J. Biochem. 126, 655-661 (1999)

Fibroblasts, Glial, and Neuronal Cells Are Involved in Extravascular Prothrombin Activation[1]

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Received May 10, 1999; accepted July 19, 1999

A membrane-associated prothrombin activator (MAPA) was found on various cultured cells derived from non-hematopoietic cells [Sekiya, F. *et al.* (1994) *J. Biol. Chem.* 269, 32441-32445]. In this study, we investigated the enzymatic properties of this enzyme using protease inhibitors. While the metalloproteinase inhibitor, *o*-phenanthroline, had no effect, some Kunitz type serine protease inhibitors attenuated MAPA activity. Recombinant tissue factor pathway inhibitor (rTFPI) also markedly reduced the activity (IC_{50}, $1.3 \pm 0.6 \times 10^{-10}$ M). MAPA activity is, therefore, most likely to be due to factor Xa. We evaluated the effect of exogenous factor Xa on MAPA activity. Factor Xa-dependent prothrombin activation was observed on fibroblast cells (apparent K_d, 1.47 ± 0.72 nM). Activation was also observed on glial and neuronal cells, which expressed MAPA activity. These results imply that membrane-bound factor Xa results in MAPA activity on these cells. Therefore, we considered the involvement of factor Va, a component of prothrombinase, in this activity. We examined whether or not the prothrombinase complex is assembled on these cells. Prothrombin was activated in a manner dependent on both exogenous factor Xa and factor Va (apparent K_d of 0.51-1.81 nM for factor Va). These results indicate that the prothrombinase complex forms specifically on various extravascular cells. Although the prothrombinase complex can be assembled on monocytes and lymphocytes, it is not known why these cells can activate prothrombin specifically. These cells which have the capacity for prothrombin activator activity could also activate factor X; *i.e.* cells with factor X activation activity were able to convert prothrombin. These observations suggest that thrombin was generated *via* two procoagulant activities; factor X activation and subsequent prothrombinase complex formation on the surface of these cells. This mechanism may explain the various pathological states involving or resulting from extravascular thrombin and fibrin formation.

Key words: extravascular thrombin formation, factor V, factor X, prothrombinase, thrombin.

In the bloodstream, zymogen prothrombin is converted to α-thrombin and activated by a prothrombinase complex composed of the enzyme factor Xa, a non-enzymatic cofactor factor Va, Ca^{2+}, and a membrane surface (*1*). Platelet-bound or phospholipid vesicle-bound factor Va forms the receptor for factor Xa in a 1:1 stoichiometric Ca^{2+}-dependent complex (*2, 3*). The thrombin generated plays many important roles in thrombosis and hemostasis. Thrombin not only plays roles in blood coagulation, but also acts multifunctionally; *e.g.* chemotaxis for monocytes (*4*), and activates venous endothelial cells (*5*).

In extravascular regions, thrombin also affects the proliferation of smooth muscle cells and fibroblasts (*6, 7*). It has been revealed that these effects are regulated *via* the thrombin receptor (*8*). The thrombin receptor is also expressed in mesenchymal cells, the heart, blood vessels, and the germinal epithelium of the hindbrain during mouse development, and therefore thrombin may be a critical factor in early mammalian development (*9*). In the nervous system, thrombin causes the inhibition of neurite outgrowth and retraction of extended neurites, and a reversal of stellation of differentiated astrocytes (*10-12*). It has been reported that thrombin and protease nexin-1, a potent thrombin inhibitor, may be involved in the pathogenesis of Alzheimer's disease (*13, 14*). Thus, it has been clearly shown that thrombin has significant effects in extravascular cells and regions. However, there have been few reports regarding how thrombin is supplied to these cells or sites

[1] This work was supported by a Research Grant for Cardiovascular Diseases (8A-1) from the Ministry of Health and Welfare to T. Morita.

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Abbreviations: Boc, *t*-butoxycarbonyl; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonic acid; IgG, immuno-globulin G; IX/X-bp, factor IX/factor X-binding protein; MAPA, membrane-associated prothrombin activator; MDCK, Madin-Darby canine kidney; PAGE, polyacrylamide gel electrophoresis; RVV-X, factor X activator from Russell's viper venom.

from prothrombin. Recently, we found a membrane-associated prothrombin activator (abbreviated as MAPA) on 8C fibroblast cells and some extravascular intact cell surfaces, but not on vascular cells (*15*). Therefore, MAPA may participate in the generation of thrombin in the extravascular sites.

