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Inhibition of factor Xa-mediated procoagulant activity of human lung fibroblasts and pleural mesothelial cells

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Inhibition of factor Xa-mediated procoagulant activity of human lung fibroblasts and pleural mesothelial cells. A. Kumar, K.B. Koenig, A.R. Johnson, D.S. Fair, S. Idell. ©ERS Journals Ltd 1995.

ABSTRACT: Extravascular fibrin deposition characterizes diverse forms of lung and pleural injury. Fibrin formation in these compartments is locally potentiated by the assembly and expression of the prothrombinase procoagulant complex (factors Xa, Va and II) at the surface of human lung fibroblasts and pleural mesothelial cells. We sought to identify structural domains on factor Xa that mediate expression of prothrombinase activity by these cells.

In order to accomplish this objective, we used panels of monoclonal antibodies (MoAbs) to factor X to block prothrombinase assembly and function on the surface of cultured human lung fibroblasts and pleural mesothelial cells.

Of 30 factor X MoAbs that recognized native factors X and Xa, 10 completely inhibited factor Xa function (prothrombin activation), and five others neutralized Xa function without affecting cell-binding, presumably by blocking the prothrombin binding site. Western blots showed that these inhibitory MoAbs reacted with the Xa heavy-chain. One MoAb that recognized the factor Xa light-chain blocked prothrombin activation at the factor Va binding site.

Our results indicate that prothrombinase activity at the surface of lung parachymal or pleural cells can be blocked by MoAbs that interact with either the heavy- or light-chain of factors X. Antibodies that neutralize cell surface-expressed prothrombin activation offer a potential means to arrest pericellular fibrin formation in the lung and pleural space.

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Expression of procoagulant activity and fibrin deposition is implicated in the pathogenesis of acute inflammation and repair in the lungs and pleural space [1–7]. Alveolar lining fluid of patients with interstitial lung diseases and adult respiratory distress syndrome (ARDS) often contain fibrin, and fibrin deposits are characteristic of pleural diseases [4, 5]. The early and persistant occurrence of tissue fibrin, the interaction of proinflammatory reactants with cells, and the relationship between tissue fibrin deposition and fibrotic sequelae emphasize the relevance of these events to pulmonary injury [1]. Coagulation at extravascular sites is initiated by expression of tissue factor and propagated through assembly of the prothrombinase complex. In earlier studies, we showed that cultured human lung fibroblasts (HLF) and human pleural mesothelial cells (HPMC) express extrinsic activation and prothrombinase complexes [4, 8], suggesting that these cells may promote fibrin deposition in pleural and parenchymal lung disease. Because activated factor X (Xa) is generated by the extrinsic activation complex and is subsequently required for functional prothrombinase assembly, we reasoned that blockade of this moiety might effectively limit the procoagulant capacity of HLF and HPMC.

Macromolecular assembly and protein substrate recognition are important aspects of interaction among coagulation factors. The rapid activation and function of coagulation serine proteases involves assembly of macromolecular complex (enzyme and substrate) on membrane surfaces in the presence of calcium and protein co-factors. Specific sites that mediate the assembly and function of coagulation serine proteases must relate to their molecular recognition sites at solvent accessible surfaces. Because antibodies to proteins recognize and combine with antigenic determinants at the hydrated surface of the antigen, we sought to target selectively functional regions of factor X with immunochemical reagents.

We used a panel of monoclonal antibodies (MoAbs) to factor X to identify molecular sites that mediate prothrombinase activity *via* interaction of Xa with components of the prothrombinase complex. From a panel of MoAbs that recognize structural epitopes of the native protein, we selected, for further study, those MoAbs that blocked functional activities of the procoagulant factors. We find that pathways of fibrin formation at the surface of HLF or HPMC can be blocked by MoAbs to factor X, and our results characterize the multiple molecular interactions through which the anticoagulant effects are achieved.

Materials and methods

Materials

Factor X [9], factor Xa [10], factor V [11], prothrombin [12] and thrombin [13] were isolated by procedures described previously. Factor Va was activated by adding thrombin to a final concentration of 2 NIH unit·mL^{-1} (1.9×10^{-8} M) and incubating at 37°C for 3 min [14]. The synthetic substrate for factor Xa, CBS 31.39 (CH_3SO_4 D-Leu-Gly-Arg-pNA AcOH), was from American Byproducts Co. (Parsippany, NJ, USA). The synthetic substrate for thrombin, S-2238, was purchased from Helena Laboratories (Beaumont, TX, USA). Protein A-agarose, BCA protein reagent and Iodo-gen iodination reagent were from Pierce Chemical Co. (Rockford, IL, USA). Carrier-free Na ^{125}I was purchased from Amersham Corp., Arlington Heights, IL, USA. Tissue culture plates and flasks, foetal bovine serum and minimum essential medium were from Gibco BRL (Gaithersburg, MA, USA). Bovine serum albumin (fatty acid free), ovalbumin (grade V), penicillin-streptomycin, trypsin (1× solution) and cephalin were from Sigma (St. Louis, MO, USA).

