Studies on the Different Modes of Action of the Anticoagulant Protease Inhibitors DX-9065a and Argatroban

II. EFFECTS ON FIBRINOLYSIS*

The accompanying paper (Nagashima, H. (2002) J. Biol. Chem. 277, 50439–50444) has demonstrated that argatroban can yield a stronger inhibitory effect on thrombin generation than DX-9065a during extrinsic pathway-stimulated human plasma coagulation, while these anticoagulant compounds have comparable abilities to prolong clot time. Since thrombin generation is known to be an important determinant for fibrinolytic resistance of clots formed during coagulation, the two compounds are compared by tissue plasminogen activator-induced clot lysis assays. The results demonstrated that, in the presence of thrombomodulin, argatroban dose dependently accelerated fibrinolysis of the clots, whereas DX-9065a did not. The activation of thrombin activatable fibrinolysis inhibitor (TAFI) determined in separate assays reflected the differential influence on thrombin generation by these compounds. Moreover, TAFI activation correlated closely with the fibrinolytic resistance observed during tissue plasminogen activator-induced clot lysis. This study demonstrates the differential effects of DX-9065a and argatroban on thrombin generation, which in turn results in a differential acceleration of fibrinolysis as well as TAFI activation in the clots formed under the influence of these compounds. The data implicate a possible difference in the antifibrinolytic properties of clots formed during treatment with these compounds.

Thrombin plays a central role in thrombosis and hemostasis. While only a small amount of prothrombin is activated at the moment of clotting, the remaining prothrombin is activated predominantly after clot formation (1). These observations are consistent with the recently proposed notion (2) that activation of the extrinsic pathway is responsible for the rapid generation of a small amount of thrombin, which is sufficient for clot formation; however, activation of the intrinsic pathway is responsible for a massive production of thrombin mostly after clot formation, which effectively augments the antifibrinolytic properties of the clot. Such an intrinsic pathway-mediated thrombin burst is critically dependent on a feedback activation mechanism mediated by thrombin-catalyzed activation of factor XI (3, 4).

TAFI1 is a zymogen that plays a key role in the regulation of fibrinolysis. During coagulation, TAFI is proteolytically activated by thrombin in a TM-dependent manner to its active form, TAFIa, which inhibits fibrinolysis by removing the carboxy-terminal lysine and arginine residues from partially degraded fibrin (5, 6). As activation of TAFI requires exposure to relatively high concentrations of thrombin, the intrinsic pathway-mediated thrombin generation plays a pivotal role in the regulation of fibrinolysis (7).

The accompanying paper (8) demonstrates the differential actions on thrombin generation by two different anticoagulant protease inhibitors DX-9065a (9) and argatroban (10). Both theoretical and experimental approaches (8) revealed that the thrombin inhibitor, argatroban, yielded a stronger inhibitory effect on thrombin generation than the fXa inhibitor, DX-9065a, while the two inhibitors prolonged clot time to a similar extent when coagulation was triggered by the extrinsic pathway stimuli of the same intensity. Since thrombin generation is known to be an important determinant of fibrinolytic resistance in clots formed during coagulation, the two compounds were compared by tPA-induced clot lysis assays. The results presented in this paper demonstrate that the two anticoagulant protease inhibitors exert differential effects on TAFI activation, thereby, differentially affecting fibrinolytic resistance of the clots formed under the influence of these inhibitors.

EXPERIMENTAL PROCEDURES

Materials—DX-9065a was obtained from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). Argatroban was obtained from Mitsubishi Pharma Corporation (Tokyo, Japan). Human thrombin was purchased from Calbiochem (California, San Diego, CA). Bovine albumin and PPACK were from Sigma. Human recombinant thromboplastin (Recombiplastin) was from Instrumentation Laboratory (Barcelona, Spain). Human recombinant soluble TM (hereinafter, simply designated as TM) was from American Diagnostica Inc. (Greenwich, CT). tPA (alteplase) was from Mitsubishi Pharma Corporation (Tokyo, Japan). Z-GGR-AMC (Z-Gly-Gly-Arg-aminomethylcoumarin) and AMC (7-amino-4-methylcoumarin) were from Bachem (Bubendorf, Switzerland). Pooled human citrated plasma was prepared as described under “Experimental Procedures” in the accompanying paper (8), distributed into plastic tubes, and stored at –80 °C until use. Analysis of tPA-induced Clot Lysis—Effects on fibrinolytic resistance were evaluated in a microtiter plate (96-well flat-bottom plate, Corning) by the following assay procedures. To 15 μl (0.05 volume of the total) of the test compound solutions in a microtiter well was added 255 μl of human citrated plasma supplemented with or without TM. The well was designated as the first well in the sample preparation procedures. The final concentration of TM added was 10 nM. Discrete drops of tPA and thromboplastin solutions, 13 μl each, were