In this study, we use several cell lines, since primary cultures may contain small but significant numbers of unfavorable cells, such as vascular endothelial cells, and culture cell lines provide an essentially pure source of various cell types.

We report here the results of detailed examination of the enzymatic properties of MAPA using protease inhibitors to determine how MAPA activity is regulated under physiological conditions. We also present a possible mechanism by which thrombin is supplied through the activation of prothrombin in extravascular regions.

METHODS

Materials—Published procedures were used to purify the following proteins: human prothrombin and factor X (*16*), bovine factor X (*17*), and factor IX/X-binding protein (IX/X-bp) from the venom of *Trimeresurus flavoviridis* (*18*). All of these proteins were homogenous, as determined by SDS-PAGE. Prothrombin and factor X were treated with di*iso*propyl fluorophosphate (DFP) and *p*-amidinophenylmethanesulfonyl fluoride (*p*-APMSF) prior to use to exclude the effect of possible contamination by thrombin or factor Xa. Factor Xa was prepared using the snake venom X activator, factor X activator from Russell's viper (RVV-X) (*19*). Bovine Gla-domainless factor X prepared by the method of Morita and Jackson (*20*) was supplied by Dr. Hideko Atoda. Human factor Va was obtained from Haematologic Technologies (VT, USA). The anti-human factor V/Va antibody was obtained from Cedarlane (Ontario, Canada). The thrombin chromogenic substrate, Boc-Val-Pro-Arg-*p*-nitroanilide (VPR-*p*NA), and factor Xa chromogenic substrate, Boc-Leu-Gly-Arg-*p*-nitroanilide (LGR-*p*NA), were purchased from Seikagaku Corporation, Tokyo. Soybean trypsin inhibitor (SBTI), aprotinin, and Bowman-Birk trypsin inhibitor (BBI) were from Sigma. Russell's viper venom inhibitor II (RVV-II) was prepared as described previously (*21*). DX-9065a was a kind gift from Daiichi Pharmaceutical, Tokyo. Recombinant human TFPI (*22*) was a kind gift from the Chemo-Sero-Therapeutic Research Institute (Kumamoto). Human urinary trypsin inhibitor (UTI) was kindly supplied by Mochida Pharmaceutical, Tokyo.

Cell Culture—8C feline kidney fibroblast cells, MDCK canine kidney (epithelial-like) cells (ECA84121903; Riken Gene Bank, Tsukuba), and T98G human glioblastoma cells (CRL 1690; American Type Culture Collection) were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% heat-inactivated fetal calf serum and 70 μg/ml kanamycin, and passaged after trypsinization as described (*15*). GOTO human neuroblastoma cells and HepG2 human hepatocellular carcinoma cells were obtained from the Riken Gene Bank (Japan). Other examined cells were from ATCC and were maintained as recommended by ATCC. These cells were selected on the basis of screening depending on an index of prothrombin activator activity.

Without serum, cells were maintained in Celgrosser-P (Sumitomo Seiyaku). Confluent cultures of adherent cells were rinsed twice with HEPES-buffered saline (HBS; 10 mM HEPES-NaOH, 150 mM NaCl, pH 7.5), and then the cells were scraped off in the absence of either trypsin or EDTA so as not to modify any cellular membrane surface. The harvested adherent and non-adherent cells were washed by centrifugation/resuspension twice at $500 \times g$ for 5 min with HBS and then suspended in HBS. Cell counts were made using a hemocytometer. Fresh cell suspensions were prepared for each experiment.

Prothrombin Activator Activity Measurements—In a typical assay, 90 μl aliquots of 1 μM human prothrombin in Tris-buffered saline (TBS; 50 mM Tris-HCl, 100 mM NaCl, pH 8.0) containing 5 mM $CaCl_2$ and 1 mg/ml bovine serum albumin (BSA) were incubated with samples at 37°C for 20 min in 96-well microtiter plates. We then added 10 μl of 2.5 mM VPR-*p*NA, and the initial rate of *p*-nitroaniline liberation was monitored at 405 nm with a kinetic plate reader (Well Reader, Seikagaku Corporation).

