terminus of F11R) and F11R-peptide 4 (amino acid sequence ^{97}KSVTREDTGTYTC^{109} present within the 1st Ig-fold of F11R), act as specific inhibitors of platelet aggregation induced by M.Ab.F11. As shown in Figure 10A, we determined that 3.32 ± 0.63 X 10^5 intact platelets adhered to 1 X 10^4 TNFα- plus INFγ-treated HUVEC (control bar). The adhesion of intact platelets to the cytokine-treated HUVEC was inhibited significantly by the presence of the soluble recombinant F11R (sF11R) as well as by F11R peptides 1 and 4.

The participation of F11R was monitored further under conditions in which cytokine-treated HUVEC were reacted with activated platelets. Under these conditions, HUVEC were pre-treated with TNFα and INFγ, whereas human platelets were activated by exposure to collagen (Fig. 10B). We determined that approximately 14.8 ± 2.49 X 10^5 of collagen-activated platelets bound to 1 X 10^4 of the TNFα- plus INFγ-treated HUVEC (control bar, Fig. 10B). The presence of the soluble recombinant F11R protein (sF11R; 10 μg/ml) significantly inhibited the adhesion of activated platelets to TNFα- plus INFγ-treated HUVEC. Furthermore, the presence of F11R peptide 1 resulted in significant inhibition of platelet adhesion to the inflamed HUVEC as did the presence of F11R peptide 4. In contrast, the addition of a peptide whose sequence of amino acids corresponded to F11R peptide 1 but was scrambled by random in-