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placed on the bottom surface of another well (designated as the second well) on the same microtiter plate. The final concentration of tPA added was 480 IU/ml. After being preincubated for 3 min at 37 °C, a 234/H9262 lo f inhibitor-plasma portion of the reaction mixture was taken from the first wells with a 12-channel pipette and transferred to the second wells, in which all of the solutions were mixed instantaneously. Immediately after being mixed by pipetting, 200 H9262 l of the solution was taken from each of the second wells and gently transferred to another vacant well (designated as the third well). This additional transfer procedure was effective in removing bubbles, which were frequently formed during sample preparation and which might otherwise cause erroneous signals during subsequent measurements. Instantaneous mixing of the final solution avoided premature contact of tPA with other components of the mixture. The microplate was then read every 30 s for 3.5 h with a photometric microplate reader (Spectra Max 190, at 405 nm) thermoregulated at 37 °C.

Determination of Clot Time, Clot Lysis Time, and Clot-to-lysis Time—In the current study, clot time is defined as the time at which the solution turbidity increases over its half-maximal values after coagulation is triggered by thromboplastin. Similarly, in the clot lysis assay,
rather than an end-point assay was carried out, whereby thrombin
activity kit from American Diagnostica Inc. according to the manufac-
the half-maximal values because of fibrinolysis after clot formation. Thus clot time and clot lysis time were determined from
turbidity measured at a wavelength of 405 nm. Since TM is
the clot lysis time is defined as the time at which the solution turbidity
depressed Thrombin Potential (ETP) from the thrombin generation
and dissolution of the clots were monitored by solution
turbidity decreases below its half-maximal values because of fibrinolysis after
clot formation. Thus clot time and clot lysis time were determined from
data points in the ascending and descending phase, respectively, in
each photometric recording from the clot lysis assays by using a linear interpolation method. The clot-to-lysis time is defined as the time
required for clot lysis after clot formation and is calculated as the
differences between clot time and clot lysis time. All of these calculations were carried out semiautomatically by the Excel spreadsheet
program (Microsoft).

**TAFI Activation Assays during Plasma Coagulation**—TAFIa activation
was assessed under the same experimental conditions as described above, except that the assay mixtures were made in 1.5-mL polypropylene tubes rather than microplate wells and tPA was omitted from
the final assay mixtures.

To 10 μL (0.05 volume) of inhibitor solution was added 180 μL (0.9 vol.)
of human citrated plasma supplemented with or without TM at a final
concentration of 10 mM. The solution was preincubated for 3 min at 37 °C. Coagulation was then triggered by addition of 10 μL (0.05 volume)
of the diluted thromboplastin solution as indicated. After incubation for
the required time interval, the reaction was terminated by addition of
50 μL of the stop solution containing 0.25 mM PPACK, 0.15 M NaCl, and
50 mM HEPES (pH 7.4) in an ice-cold water bath. The mixtures were then centrifuged at 25,000 × g for 15 min at 4 °C. Fractions of the
supernatant were analyzed for TAFIa activity as described below.

TAFIa activity was determined by the ACTICHROME plasma TAFI activity kit from American Diagnostica Inc. according to the manufacturer’s instructions with the following modifications: a kinetic assay
rather than an end-point assay was carried out, whereby thrombin cleavage of a chromogenic substrate was registered at 420 nm every 20 s
for 15 min in a thermoregulated photometric microplate reader (Spectra
Max 190, at 37 °C). This relatively short incubation period was chosen
to prevent inactivation of TAFIa, since TAFIa is known to be thermo-
labile when incubated at 37 °C (11).