Assay for Effects of Protease Inhibitors—CHAPS-solubilized enzyme fractions (fraction C) and phospholipid vesicles were prepared as described previously (*23*). Solubilized fractions were incubated with inhibitors for 30 min at 37°C, and then residual prothrombin activator activity was measured in the presence of 0.1 mg/ml phospholipid vesicles (PC/PE/PS=5:3:2) as described above. The inhibitory activity of rTFPI was evaluated in the presence of 1 unit/ml unfractionated heparin (Sigma). The inhibitors did not affect α-thrombin activity at the concentrations used. The effects of habu IX/X-bp and DX-9065a were investigated by the same method, except for the use of a cell lysate.

Assay for Factor Xa-Dependent Prothrombin Activation—A cell lysate (1.0 mg protein/ml, prepared as described previously) (*15*) was washed by centrifugation four times at $5,000 \times g$ for 20 min with TBS. The precipitate was resuspended in TBS containing factor Xa and 1 mM $CaCl_2$, and then incubated at 37°C for 10 min. The lysate was washed by centrifugation three times at $5,000 \times g$ for 20 min with TBS containing 1 mM $CaCl_2$. Then, the prothrombin activator activity of the lysate was measured as described above. As a control, a cell lysate was first incubated for 10 min at 80°C and then subjected to the following experiments in the same manner. This treatment completely abolished MAPA activity.

Functional Binding Assay for Factor Va with Various Cells—A serum-free cultured cell suspension (1×10^4 cells/ml) was incubated with various concentrations of human factor Va and factor Xa (5 nM; saturation) in HBS containing 1 mg/ml BSA and 2 mM $CaCl_2$ at 37°C for 2 min. Then the reaction mixture was assayed for prothrombin (1 μM) activator activity. In the factor Va-dependent functional factor Xa binding assay, cells were first treated with the saturation concentration of human factor Va (5 nM), varying amounts of human or bovine factor Xa, and 2 mM $CaCl_2$ at 37°C for 2 min, and then prothrombin (1 μM) activator activity was evaluated. Prothrombin activation was measured as described above (see "*Prothrombin Activator Activity Measurements*"). These methods were based on the observation that deletion of the catalytic membrane surface results in a 1,000-fold decrease in the reaction rate (*2*).

Treatment with Anti-Human Factor V/Va IgG—To

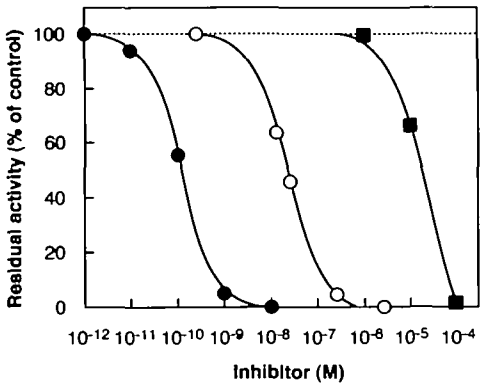

Fig. 1. **Effect of recombinant TFPI on MAPA activity.** CHAPS-solubilized 8C fibroblast enzyme fractions were incubated with various amounts of rTFPI (●), SBTI (○), or aprotinin (■) for 30 min at 37°C. Residual prothrombin (1 μM) activator activity was then measured in the presence of 0.1 mg/ml phospholipid (PC/PE/PS = 5:3:2). The assay for rTFPI was performed in the presence of 1 unit/ml unfractionated heparin. Note that a urinary trypsin inhibitor, a physiological Kunitz-type inhibitor, had no effect at all. Bowman-Birk-type trypsin inhibitor from soybeans (BBI) and RVV-II, a Kunitz-type trypsin inhibitor from the snake venom, reduced MAPA activity, with IC_{50} of 5×10^{-7} and 2×10^{-6} M, respectively.

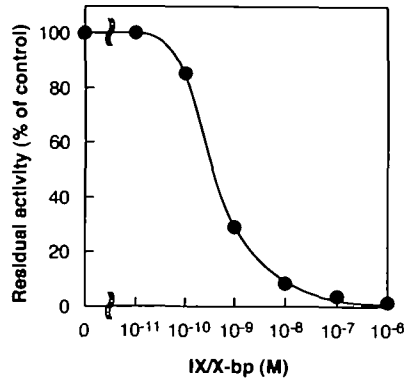

Fig. 2. **Effect of IX/X-bp on MAPA activity.** An 8C fibroblast cell lysate was incubated with various amounts of IX/X-bp in the presence of 5 mM Ca^{2+} ions for 30 min at 37°C. Residual prothrombin (1 μM) activator activity was then measured.