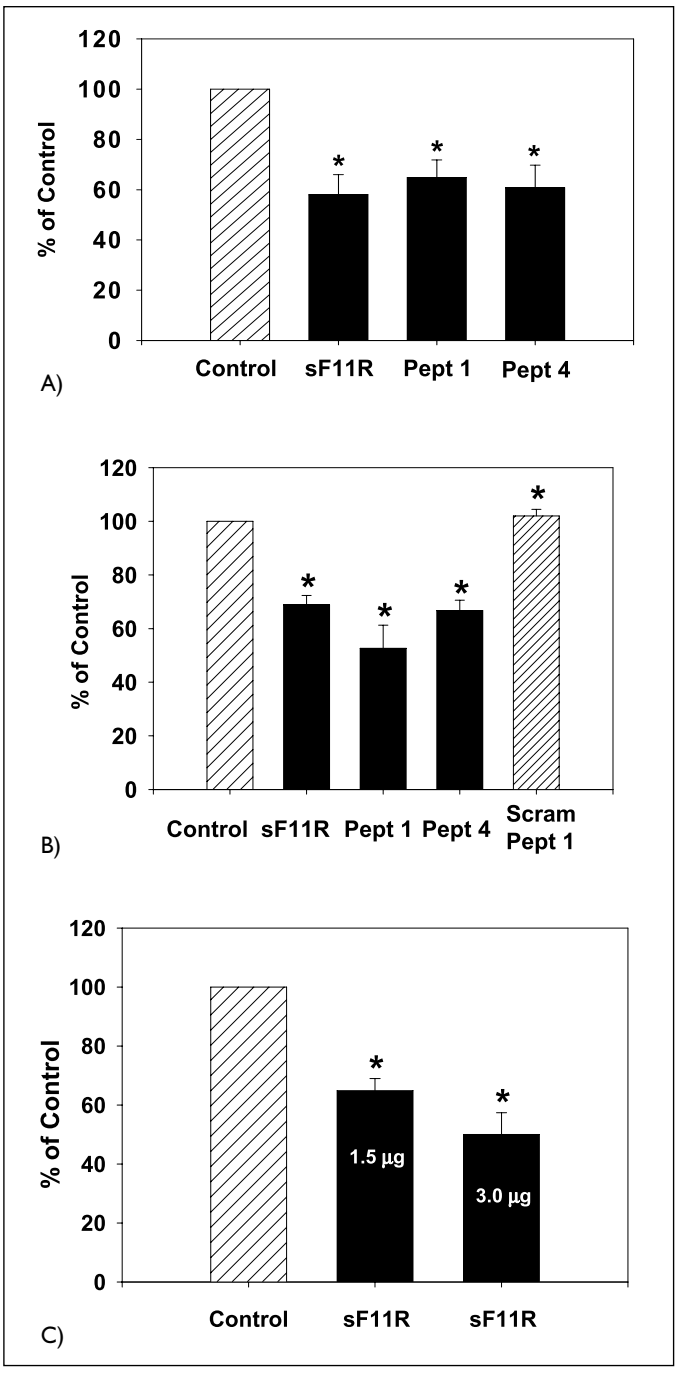

Figure 10: Inhibition of platelet adhesion to HUVEC treated with combination of cytokines TNFα and INFγ. A) Non-activated platelets: Isolated platelets were applied onto HUVEC previously treated with cytokines TNFα (100 u/ml) and INFγ (200 u/ml). Control, no additions: Platelet adhesion to cytokine-treated HUVEC in the absence of F11R inhibitors. Value set at 100% represents the binding of 3.32 ± 0.63 X 10^5 non-activated platelets per 1 X 10^4 TNFα- + INFγ-treated EC. Plus sF11R: Platelet adhesion to TNFα- plus INFγ-treated HUVEC in the presence of 10 μg/ml soluble recombinant F11R (sF11R). Values, mean ± SEM, *P<0.05. Plus F11R peptide 1: Platelet adhesion to TNFα- + INFγ-treated HUVEC in the presence of 500 μM peptide 1. *P<0.05. Plus F11R peptide 4: Platelet adhesion to TNFα- + INFγ-treated HUVEC in the presence of 500 μM peptide 4. *P < 0.05. B) Activated platelets: Isolated platelets were activated with collagen (10 g/ml) and applied onto TNFα (100 u/ml) plus INFγ (200 u/ml)-treated HUVEC. Control, no additions: Adhesion of activated platelets to cytokine-treated HUVEC, in the absence of F11R inhibitors. The 100% value represents binding of 14.8 ± 2.49 X 10^5 activated platelets per 1 X 10^4 treated HUVEC. Plus sF11R: Adhesion of collagen-activated platelets to TNFα- plus INFγ-treated HUVEC in the presence of soluble F11R recombinant protein (sF11R) (10 μg/ml). *P<0.05. Plus peptide 1: Adhesion of activated platelets to cytokine-treated HUVEC in the presence of 500 μM peptide 1. *P<0.05. Plus peptide 4: Adhesion of activated platelets to cytokine-treated HUVEC in the presence of 500 μM peptide 4. *P<0.05. Plus scrambled peptide 1: Adhesion of activated platelets to cytokine-treated HUVEC in the presence of 500 μM scrambled peptide 1. *P<0.05, significantly different from F11R peptides 1, 4 and sF11R. C) Adhesion of activated platelets to VEGF-treated HUVEC. Control, no additions: The adhesion of collagen (10 μg/ml)-activated platelets to HUVEC treated with VEGF (10 ng/ml) for 24 hat 37°C. The 100% value represents adhesion of 10.4 ± 1.7 X 10^5 activated platelets to 1 X 10^4 VEGF-treated HUVEC. Plus sF11R (1.5 μg): Adhesion of activated platelets to VEGF-treated HUVEC in the presence of soluble F11R recombinant protein (sF11R) (250 μM). *P<0.05. Plus sF11R (3.0 μg): Adhesion of activated platelets to VEGF-treated HUVEC in the presence of soluble F11R recombinant protein (sF11R) (500 μM). *P<0.05.

sertion of amino acids during the synthesis of this peptide (scrambled peptide 1) did not inhibit the adhesion of activated platelets to the cytokine-treated HUVEC. Finally, we monitored the effect of F11R in the adhesion of platelets to HUVEC treated for 24 h with the growth factor VEGF (10 ng/ml). Figure 10C demonstrates that, whereas $10.4 \pm 1.7 \times 10^5$ collagen-activated platelets adhered to 1×10^4 VEGF-treated HUVEC (control bar); the presence of sF11R (1.5–3.0 μg/ml) resulted in the inhibition (by about 35% and 50%, respectively) of the adhesion of collagen-activated platelets to the VEGF-treated EC.

Discussion

Recent studies have demonstrated that platelets adhere to an inflamed endothelium even prior to the invasion of leukocytes and before lesions become detectable, suggesting that this adhesion is an initial step in the development of plaques in atherosclerosis (23–25). The results of the study reported here provide new insights into the molecular mechanisms by which inflammatory agents initiate the interaction of platelets with endothelial cells, leading to formation of thrombotic plaques. Under normal physiological conditions, endothelial cells (EC) are non-thrombogenic (26–28). However, the non-thrombotic surface of EC can be transformed to a prothrombotic surface following their exposure to inflammatory agents, resulting in procoagulant activity and predisposition to thrombosis (28–30). Previous studies of the adhesion, accumulation and recruitment of platelets onto the inflamed surface of cytokine-stimulated EC have implicated P-selectin (24, 31), the P-selectin glycoprotein ligand (PSGL-1) (31, 32), platelet endothelial cell adhesion molecule-1 (PECAM-1) (33 –35), beta-1 integrin (30, 31, 36, 37), tissue factor (29), von Willebrand factor (24, 30), and GPIIb/IIIa (31) in this process. Recently, the F11R/JAM-A protein has been described with properties indicating its potential role in triggering the pathogenesis of inflammatory thrombosis and atherosclerosis (6). The purpose of the study reported here was to provide additional evidence for this role.