For TAFI assays, coagulation was run singly, but the TAFIa activity
in the supernatants from the coagulating mixtures was determined in
duplicate. The results are indicated as averages of the duplicate values.

**Results**

**Effects on Clot-to-Lysis Time and Plasma TAFI Activity**—Since thrombin generation affects the fibrinolytic resistance of
clots through TAFI activation, it has been predicted that DX-
9065a and argatroban can exert differential effects not only on
thrombin generation, but also on TAFI activation and fibrinol-
ysis. To study effects on fibrinolysis, the two compounds were compared by clot lysis assays, in which thromboplastin-induced
clotting in the plasma mixtures was followed by tPA-mediated fibrinolysis of the clot. Human recombinant tPA was added to the
human citrated plasma at a concentration of 480 IU/ml in the final coagulation mixtures together with thromboplastin to
trigger both coagulation and fibrinolysis. The kinetics of for-
mation and dissolution of the clots were monitored by solution

**Effects of TM and/or Thromboplastin Concentration on TAFI activation.** Coagulation was triggered at a high or low
concentration of thromboplastin with or without TM. TAFIa was determined after the reaction was quenched with PPACK. TM was added at a final
concentration of 10 nM. The solution was preincubated for 3 min at 37 °C,
and was then centrifuged at 23,000 ×g for 15 min at 4 °C. The mixtures were

**Fig. 5.** Effects of TM and/or thromboplastin concentration on
TAFI activation. Coagulation was triggered at a high or low
concentration of thromboplastin with or without TM. TAFIa was determined after the reaction was quenched with PPACK. TM was added at a final
concentration of 10 nM. The solution was preincubated for 3 min at 37 °C,
and was then centrifuged at 23,000 ×g for 15 min at 4 °C. The mixtures were

**Fig. 4.** Differential effects of DX-9065a and argatroban on tPA-induced clot lysis in the extrinsically triggered human plasma. Clot
time (A) and clot-to-lysis time (B) were examined with or without TM, and at a high (∗1/10) or low (∗1/100) concentration of thromboplastin. The
bar graphs indicate means ± S.E. (n = 5–6).

**RESULTS**

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mation and dissolution of the clots were monitored by solution

**Effects of TM and/or TF Concentrations on Fibrinolysis**—To
determine whether TAFI activation and its antifibrinolytic actions
were indeed altered by the addition of TM and by changing
TF concentrations, clot-to-lysis time was examined with or
without TM at high or low thromboplastin concentrations. The
thromboplastin samples used to trigger plasma coagulation in
these experiments were prepared by diluting its stock solution
with 0.5 M CaCl₂ at a dilution rate of 1/10 or 1/100, thereby
yielding high or low TF concentration samples, respectively. Fig. 3 shows examples of photometric tracings from such clot
lysis assays, and Fig. 4 summarizes the results from these experiments. As shown in Fig. 3, A–D, DX-9065a and argatroban prolonged clot time by the same extent under each experimental condition; neither the alteration in TF concentration nor the addition of TM altered the relative effect on clot time by these compounds; however, they did affect fibrinolysis of the clots differently. Argatroban strongly accelerated fibrinolysis in the presence of TM irrespective of the concentration of thromboplastin. In the absence of TM, however, such a profibrinolytic effect of argatroban was less evident, especially at the lower TF concentration (Figs. 3, A and B and 4B). These results suggest that not only TM concentration but also TF concentration is an important determinant for the profibrinolytic effects observed with argatroban.

Fig. 5 shows a time course of TAFI activation in the absence of the anticoagulant inhibitors, representing the effect of the addition of TM and/or the effect of varying TF concentrations. TM greatly enhanced TAFI activation at both high and low thromboplastin concentrations. Thus, there was a good correlation between the observed TAFI activation and antifibrinolytic effects of TM. However, the effect of TF concentrations on TAFI activation was less evident than did TM.

TM is an endothelial receptor, which after forming the complex with thrombin can accelerate not only TAFI activation but also protein C activation. It has been reported that, at a TM concentration higher than 5 nM, the protein C activation may in turn reduce the rate of thrombin generation and eventually reduce TAFI activation (13). To confirm such an effect of TM on thrombin generation, DX-9065a and argatroban were compared with (10 nM) and without added TM in the thrombin generation assays (Fig. 6). There was a reduction not only in ETP but also in the degree to which the two compounds differ in the ability to reduce ETP when TM was added to the plasma mixtures. Nevertheless, even with the added TM, the differential effects on ETP between the two inhibitors were still apparent.