determine the role of cell-bound factor Va in MAPA activity and functional factor Xa binding ability, we performed pretreatment with anti-human factor V/Va IgG, that neutralizes factor Va cofactor activity. A cell lysate was incubated with anti-human factor V/Va IgG (100 μg/ml) at 37°C for 30 min, and then the residual MAPA activity was measured. With thrombin-activated platelets, the same treatment reduced factor Va cofactor activity. To determine the effects on functional factor Xa binding, a cell lysate was incubated with bovine factor Xa (3 nM) in the presence of anti-factor V/Va IgG (100 μg/ml) and $CaCl_2$ (1 mM) for 10 min at 37°C. After washing three times by centrifugation/resuspension, the lysate was examined for prothrombin activator activity.

Factor X Activator Activity Measurements—Cell suspensions (1×10^6 cells/ml) were assayed for factor X activator activity with 100 nM factor X and 5 mM $CaCl_2$ as described above (see "*Prothrombin Activator Activity Measurements*"). Factor Xa formation was monitored with LGR-*p*NA. As the activation of human factor X was weaker (at least 10-fold) than that of bovine factor X, the cells were assayed using bovine factor X in this study.

Other Methods—SDS-PAGE was performed by the method of Laemmli (*24*). Protein concentrations were determined with a BCA protein assay kit (Pierce).

RESULTS

Effects of Protease Inhibitors on MAPA Activity—Previously we showed that exposure of MAPA to EDTA results in an irreversible loss of activity, and that potent factor Xa inhibitors such as antithrombin III, *p*-amidinophenylmethanesulfonyl fluoride (*p*-APMSF), or 1,5-dansyl-Glu-Gly-Arg chloromethyl ketone (Dns-EGRck) did not abolish MAPA activity (*15*). The enzyme was, therefore, suggested to be different from factor Xa, and also likely to be a metalloproteinase.

Since the prothrombin activator activity of 8C fibroblasts detected was strong MAPA activity, we characterized the activity in detail using protease inhibitors in this study. While the metalloproteinase inhibitor, *o*-phenanthroline (1 mM), had no effect (data not shown), some Kunitz-type trypsin inhibitors, *i.e.* soybean trypsin inhibitor (SBTI) and aprotinin, reduced MAPA activity (IC_{50}, 4.0×10^{-8} and 1.4×10^{-5} M, respectively) (Fig. 1). The effects of recombinant TFPI (rTFPI), a well-known physiological Kunitz-type factor Xa inhibitor (*22, 25*), were also investigated. rTFPI showed more marked inhibition of MAPA activity than SBTI, with an IC_{50} of $1.3\pm0.6\times10^{-10}$ M ($n=3$) (Fig. 1). IX/X-bp (*26, 27*), an anticoagulant protein from snake venom which recognizes the Gla domain of factors IX and X (*28*), abolished MAPA activity completely (IC_{50}, 5×10^{-10} M) (Fig. 2). DX-9065a, a selective and potent inhibitor of factor Xa (*29*) attenuated the prothrombin activator activity (IC_{50}, 3×10^{-8} M) (data not shown). These observations indicated that MAPA is most likely factor Xa.

Activation of Prothrombin by Various Cells Depending on Factor Xa—Since rTFPI completely abolished MAPA activity, it appears that MAPA may consist of membrane-bound factor Xa or a factor Xa-like molecule. We next investigated the effects of exogenous factor Xa on MAPA activity. Factor Xa-dependent prothrombin activation was examined using bovine factor Xa in the presence of 8C fibroblasts and Ca^{2+} ions. A non-treated cell lysate activated prothrombin in an added factor Xa-dependent and saturable manner, but no marked factor Xa-dependent prothrombin activation was observed on the heat-treated cell lysate (Fig. 3a). Human factor Xa with fibroblasts and Ca^{2+} ions also activated prothrombin (Fig. 3b). Similar factor Xa (bovine)-dependent prothrombin activation was observed in the experiment involving intact 8C fibroblasts (not shown). These results suggest that factor Xa binding sites are located on the fibroblast cell surface. The apparent K_d values of the 8C cells for human and bovine factor Xa were 10.6 ± 1.5 nM ($n=4$) and 1.47 ± 0.72 nM ($n=4$), respectively. The optimal apparent factor Xa-binding required 1 mM Ca^{2+}. Other cell lines which expressed MAPA activity were also investigated (MDCK kidney cell, T98G glioblastoma, and GOTO and Neuro-2a neuroblastoma), as shown in Table I. These cells also activated prothrombin in a human and bovine factor Xa and Ca^{2+}-