Immunization and preparation of hybridomas

Purified native human factor X and a mixture of reduced, alkylated factor X were used as immunogens. Female Balb/c mice were injected subcutaneously with 5 µg of factor X in Ribiadjuvant. Fourteen days later, each was injected subcutaneously with 5 µg of factor X in Ribiadjuvant, and again on day 28 intraperitoneally with 33 µg of factor X in complete Freund's adjuvant. On days 154 and 155, mice received 10 µg of factor X intravenously prior to harvesting of spleens on day 156. Spleen cells were fused to the P3Ag8.653.1 myeloma cell line, and hybridomas were isolated as described previously [15, 16]. Positive clones were selected by solid-phase radioimmunoassay (SPRIA) as described previously [17]. Immunopurified goat anti-mouse immunoglobulin G (IgG) (method A) or factor X (method B) at a concentration of 2.5 µg·mL^{-1} were attached to 96 well polystyrene microtitre plates. Method B was used for clonal selection of all antibodies, and method A was used for determination of Ka values. Nonspecific proteinbinding was blocked by 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 30 min at room temperature. After removal of this solution, 100 µL of 1/100 dilution of culture supernatant was added and incubated for 90 min at 37°C. The plates were washed, the wells cut out and counted in a gamma radiation counter.

Each hybridoma was recloned at least twice. Isotypes were determined with an enzyme-linked immunosorbent assay (ELISA) test (Hyclone Laboratories, Logan, UT, USA). Hybridoma culture supernatants were used for initial screening experiments, ELISA, western and dot blot assays. Purified antibodies were used for all the latter assays. Antibody was purified from ascites fluid using affinity chromatography on immobilized *S. aureus* protein A (MAPS II system; BioRad Laboratories, Richmond, CA, USA).

Western and dot blot assays

For immunoblots, 10 µg of factor X were dissolved in sample buffer (2% sodium dodecyl sulphate (SDS), 50 mM dithiothreitol, 10% glycerol, 125 mM Tris-HCl pH 6.8) and boiled for 5 min. The factors were then separated by SDS-polyacrylamide gel electrophoresis (PAGE) [18] and transferred onto nitrocellulose [19]. The blot was blocked with a solution of 5% powdered nonfat milk in Tris-buffered saline (TBS) and clamped into a manifold (Miniblotter; Immunetics, Cambridge, MA, USA). Hybridoma supernatants were loaded into each manifold slot and incubated for 1 h at 37°C, after which the blot was removed and rinsed with TBS. Bound antibody was detected by an alkaline phosphatase-conjugated second antibody developed with a synthetic substrate (Protoblot; Promega Biotech, Madison, WI, USA). For dot blots, 10 µg of factor X in TBS, factor X heavy-chain (HC), light-chain (LC), prothrombin (FII), factor IX (FIX) or protein S (PS), were dried onto nitrocellulose paper, blocked with 5% powdered nonfat milk/TBS, reacted with hybridoma supernatant diluted in the same solution, and developed as for the Western blots above.

Cell cultures

Mesothelial cells (HPMC) and lung fibroblasts (HLF) were cultured as previously described [4]. Briefly, mesothelial cells were obtained from pleural fluids and fibroblasts were obtained from surgical specimens of lung tissue removed from adult patients. The cultures were maintained in RPMI medium (Gibco) supplemented with 10%, foetal calf serum and 1% antibiotics. Cells for experiments were transferred into multiwell culture plates and allowed to reach confluency. Cultures were used between the third to fifth passage.

Functional assays

Rates of prothrombin activation were determined by amidolytic assays in a purified system as described previously [20, 22]. For prothrombin activation, factor Xa (10 nM) was incubated for 2 h with MoAbs (10 µg·mL^{-1}) at 37°C, then mixed with or without factor Va (1.5 nM) in the presence of cephalin (75 µL), prothrombin (0.34 µM) and 5 mM $CaCl_2$; and factor Xa activity was measured. Data are expressed as the percentage of the rate of factor Xa or thrombin formation in the presence of normal mouse IgG.

Prothrombin activation

The rate of prothrombin activation dependent on factor Xa was analyzed in a two stage assay using a thrombin

specific synthetic substrate, S-2238 [17, 22]. Duplicate wells of confluent cells were preincubated with varying concentrations of factor X, factor Xa and/or factor V/Va in buffer A^+ at room temperature, with constant agitation for 3 min. The reaction was initiated by addition of prothrombin. At defined intervals (10 and 20 min), 25 μL aliquots were removed, 0.2 mM synthetic substrate (S-2238) was added, and the rate of change in the absorbance at 405 nm was monitored for 10 min in a Vmax Kinetic Microplate Reader (Molecular Devices, Palo Alto, CA, USA). Data are expressed as rate of thrombin formation·min^{-1}.