**DISCUSSION**

TAFI is activated by relatively high concentrations of thrombin generated via an intrinsic pathway-mediated secondary burst of thrombin generation (2). The present study examined the effects of DX-9065a and argatroban on TAFI activation in relation to the antifibrinolytic resistance of a clot formed during coagulation. The clot-to-lysis time, which was determined as the time required for clot dissolution after clot formation, was significantly shortened by argatroban but not by DX-9065a. The observed inhibitory effect of argatroban on TAFI activation (Fig. 2B) can provide a clear explanation for why this compound accelerates dissolution of clots during tPA-induced fibrinolysis (Fig. 1B).

It has been reported previously that TM can accelerate thrombin-mediated TAFI activation by more than 1000-fold (12). Fig. 4 demonstrates such a TM-dependent activation of TAFI during plasma coagulation. Addition of TM at a concentration of 10 nM greatly enhanced TAFI activation in plasma at both high and low TF concentrations. However, when TM was not added to the plasma mixtures TAFI activity increased only marginally, and this could only be observed at the higher TF concentration (Fig. 5, open circles). Although assessed separately, TAFI activation (Fig. 5) and the corresponding clot-to-lysis times (indicated as the values without inhibitors in Fig. 4B) showed a correlation such that an increase in TAFI activity was associated with increased clot-to-lysis times. The two compounds showed only a marginal difference in clot-to-lysis time when they were compared without TM at the lower TF concentration (Figs. 3A and 4B), the experimental conditions corresponding to those when TAFI was not activated to a significant extent (Fig. 5, open triangles). These observations are consistent with a view that the two compounds can produce differential fibrinolysis mainly through TAFI activation.

As shown in Fig. 6, DX-9065a and argatroban differentially affected thrombin generation both in the absence and presence of TM, although the differences observed with the added TM were somewhat smaller than those seen without. Similarly, the relationship seen in the ETP-clot time curves obtained with varying tissue factor concentrations (see Fig. 4 in the accompanying paper (8)) shows that the differences between the two compounds were less evident at lower tissue factor concentrations and, therefore, with smaller ETP values. However, the differential effects on ETP were still sufficiently evident with added TM (Fig. 6B), in agreement with those effects observed on fibrinolysis as well as on TAFI activation in the presence of TM. Thus these observations support the view that thrombin generation is an important determinant for TAFI-mediated regulation of fibrinolysis.

By virtue of the activation mechanism of TAFI by thrombin generation, one may choose two different approaches for anti-coagulation therapy. One approach utilizes the anticoagulants,
such as argatroban, that can accomplish not only anticoagulation but also suppression of TAFI activation leading to accelerated fibrinolysis of the clot formed during drug treatment. An alternative approach utilizes other anticoagulants, such as DX-9065a, that can accomplish anticoagulation with less effects on fibrinolysis as well as on TAFI activation. At least in theory, the former approach might serve to facilitate fibrinolytic therapy, whereas the latter approach would be associated with a reduced risk for bleeding complications. Earlier studies also showed that argatroban accelerates clot lysis both in vitro (14) and in vivo (14, 15). It seems probable that argatroban may result in clots that are more susceptible to fibrinolysis and, thereby, may be beneficial for fibrinolytic therapy. However, bleeding could be a serious drug-related complication associated with such a combination therapy.

Previous data on animal experiments have demonstrated that synthetic, low molecular weight FXa inhibitors, such as DX-9065a, can exert antithrombotic effects with little prolongation of a bleeding time assay compared with the other currently used anticoagulants (16–19). It seems possible that TAFI activation might contribute to clot protection particularly when blood is exposed to high concentrations of thrombomodulin and/or TF in the damaged tissues; situations that are conceivable in bleeding time assays. However, this explanation presently awaits further verification. So far there have been few studies that demonstrate the relationship between fibrinolysis and bleeding time assays. Thus further studies are needed to determine whether the TAFI-mediated inhibition of fibrinolysis is a mechanism for the lower bleeding times observed with the synthetic FXa inhibitors.

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