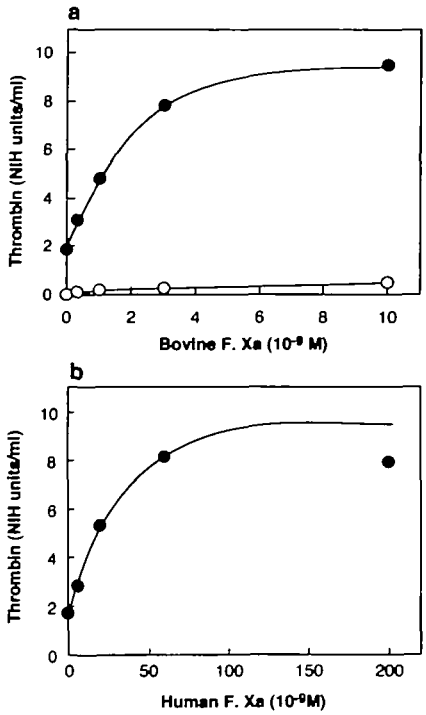

Fig. 3. **Factor Xa-dependent prothrombin activation in the presence of fibroblast cells.** An 8C cell lysate (1.0 mg protein/ml) was washed by centrifugation/resuspension four times at 5,000×*g* for 20 min with TBS. The cell lysate was then incubated with various concentrations of bovine (a) or human (b) factor Xa and 1 mM Ca^{2+} ions for 10 min at 37°C. After incubation, the cell lysate was washed with TBS containing 1 mM Ca^{2+} ions. The prothrombin (1 μM) activator activity of the lysate was then measured (●). A heat-treated lysate was also assayed as described above (○).

TABLE I. **Activation of prothrombin by various cells dependent on human and bovine factor Xa.** A cell lysate (1.0 mg protein/ml) was incubated with factor Xa (human or bovine) and Ca^{2+} (1 mM) for 10 min at 37°C. The lysate was then washed by centrifugation as described under "METHODS." Prothrombin (1 μM) activator activity was then measured. The apparent K_d of factor Xa was obtained from a Lineweaver-Burk plot.

Cell line	Origin	App. K_d (nM)	
		Human	Bovine
8C	Fibroblast (cat)	10.6	1.47
MDCK	Kidney (dog)	3.1	0.73
T98G	Glioblastoma (human)	7.5	0.50
GOTO	Neuroblastoma (human)	0.37	0.78
Neuro-2a	Neuroblastoma (mouse)	1.6	0.65

dependent and saturable manner. To determine whether or not the binding of factor Xa was dependent on the Gla domain, as the binding was completely Ca^{2+}-dependent, we examined whether or not Gla-domainless factor X bound to cells. Factor Xa-dependent prothrombin activation by fibroblasts was not affected by the addition of 300 nM Gla-domainless factor X, a 100-fold molar excess over factor Xa. These observations indicated that fibroblasts (MDCK), and GOTO and Neuro-2a neuroblastoma, and T98G glioblastoma cells express high-affinity and Ca^{2+}-dependent (*via* Gla domain) factor Xa-binding sites on their surface. MAPA activity, therefore, seems to be due to a

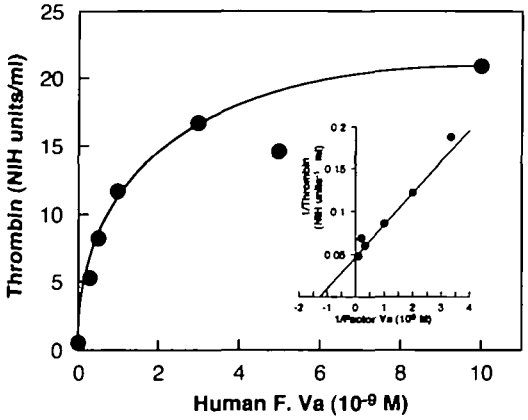

Fig. 4. **Factor Va-fibroblast binding, as determined by the kinetics of prothrombin activation.** An 8C cell suspension (1×10⁴ cells/ml, cultured without serum) was incubated with various amounts of human factor Va, human factor Xa (5 nM), and Ca^{2+} ions (2 mM) for 2 min at 37°C. Prothrombin (1 μM) activator activity was then measured. The apparent K_d of factor Va was obtained from a Lineweaver-Burk plot (inset).

membrane-bound form of factor Xa or a factor Xa-like enzyme.