Inhibition assays

Inhibition of factor Xa functional activity on cultured cell surfaces by MoAbs was determined by measurement of the thrombin formed after addition of Xa/MoAb mixture to confluent cell cultures. Factor Xa (10 pM) was preincubated with each of the MoAbs (20 μg·mL^{-1}) for 2 h at 37°C followed by 2 h incubation with the cells at 4°C. The plates were washed and the rate of thrombin formation in the absence of factor V was measured as described above, except that cephalin was not added to the reaction mixture. Data are expressed as the percentage of thrombin activity determined in the presence of normal mouse IgG.

Coagulation assays

The effect of MoAbs on factor Xa coagulation activity was determined by clotting assay as described previously [20]. Affinity-purified MoAbs (10 μg·mL^{-1}) were incubated individually with factor Xa in the presence of calcium at 37°C for 2 h. Clotting assays were performed with 100 μL of preincubated mixture, 75 μL of factor X deficient human plasma (George King Bio-Medical Inc.). For factor Xa clotting 75 μL of TBS-BSA and 50 μL of 25 mM $CaCl_2$ were added, and the time required for clot formation was measured in a fibrometer (BBL Microbiology Systems, Cockeysville, MD, USA). Activity of factors after incubation with MoAbs was quantitated by comparison with a standard curve constructed from the log of the clotting time *versus* the log of known concentrations of factors. The results of antibody-inhibited factor Xa were compared with those obtained using nonspecific mouse IgG as a control.

Radiolabelling of coagulation proteins

Human factor X and factor Xa were each radiolabelled to a specific activity of 1–2 μCi·$μg^{-1}$ using the method of FRAKER and SPECK [23]. Iodogen was prepared according to the manufacturer's instructions, and 10 μg was dried in a glass tube. Each coagulation factor, 5 μg in 100 μL of 0.1 M hydroxyethylpiperazine ethanesulphonic acid (HEPES) buffer (pH 7.45) and 0.5 mCi of Na ^{125}I (5 μL) was added to an iodogen tube and allowed to react at 4°C for 5 min. The reaction was stopped by addition of 1% KI and 1% ovalbumin (50 μL each). Free iodine was separated from ^{125}I-labelled protein by gel filtration in a 12 mL column of Sephadex G-25 equilibrated with HEPES buffered saline containing 1% BSA. The radiolabelled proteins retained complete activity, as assessed by one-stage clotting assays, and were used within 2 weeks of labelling.

Direct binding of factor Xa

Binding of ^{125}I-labelled factors to HLF and HPMC was similar to that described previously [8]. Duplicate wells of confluent cells were washed three times with 1 mL of buffer A, and preincubated with ^{125}I-protein in buffer A supplemented with 0.5% BSA and 5 mM $CaCl_2$ for 2 h at 4°C with constant agitation. The plates were then rapidly washed five times with TBS containing 5 mM $CaCl_2$. The amount of cell-bound radiolabelled protein was determined by lysing the cells in 0.5 mL of 200 mM NaOH with 1% SDS and 10 mM ethylenediamine tetra-acetic acid (EDTA). Radioactivity was measured in a gamma radiation counter. Nonspecific binding was determined by inclusion of 100 fold molar excess unlabelled ligand. Inhibition of direct binding of Xa (0.28 μM final concentration) by MoAbs was determined by preincubating affinity purified MoAbs (20 μg·mL^{-1}; 0.12 μM) with ^{125}I-labelled factor Xa in buffer A^+ for 2 h at 37°C. The preincubated mixtures were added to replicate wells and incubated for 1 h longer at 4°C. Specific cell-associated protein was determined by subtracting radioactivity bound in the presence of mouse IgG.

Results

Isolation and initial characterization of antibodies to factor X

Thirty MoAbs to human factor X were used in this study. Our first series of MoAbs (Nos 1–15) were developed against native factor X. Later, we obtained 15 others from immunizations with a mixture of fully reduced and alkylated factor X. Competitive inhibition studies indicate that all MoAbs specifically recognized factors X and Xa. Affinity constants were estimated by competitive inhibition between ^{125}I factor X and unlabelled factor X. Specificity of factor X MoAbs towards other coagulation factors was determined by dot blot. Most of the MoAbs recognized exclusively both factor X and Xa, except for six that cross-reacted with factor IX and prothrombin (table 1).

Inhibition of factor Xa activity by factor X antibodies

The effect of anti-factor X antibodies on factor Xa function was determined initially in cell-free systems by measurement both of plasma clotting activity and prothrombin activation in the presence or absence of factor Va. The Ka values for most MoAbs were in the 1–10 nM range; we used 10–50 fold excess concentrations of

Table 1. – Summary of the factor X monoclonal antibodies (MoAbs)