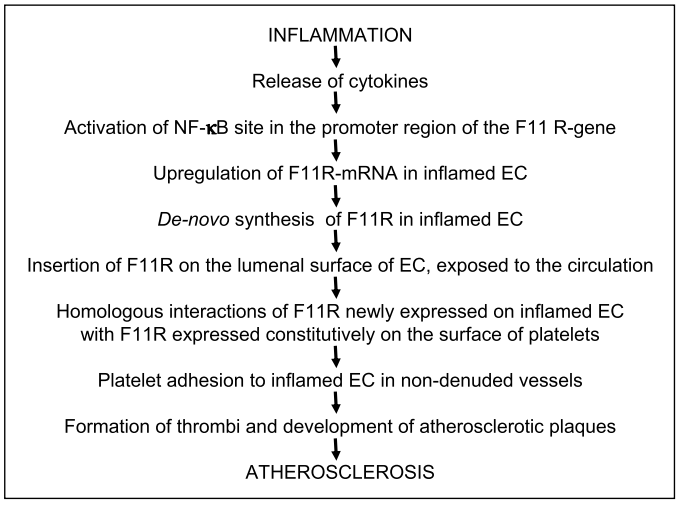

Figure 11: Schematic presentation of the role of F11R/JAM-A in triggering inflammatory thrombosis with the formation of platelet thrombi underlying the pathogenesis of atherosclerosis. Sequence of events in inflammatory thrombosis that involve the upregulation of F11R-mRNA and induction of the F11R protein in cytokine-inflamed EC, leading of the initial formation of a platelet thrombus on the luminal surface of inflamed EC with eventual progression to the development of atherosclerotic plaques. THe arrow (↓) indicates causal effect. The experimental evidence indicating this chain of events and the references to the studies reporting this evidence are outlined in the text of the *Discussion*.

The results reported here demonstrate that upregulation of F11R mRNA is induced by cytokines in EC both from venal and arterial vessels, and that the newly-induced F11R/JAM-A protein has a significant role in the adhesion of platelets to the inflamed endothelium. The combined cytokine treatment utilized in the present study was critical for the induction of maximal changes in the redistribution of F11R/JAM-A to the vessel lumen. Therefore, these experimental conditions are most optimal for examining the role of F11R/JAM-A in platelet adhesion to EC under inflammatory conditions, that are known to involve the action of both TNFα and INFγ (38). Experiments utilizing specific F11R/JAM-A inhibitors determined that over 50% of the force of platelet adhesion to EC inflamed by exposure to [TNFα + INFγ] is exerted by F11R/JAM-A. We conclude that F11R/JAM-A-mediated adhesion of platelets to EC is a critical event in the inflammatory process that leads to thrombosis in non-denuded vessels. We propose that circulating platelets, which constitutively express the F11R/JAM-A molecule on their surface (1) adhere directly to the inflamed endothelium by virtue of their ability to interact with F11R/JAM-A molecules that are newly expressed on the exposed surface of inflamed EC. This homologous interaction (6) could cause the initial adhesion of platelets to endothelial cells in an early stage, thus triggering the process of inflammation-induced atherogenesis. Figure 11 depicts a schematic presentation of the proposed steps of this process, which is initiated by the activation of the transcription factor NF-κB in EC by cytokines, followed by the interaction of this transcription factor with specific binding segments for NF-κB present in the promoter region of the F11R gene (9). This sequence of events is consistent with the reports that platelets adhere to the inflamed endothelium even prior to the invasion of leucocytes and the detection of lesions (23–25). Platelet thrombi are then stabilized also by other CAMs and integrins (24, 29–37) are formed and lead to the development of atherosclerotic lesions and atherothrombosis (23). Figure 11 also serves to point out specific molecular events, to be studied *in vivo* under flow conditions, in order to provide final proof for the role of F11R in initiating and/or promoting atherogenesis and atherosclerosis.

In parallel experiments of the study reported here, we have begun to examine the involvement of F11R/JAM-A in the formation of atherosclerotic plaques *in vivo*. In the first of these experiments we demonstrated a highly enhanced expression of F11R/JAM-A in the aorta of 12-week-old $apoE^{-/-}$ mice, an advanced age in which atherosclerotic plaques are well established (39). The expression of F11R/JAM-A on the atherosclerotic endothelium of carotid arteries of $apoE^{-/-}$ was observed previously by others (18). However, these animals were studied at an earlier stage of development (6 weeks) and fed an atherogenic diet with a high fat content. Since the mice in our study were maintained on a regular chow diet without a high-fat content, we conclude

that the advancing age of these animals is causing a spontaneous upregulation of F11R mRNA-transcripts and induction of F11R/JAM-A protein expression in cellular components of the plaques developing in these animals.

Similar to the results with apoE$^{-/-}$ mice, our examination of F11R/JAM-A expression in plaques developed *in vivo* in the arteries of atherosclerotic human patients revealed abnormally-high levels of F11R/JAM-A mRNA and F11R/JAM-A protein. A recent study (20) of forty-six human atherosclerotic patients reported of enhanced expression of F11R/JAM-A in areas of instability of the atherosclerotic plaque, which are associated with the rupture of plaques and with embolic complications that can lead to myocardial infarcts and/or occlusions in the cerebral vasculature. The results of the in-vivo studies with animals and human patients are consistent with the in-vitro data on the critical role of F11R/JAM-A in the initial adhesion of platelets to inflamed EC. Thus, the sequence and structure of the peptides and recombinant protein which specifically antagonize the action of F11R can provide the basis for the design of novel drugs for the prevention and treatment of atherosclerosis, thrombosis, heart attacks and stroke.

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