Functional Binding of Factor Va to Fibroblasts, and Neuroblastoma and Glioblastoma Cells—Factor Xa-dependent prothrombin activation was observed in various cell types such as fibroblasts, and neuroblastoma and glioblastoma cells. We hypothesized that the irreversible inhibition of MAPA activity by EDTA was caused by the release of factor Xa from the 8C cell surface. We next evaluated the effects of EDTA on factor Xa-dependent prothrombin activation. No factor Xa-dependent prothrombin activation was observed on EDTA (10 mM)-treated 8C cells. This indicated that the irreversible inhibition by EDTA is caused by not only dissociation of factor Xa from the 8C cell membrane, but also an inhibitory effect on factor Xa binding sites. Factor Va is a possible factor Xa-binding site, participating in the formation of the factor Xa-receptor and generating thrombin by forming a prothrombinase complex (factor Xa, factor Va, anionic phospholipids, and Ca^{2+} ions) on various vascular cell surfaces. As described above, the anticoagulant protein, IX/X-bp, inhibited MAPA activity. IX/X-bp completely attenuates the prothrombinase activity by binding to the Gla domain of factor Xa (*18*). These combined results also indicate MAPA activity resembles prothrombinase activity. Prothrombin activation dependent on the prothrombinase complex on cells such as fibroblasts, and neuronal and glial cells has not been reported. To determine whether these cells cultured in serum-free medium propagate the prothrombin activation through the assembly of a functional prothrombinase complex, the activation of prothrombin by 8C fibroblast cells was determined in the presence of various concentrations of factor Va, factor Xa (5 nM), prothrombin (1 μM), and Ca^{2+} ions. Thrombin was generated in a factor Va-dependent and saturable manner, with an apparent K_d of 0.80 nM (Fig. 4). This reaction depended on both exogenous factor Va and factor Xa. A similar factor Va-dependent prothrombinase complex was found on GOTO neuroblastoma and T98G glioblastoma cells (apparent K_d, 0.51 and 1.81 nM, respectively). In addition, no prothrombin ac-

TABLE II. **Human and bovine factor Xa-binding affinity to various cell types dependent on exogenous factor Va, as determined by the kinetics of prothrombin activation.** A freshly prepared cell suspension (1×10^4 cells/ml, serum-free cultured) was incubated with factor Xa (human or bovine), human factor Va (5 nM), and Ca^{2+} (2 mM) for 2 min at 37°C. Prothrombin (1 μM) activator activity was then measured. The apparent K_d of factor Xa was obtained from a Lineweaver-Burk plot.

Cell line	Origin	App. K_d (10^{-10} M)	
		Human	Bovine
8C	Fibroblast (cat)	5.3	2.6
MDCK	Kidney (dog)	5.6	5.6
T98G	Glioblastoma (human)	20.4	12.5
GOTO	Neuroblastoma (human)	1.5	ND[a]
Neuro-2a	Neuroblastoma (mouse)	5.6	3.3

[a]ND, not determined.

tivator activity was observed in serum-free cultured cells (data not shown).

In the following experiments, we investigated the effects of the anti-human factor V/Va antibody (100 μg/ml) on the prothrombin activator MAPA activity and functional factor Xa binding on 8C cells to assess the involvement of the factor V/Va molecule in this system. The prothrombin activator activity and factor Xa-dependent prothrombin activation on 8C feline kidney cells were not affected by the anti-human factor V/Va antibody (data not shown). These results indicate that the anti-factor V/Va antibody does not cross-react with factor X/Xa, since the anti-factor V/Va antibody apparently did not inhibit factor Xa-binding to 8C cells.

Human factor Va-dependent functional factor Xa binding on these extravascular cells was investigated. Both human factors Xa and bovine factor Xa bound to these cells in a factor Va-dependent manner (as summarized in Table II), compared with previous results (Table I). These findings suggest that the bovine factor Va molecule serves as a factor Xa-receptor on cells in serum (bovine)-containing cultured medium.