MoAb No.	Clone	Isotype	Specificity (dot-blot)						
			FX	FXa	HC	LC	FII	FIX	PS
1	F6-lOC10-1.1	γ1 κ	++	++	-	-	-	-	-
2	F6-lOC10-6.2	γ1 κ	++	++	-	-	-	-	-
3	F7-3B8-1.2	γ1 κ	+	+	-	-	-	-	-
4	F7-1G7-1.8	γ1 κ	+	+	-	-	-	-	-
5	F7-3C3-1.6	γ1 κ	+	+	-	-	-	-	-
6	F8-lF9-5.3	γ1 κ	++	++	-	-	-	-	-
7	F8-2E5-11.3	γ1 κ	++	++	-	-	-	±	-
8	F8-lCll-1.1	γ1 κ	++	++	-	-	-	±	-
9	F11-6H5-4.2	γ1 κ	++	++	-	-	-	-	-
10	F12-lOC8-3.6	γ1 κ	++	++	-	-	-	-	-
11	F12-10F3-4.5	γ1 κ	++	++	-	-	-	++	-
12	F12-12G10-4.5	γ1 κ	++	++	-	-	-	++	-
13	F12-24C11-7.4	γ1 κ	++	++	-	-	-	-	-
14	F12-12H8-4.4	γ1 κ	++	++	-	-	++	-	-
15	F12-21D12-1.1	γ2b κ	++	++	-	-	-	+	±
16	F15-4F5-1.2	γ2b κ	++	++	-	-	-	-	-
17	F15-9G1-4.2	γ1 κ	++	++	-	-	-	+	±
18	F17-1H2-1.2	γ1 κ	+	+	-	-	-	-	±
19	F17-1H12-3.2	γ2b κ	++	++	-	-	-	-	-
20	F17-3E2-4.3	γ2b κ	++	++	-	-	-	-	-
21	F17-3B11-6.3	γ1 κ	++	-	-	-	-	-	-
22	F17-4Al-7.4	γ1 κ	++	++	-	-	-	-	-
23	F17-5Dl-9.1	γ1 κ	++	++		-	-	-	-
24	F17-5D6-10.2	γ1 κ	++	++	-	-	-	-	-
25	F17-7Gl-12.4	γ2b κ	++	++	-	-	-	-	-
26	F17-7D7-13.3	γ1 κ	++	++	-	-	-	-	±
27	F21.1.2B	γ1 κ	++	++	-	-	-	-	-
28	F21-2. X	γ1 κ	++	++	-	-	-	-	-
29	F21-3.1	γ1 κ	++	+	-	-	-	-	-
30	F21-4.2C	γ1 κ	++	++	-	-	-	-	-

++: 100% positive; +: <50% positive; ±: >10% positive; -: no cross reactivity. FX: factor X; FXa: factor Xa; HC: factor X heavy-chain; LC: factor X light-chain; FII: prothrombin; FIX: factor IX; PS: protein S.

antibodies above the Ka values for inhibition experiments. Affinity purified MoAbs were incubated with factor Xa at 37°C for 2 h, and the factor Xa-antibody complex was tested for prothrombin activation and plasma clotting activity (fig. 1a). Of 30 MoAbs tested for inhibition, factor Xa clotting activity was completely inhibited by six partially by two, and not at all by the rest. Inhibition of prothrombin activation by factor Xa in the presence and absence of factor Va had a similar profile for selected MoAbs (fig. 1b). Most MoAbs inhibited prothrombin activation to a similar extent whether factor Va was present or not, but there were two exceptions (Nos. 8 and 23). Comparison of factor Xa activity in the prothrombin activation assays shows that the same five MoAbs (Nos. 9, 27–30) almost completely neutralized factor Xa activity in both assays. MoAbs Nos. 1, 2 and 10, however, inhibited prothrombin activation but did not inhibit clotting activity; also MoAbs Nos. 5 and 6 inhibited clotting activity but failed to inhibit prothrombin activation. These differences in the inhibition of factor Xa activity may be due to complexity of plasma protein interactions in the clotting assay compared to that of purified proteins in the prothrombin assay. Interaction with clotting factors/ inhibitors in the plasma may affect affinities of MoAbs for factor Xa, probably accounting for the disparate findings in the different assay systems. Because the clotting assay more closely approximates physiological conditions, it is more useful for selection of MoAbs for potential clinical application.

The relative efficacy of nine selected MoAbs for inhibition of prothrombin activation was determined by preincubating varying concentrations of MoAbs with factor Xa, and the rate of thrombin formation was compared to controls containing mouse IgG. Eight of the antibodies completely or nearly completely inhibited prothrombin activation. Another (No. 8), which had strong affinity for factor X but showed no inhibition of prothrombin activation in the absence of factor V, was chosen for comparison. All nine MoAbs inhibited factor Xa activity in a concentration-dependent manner (fig. 2). The median inhibitory concentration (IC_{50}) for four MoAbs (Nos. 9 and 27–29) was less than 10 $ng \cdot mL^{-1}$, for three others (Nos. 1, 2 and 30) it was less than 1 $\mu g \cdot mL^{-1}$, and for the remaining MoAbs it was greater than 10 $\mu g \cdot mL^{-1}$.