Factor X Activation by Various Cell Types—It was reported previously that prothrombinase complex assembly was also observed on hematopoietic cells such as monocytes and lymphocytes (*30*), although little prothrombin activator (MAPA) activity was observed on HL-60 promyelocytic leukemia, U-937 histiocytic lymphoma or Molt-4 lymphoblastic leukemia cell lines in our study (Fig. 5a). HL-60 and U-937 cells also have monocytic properties, which are also thought to contribute to extravascular coagulation and fibrin deposition (*30*). This raises the question of why the activation of prothrombin specifically occurs on some types. We demonstrated previously that 8C fibroblasts activate factor X (*15*). We supposed that the prothrombin activator activity of 8C cells is due to the factor X activator expressed on their surface. Figure 5b shows the results of screening for the factor X activator on several cell types. 8C, MDCK, T98G, GOTO, and Neuro-2a cells activated factor X in a Ca^{2+}-dependent manner, while no factor X activation was found on HL-60, U-937, or Molt-4 cells; *i.e.* the expression of prothrombin activator (MAPA) activity was related to that of X activator activity (Fig. 5, a and b). This implies that factor Xa generated by the factor X activator is assembled with factor Va and Ca^{2+} ions and forms a prothrombinase complex on the membranes of these cells, and this complex serves to generate

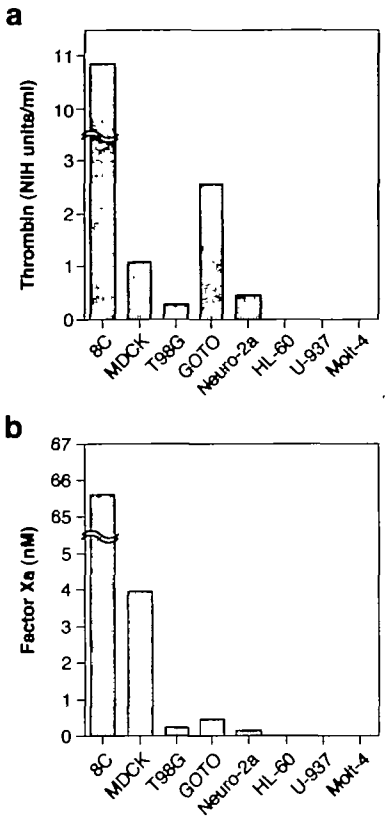

Fig. 5. **Screening for MAPA activity (a) and factor X activator activity (b) on several cell lines.** (a) A cell suspension (1×10^5 cells/ml, cultured with serum) was incubated with human prothrombin (1 μM) and Ca^{2+} ions (5 mM) for 20 min at 37°C. Prothrombin activation activity was then measured. (b) A cell suspension (1×10^6 cells/ml, cultured with serum) was incubated with bovine factor X (100 nM) and Ca^{2+} ions (5 mM) for 20 min at 37°C. Factor X activator activity was then measured. The cells used and their origins were: 8C, cat fibroblasts; MDCK, dog kidney; T98G, human glioblastoma; GOTO, human neuroblastoma; Neuro-2a, mouse neuroblastoma; HL-60, human promyelocytic leukemia; U-937, histiocytic lymphoma; and Molt-4, human acute lymphoblastic leukemia.

thrombin. The activator on these cells showed a marked decrease in activity on culture in serum-free medium.

DISCUSSION

The prothrombinase complex on several vascular and some tumor cells has been reported previously (*3, 30-33*). In the present study, the prothrombinase complex was shown to occur on fibroblasts, neuronal and glial cell surfaces with a similar affinity to platelets (*3*), monocytes, lymphocytes (*30*), tumor cells (*33*), and tracheal epithelial cells (*34*). This is the first report indicating the mechanism by which these extravascular cells, such as fibroblasts, and neuronal and glial cells, encounter thrombin on extravascular surfaces. Moreover, we have shown that extravascular cells generate thrombin *via* two procoagulant activities, factor X activator and subsequent formation of the prothrombinase complex. Our present study also indicates the possibility that MAPA activity, found on several extravascular cells, reflects this system. This mechanism will play an important role in the supply of thrombin to extravascular cells or regions, although further studies on factor X activator are