Inhibition of cellular prothrombinase activity by factor Xa antibodies

Prothrombin activation by factor Xa on cell surfaces is mediated by two pathways. One is through assembly of the prothrombinase complex and requires factor

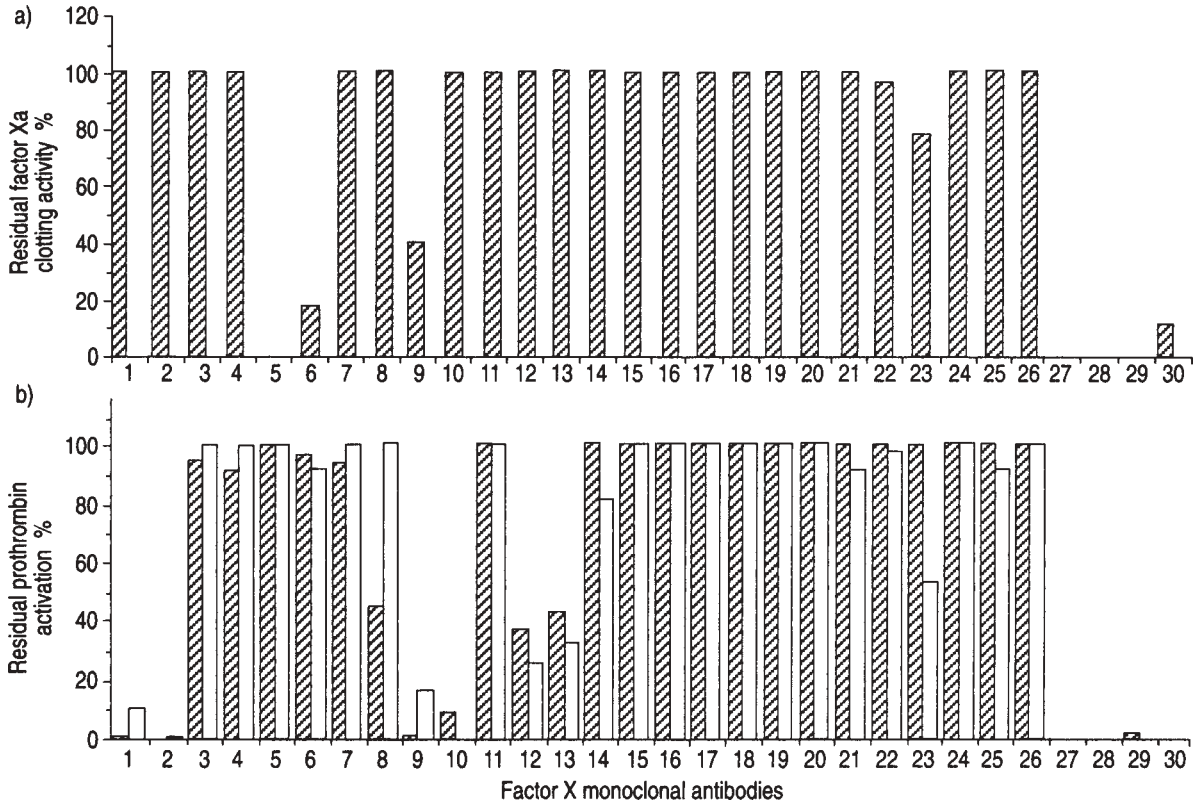

Fig. 1. – Inhibition of factor Xa clotting activity and prothrombin activation by factor X monoclonal antibodies (MoAbs). a) Factor Xa (10 nM) was incubated for 2 h with MoAbs (10 μg·mL^{-1}) at 37°C, then mixed with cephalin (75 μL) and factor X deficient plasma (75 μL) in the presence of 5 nM $CaCl_2$, and the clotting time was measured using a fibrometer. b) Factor Xa (10 nM) was incubated for 2 h with MoAbs (10 μg·mL^{-1}) at 37°C, then mixed with or without factor Va (1.5 nM) in the presence of cephalin, prothrombin and 5 mM $CaCl_2$, and the thrombin activity, an index of residual factor Xa activity, was measured by chromogenic assay. Data are plotted as percentage of activity determined in the presence of normal mouse immunoglobuin G (IgG). ▨: factor Xa/factor Va; ▭: factor Xa.

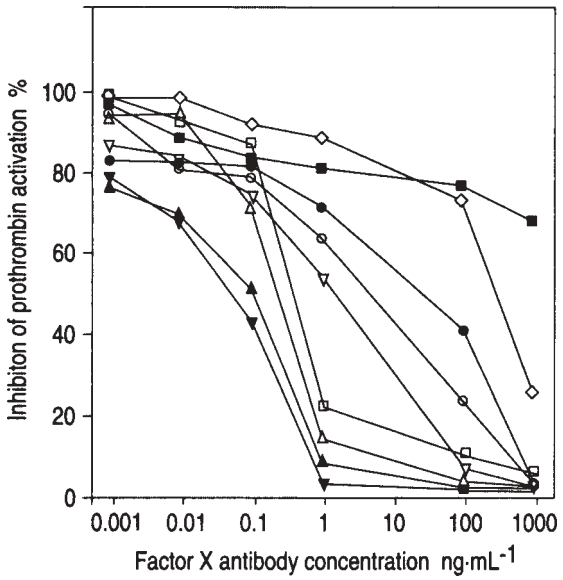

Fig. 2. – Concentration-dependent inhibition of the rate of prothrombin activation by factor X monoclonal antibodies (MoAbs). Varying concentrations of the MoAbs indicated were incubated with factor Xa (10 nM) for 2 h at 37°C, then mixed with factor Va, cephalin and prothrombin in the presence of 5 mM $CaCl_2$, and the thrombin activity measured. Data are plotted as percentage of factor Xa activity determined in the presence of normal mouse immunoglobulin G (IgG). —●— : No. 1; —○— : No. 2; —■— : No. 8; —□— : No. 9; —▲— : No. 27; —△— : No. 28; —▼— : No. 29; —▽— : No. 30; —◇— : No. 10.

Va; the other is through direct activation by cell surface-assembled factor Xa and is independent of factor Va. We examined whether MoAbs blocked assembly and function of factor Xa in the prothrombinase activation complex on the cell surface. Inhibition of factor Xa binding to the cell surface is reflected by the decreased amount of cell-associated factor Xa to activate prothrombin.

Direct binding of ^{125}I-factor Xa to cultured HLF and HPMC in the presence of antibodies was used to probe functional sites on factor Xa that interact with its specific receptor on cell surfaces. Figure 3a shows inhibition of binding of ^{125}I-factor Xa to HLF and HPMC by MoAbs. As with inhibition of factor Xa activity, many of the MoAbs inhibited binding of radiolabelled factor Xa to a different extent. MoAb (No. 8), which inhibited factor Xa activity only in the presence of factor Va, did not block binding of factor Xa to HLF and HPMC surfaces. Antibodies Nos 26–30 likewise inhibited Xa-mediated conversion of prothrombin to thrombin both by HLF and HPMC but did not inhibit Xa binding, whilst the effects of antibodies Nos 1, 2, 9 and 10, in particular, appeared to involve cellular Xa binding sites.

Figure 3b shows that several MoAbs to factor Xa decreased the rate of thrombin formation by HLF and HPMC. Prothrombin activation by HLF and HPMC surface-associated factor Xa was neutralized by the same set of MoAbs that inhibited prothrombin activation without