required. The origins of factors X and V have not yet been determined. Some investigators observed that factor X is expressed in the kidney, lung and heart, and not only in the liver among adult human tissues (*35*). Factor V is synthesized by pig megakaryocytes (*36*), vascular smooth muscle cells (*37*), and HepG2 cells (*38*). Fibroblasts, and neuronal and glial cells may equally exhibit these factors, but we cannot directly rule out the possibility of derivation from the culture medium. Likewise, the factor X activator on these cells should be a culture serum-derived factor(s), such as factor VIIa or factor IXa/factor VIIIa (Xase complex). Fibroblast cells exhibit a high degree of tissue factor (*39*), a membrane-anchored protein acting as a cofactor for factor VIIa (*40*); accordingly, 8C cells may activate factor X as well as prothrombin.

Recently, a MAPA-like enzyme were found on lipopolysaccharide (LPS)- or interleukin-1 (IL-1)-treated human venous endothelial cells (*41*). The enzymatic properties of this enzyme are remarkably similar to MAPA activity.

Bouchard *et al.* (*42*) reported that expression of the factor Xa receptor effector cell protease receptor-1 (EPR-1) by activated platelets substantially regulates the assembly and function of the platelet prothrombinase complex, and coordinates thrombin generation. Similar observations were also made for human brain pericytes (*43*). Although EPR-1 is widely expressed on several vascular cell types, *e.g.* monocytes, leukocytes, and endothelial cells (*44*), it has not been determined whether or not extravascular cells also express EPR-1 on their surfaces. In this experiment, we observed that factor Xa bound to extravascular cells with an apparent K_d of $\sim 10^{-9}$ M, while K_d for factor Xa as to EPR-1 on vascular cells was 10-30 nM (*45, 46*). Furthermore, the binding of factor Xa as to EPR-1 was evaluated on EDTA-treated cells (*45, 46*). Similar treatment of EDTA-treated cells caused a significant decrease of apparent factor Xa-binding to extravascular cells in our experiment (see "*Functional Binding of Factor Va to Fibroblasts, and Neuroblastoma and Glioblastoma Cells*"). These data imply that the EPR-1 molecule did not contribute to extravascular prothrombin activation in our experiment.

Recently, we also detected MAPA-like activity in the liver, kidney, and lung in normal mice, and observed specific increases in the activity in the injured liver and kidney (*23*). The prothrombinase complex is possibly assembled on the extravascular regions of these tissues, and serves for thrombin formation leading to the fibrin deposition characteristic of some inflammatory lesions in extravascular sites (*23*). Inflammatory cytokines, such as interleukine-1 or tumor necrosis factor-α, increase the procoagulant activity of some cell types *via* tissue factor expression (*47, 48*). These cytokines possibly affected the extravascular prothrombin activation in the present study, however, there remains room for further investigation.

We showed here that neuronal cells, *i.e.* GOTO and Neuro-2a cells, in addition to glial cells, also provide thrombin. Most recently, factor X/Xa-dependent prothrombin activation was observed on GOTO human neuroblastoma cells (*49*). Thrombin affects cultured neuronal cells by inhibiting neurite outgrowth and the retraction of extended neurites (*10, 11*). It was revealed that prothrombin also affects neurite outgrowth through cell-mediated conversion to thrombin (*50*). Thus, the ability of neuronal cells to express two enzymes leading to thrombin formation will play a critical role in the control of neuronal damage and neuronal differentiation (*10, 11*). In addition, human factor Xa is bound to GOTO human neuroblastoma cells with high affinity similar to bovine factor Xa, although the cells are maintained in a serum-containing medium (Table I). While these cells have strong prothrombin activator activity, they do not show factor X activation (Fig. 5, a and b). This suggested that GOTO cells constitutively synthesize factor V and serve as a source of thrombin in the nervous system. These findings indicated that dual activation mechanisms, factor X activation and subsequent prothrombin activation, occur on neuronal cell surfaces.

In conclusion, we have demonstrated in this study that two membrane-associated procoagulant activities, factor X activator and the subsequent prothrombinase complex assembly, generate thrombin on extravascular cells such as fibroblasts, and glial and neuronal cells. This is the first evidence of how cells in the extravascular regions can activate prothrombin to supply thrombin. Our observations strongly suggest that extravascular cells may also play an important role in regulating thrombin generation.

We are grateful to Dr. Yongchol Shin for the helpful suggestions. We would also like to thank Emi Yokota and Satsuki Hori for their technical assistance.

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