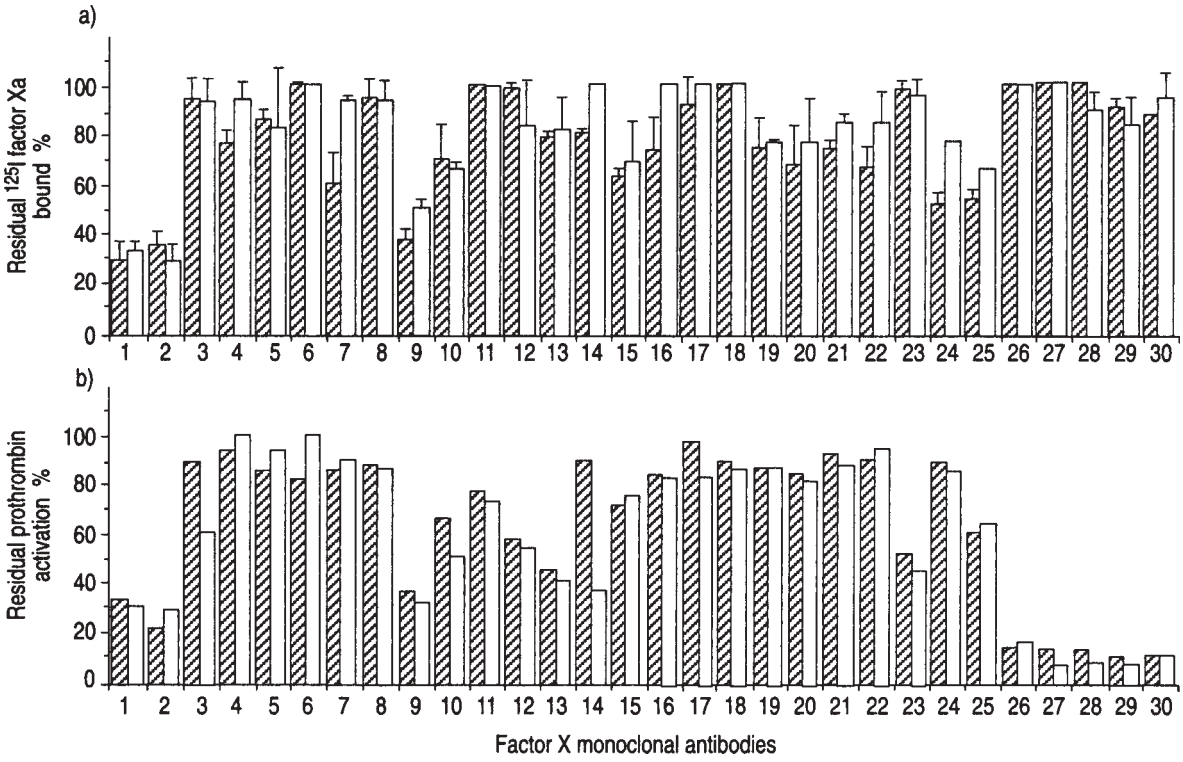

Fig. 3. – a) Inhibition of ^{125}I-factor Xa binding to HPMC and HLF by factor Xa MoAbs. ^{125}I-factor Xa (1 nM) was preincubated with each of the MoAbs as described in the text. The plates were washed and the amount of bound radioactivity measured. Data are plotted as percentage of ^{125}I-factor Xa bound determined in the presence of normal mouse IgG, and represents mean±SD of 4 experiments. b) Inhibition of prothrombin activation at HLF and HPMC surfaces by factor X MoAbs. Thrombin formation, dependent on factor Xa activity expressed in the presence of MoAbs, was measured as described in the text. The data show the percentage of thrombin activity determined in the presence of normal mouse IgG. HPMC: human pleural mesothelial cells; HLF: human lung fibroblasts; MoAbs: monoclonal antibodies; IgG: immunoglobulin G. ▨ HLF; ▭ : HPMC.

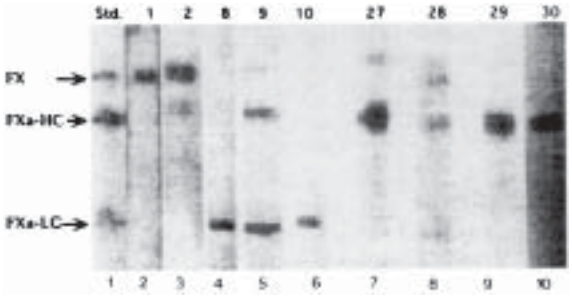

Fig. 4. – Western blot analysis of inhibitory monoclonal antibodies (MoAbs) to factor X. Factor X (10 µg) was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence or absence of 2-mercaptoethanol. The protein was transferred to nitrocellulose, blocked with a solution of 5% powdered nonfat milk in TBS and treated with purified MoAbs for 2 h at 37°C. The antibody bound was visualized using an alkaline phosphatase-conjugated secondary antibody. Lane 1: standard (Std); lanes 2–10 MoAbs, as indicated at the top of each lane. Lane numbers indicated below. FX: factor X; FXa: activated factor X: HC: heavy-chain; LC: light-chain; TBS: Tris-buffered saline.

factor Va (fig. 1b). The same MoAbs inhibited factor Xa activity to a similar extent both in HLF and HPMC. These data suggest that cellular binding factor of Xa is independent of factor Va and that the same functional domain on factor Xa interacts with both cell lines.

To map antibody recognition sites of factor Xa, inhibitory MoAbs were blotted to factor X, separated by SDS-PAGE in the presence or absence of 2-mercaptoethanol, and transferred to nitrocellulose (fig. 4). MoAbs Nos. 1 and 2 recognize specifically nonreduced factor X but not light- or heavy-chains of the factor under reducing conditions. Three inhibitory MoAbs (Nos. 8, 9 and 10) recognized only the Xa light-chain, and four others (Nos. 27–30) recognized the heavy-chain.

Discussion

The prothrombinase complex is a critical participant in the molecular events leading to thrombin formation and fibrin deposition. The assembly of this complex on cell surfaces appears to be an integral part of coagulation that occurs in haemostasis, wound healing, inflammation, thrombosis and atherosclerosis [1, 6]. Coagulation at the cell surface is initiated by expression of tissue factor, and propagates through assembly of the prothrombinase complex. Studies from several laboratories, including our own [2, 24], implicate these events in the pathogenesis or resolution of various types of lung injury [1–8].

Although all of our MoAbs to factor X recognized native factor Xa antigen, not all of them neutralized factor Xa functional and cell-binding activity to the same extent. Our observations suggest that the inhibitory MoAbs interact with various structural epitopes that mediate interaction of factor Xa with components of the prothrombinase complex. Noninhibitory MoAbs appear to bind to sites on factor Xa irrelevant for assembly or activity.

Inhibition of factor Xa by MoAbs could block Xa-mediated conversion of prothrombin to thrombin and Va-mediated augmentation of this activity at the cell surface. We found that prothrombin activation by factor Xa treated with MoAbs was neutralized to a similar extent in the presence or absence of co-factor (factor Va) by all of the MoAbs except one. Factor Va is essential for factor Xa binding to platelet membranes as well as other cell surfaces [25], and promotes the increased conversion of prothrombin to thrombin in the presence of factor Xa by 40–50 fold [4, 8]. However, recent studies with cultured endothelial cells [26] and human tumour cell lines [27] have shown that, contrary to observations with platelets or peripheral blood cells, factor Xa binding did not require factor Va. We previously showed that binding of Xa to HPMC or HLF surfaces does not require factor Va, but factor Va, when added to Xa, promotes conversion of prothrombin to thrombin. This reaction at the HPMC or HLF surface is comparable to that reported in other cell types [4, 8]. MoAb No. 8 neutralized prothrombin activation mediated by factor Xa in the presence of Va, but it did not affect prothrombin activation in the absence of Va, as determined with either purified components or cultured cells. Furthermore, MoAb No. 8 failed to inhibit direct binding of ^{125}I-factor Xa to cultured cells. Together, the data suggest that the MoAb interferes with factor Va binding sites on factor Xa. A Western blot of factor Xa treated with MoAb No. 8 specifically recognized the light-chain of factor Xa. This observation indicates that the light-chain of factor Xa is involved in co-factor (factor Va) binding, and agrees with an earlier report [28] that factor Va interacts with the light-chain of factor Xa.

Four of our MoAbs (Nos. 27–30) completely neutralized both factor X clotting activity and prothrombin activation, without affecting direct binding of ^{125}I-factor Xa to cultured cells. Because cell-bound factor Xa is functionally active, these MoAbs probably interact with substrate binding sites on factor Xa, where they might not interfere with cell-binding. Furthermore, western blots of MoAbs Nos. 27–30 reacted with factor Xa which showed that the antibodies recognized the factor heavy-chain. This confirms that the epitope on the heavy-chain of factor Xa recognized by these MoAbs is involved in an interaction with prothrombin and not in cell-binding. Along these lines, previous studies using synthetic peptides of factor X also indicate that the heavy-chain of factor Xa interacts with prothrombin [22].

The effects of MoAbs on assembly and functional properties of the prothrombinase complex of either HLF or HPMC were similar. This observation implies that initiation and propagation of coagulation involves components common to the cell membranes of these lung parenchymal and mesothelial cells. Our data suggests that the same MoAbs could be used to inhibit pericellular fibrin deposition surrounding lung fibroblasts or mesothelial cells. The antibodies could, potentially, be used clinically to block extravascular fibrin and formation and organization in the interstitial compartment or the pleural space.

Previous reports described the inhibition of procoagulant activity in bronchoalveolar lavage fluids of patients with ARDS and interstitial lung diseases by antibodies to tissue factor [7, 24]. In lavage fluids from patients with ARDS, a small amount of the procoagulant activity was prothrombinase-like and was inhibitable by an antibody to factor X [2]. A similar situation exists in the setting of pleural diseases. The pleural exudates contain increased tissue factor activity and/or fibrinopeptide A concentrations, reflecting increased coagulation. Procoagulant activity of pleural effusions contributed by prothrombinase-like activity is inhibitable by an antibody to factor X [2]. Thus, it should also be possible to block fibrin deposition in exudative injury of the lungs or pleural space by inhibiting fluid-phase procoagulant activity by MoAbs to factor X.

MoAbs to factor X would be expected to block activation of factor X by tissue factor associated with factor VII, but Xa formation may be accomplished by mechanisms other than formation of the extrinsic activation complex at the cell surface. For example, factor X can be activated at the surface of monocytes by interaction with the Mac-1 receptor [29]. McGee and Li [30] showed that monocytes support the activation of factor X through the intrinsic pathway activators, factors IX and VIIIa. Finally, factor X may be activated in neoplastic disease by a cysteine protease activator of factor X called cancer procoagulant [31]. The role of cancer procoagulant in other diseases is currently unknown, but the protein has been identified in lavage fluids from patients with ARDS (personal communication, S. Gordon). Monoclonal antibodies to factor X could effectively block factor Xa formation predicted on any of these pathways; this effect may have clinical value in situations, such as inflammation or neoplasia, where several of these pathways are concurrently activated. Blockade of transitional fibrin in these settings might be used to interrupt local organization and subsequent scarring or neoplastic spread [1, 32]. Selective blockade of procoagulant activity might mitigate the potential for bleeding complications associated with the use of broad-spectrum anticoagulants, such as heparin or coumadin.

The MoAbs that we describe could interact with and complement the effects of endogenous anticoagulants. Tissue factor pathways inhibitor (TFPI), in particular, would probably be influenced by and interactive with the MoAbs. TFPI inhibits factor Va/tissue factor activity by forming a quarternary Xa/TFPI/VIIa complex [33]. It has also been suggested that TFPI at high concentration inhibits VIIa/tissue factor in the absence of Xa [34]. The antibodies might limit availability of factor Xa participation in the complex but would otherwise be expected to limit availability of Xa for assembly of the distal prothrombinase complex. TFPI was also reported to inhibit activated factor Xa directly, suggesting that the MoAbs could augment this particular TFPI-mediated effect.

In summary, we determined structural and functional interactions of MoAbs that inhibit the assembly and function of the prothrombinase complex. Procoagulant activity may be selectively and completely inhibited by several different MoAbs to factor X. Possible ramifications include application to *in vivo* systems. The utility of

antibody reagents in clinical medicine seems assured, particularly in clinical intervention trials involving ARDS and sepsis patients. Our characterization of MoAbs that inhibit procoagulant activities in purified protein assay systems and cell cultures provides the rationale for extension to animal models of pulmonary or pleural inflammation. Using such reagents, the effect of selective inhibition of fibrin formation on remodelling of the extracellular matrix and the course of lung or pleural injuries could be addressed.

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