therapy, respectively. Minimal residual disease (MRD) in the graft collection was evaluated using FCM analysis of $CD20^+$ cells, Southern blot analysis, and polymerase chain reaction (PCR) analysis of immunoglobulin heavy chain (IgH) rearrangement. Primers for the PCR assay were made for each patient, according to their lymphoma-cell specific base sequence of the third complementarity-determining region (CDR-III) of IgH. PCR assays were carried out at Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan.

Statistical methods

Statistical comparisons were made between the CHOP and CHOP-R groups. The Mann–Whitney *U*-test was used for comparisons regarding $CD34^+$ cell mobilization, CFU-GM, and BFU-E in PB samples or apheresis products. Comparisons of the number of $CD20^+$ cells in PB before and after rituximab administration were made using the Wilcoxon signed-ranks test. *P*-values <0.05 were considered significant.

Results

Mobilization of circulating $CD34^+$ cells (Table 2)

Mobilization kinetics of PB $CD34^+$ cells in the CHOP and CHOP-R groups are shown in Table 2. The median PB $CD34^+$ cell count on day 14 of CHOP therapy was 27.0×10^6/l, and 39.1×10^6/l in the CHOP and CHOP-R groups, respectively. These numbers of mobilized PB $CD34^+$ cells were sufficient for leukapheresis. Rituximab does not seem to adversely affect mobilization kinetics.

PBSC collection (Table 3)

PBSC collection data are summarized in Table 3. The total doses of G-CSF given for mobilization were not significantly different between groups. The target $CD34^+$ cell number was collected from all eight patients in the CHOP-R group in a single leukapheresis. For three of eight patients in the CHOP group, two leukaphereses were required. The median $CD34^+$ cells yield in the graft collection was 2.29×10^6/kg in patients on CHOP-R compared with 2.90×10^6/kg for the CHOP group. Colony assays of CFU-GM and BFU-E from stem cell grafts in the CHOP-R group are not significantly different from those assayed in the CHOP group.

Engraftment (Table 4)

Engraftment data are summarized in Table 4. Eight patients in the CHOP-R group achieved neutrophil engraftment at a median of 10 (range 9–11) days and platelet transfusion independence at a median of 15.5 (range 10–30) days. There was no significant difference in time to neutrophil engraftment or platelet independence and transfusion requirements among patients mobilized with or without rituximab.

Table 2 Mobilization kinetics of peripheral blood $CD34^+$ cells

	CHOP (n=8)	*CHOP-R (n=8)*
$CD34^+$ cells ($\times 10^6$/l)		
Day 12		
Range	0.7–24.0	0.8–40.6
Median	2.0	9.9
Day 13		
Range	1.8–115.0	3.0–112.1
Median	14.4	26.0
Day14		
Range	10.9–77.9	18.4–115.6
Median	27.0	39.1

Table 3 PBSC data

	CHOP (n=8)	*CHOP-R (n=8)*
Total doses of G-CSF given for mobilization (μg)		
Range	1400–2100	1150–2500
Median	1750	1725
No. of days of apheresis required for collection		
1	5	8
2	3	0
$CD34+$ graft yield ($\times 10^6$/kg)		
Range	1.58–5.58	1.94–5.12
Median	2.90	2.29
CFU-GM ($\times 10^5$/kg)		
Range	1.85–10.01	2.73–12.35
Median	4.63	7.14
BFU-E ($\times 10^5$/kg)		
Range	5.19–12.44	3.28–9.29
Median	7.65	7.77

Table 4 Engraftment times and transfusion requirement

	CHOP (n=8)	*CHOP-R (n=8)*
No. of days to neutrophil engraftment (neutrophil count $>0.5 \times 10^9$/l)		
Range	9–10	9–11
Median	10	10
No. of days to platelet independence (platelet count $>50 \times 10^9$/l)		
Range	11–31	10–30
Median	14	15.5
Red blood cell transfusion (unit)		
Range	0–10	4–12
Median	6	6
Platelet transfusion (unit)		
Range	40–160	80–160
Median	67.5	95

Toxicity

There was no significant toxicity during mobilization with CHOP-R. Five patients experienced grade 1 fever. One patient experienced mild tightness sensation in the throat and another patient experienced eyelid edema, which resolved on decreasing the infusion rate of rituximab.

Purging efficiency with rituximab

$CD20^+$ cells in the PB after rituximab (day 14 after CHOP) decreased significantly ($P = 0.018$) compared with findings before the use of rituximab (day 12 after CHOP). In all patients on CHOP-R, $CD20^+$ cells and IgH rearrangement were undetectable in the graft collection by FCM and Southern blot analysis, respectively. For eight patients receiving CHOP-R, two were positive for IgH rearrangement on PCR analysis of the graft collection.

Discussion

Rituximab caused a rapid depletion of PB B-cells.[7] Recent studies show that rituximab has the potential, in conjunction with chemotherapy, to allow harvesting of tumor-free progenitor cells for patients with B-cell NHL.[8–10] However, reports of its effects on stem cell mobilization kinetics and engraftment are few.[19] Our study shows that stem cell mobilization with CHOP-R therapy is safe and does not adversely affect the number or function of stem cells, compared with mobilization with CHOP therapy without rituximab.

It is important to know the stem cell mobilization kinetics in the course of a mobilization regimen to set up the day of leukapheresis in advance. In almost all reported mobilization regimens, the day of leukapheresis was determined by monitoring PB $CD34^+$ cells number, neutrophils or platelet recovery in PB; so it differed with each case.[8–10] Buckstein *et al*[19] reported the stem cell mobilization kinetics after 5 consecutive days of administration of G-CSF, and they started leukapheresis from day 5. They collected sufficient number of stem cells, but some patients needed two or three leukaphereses to meet the target number of stem cells. In our study, a single leukapheresis (day 14 after CHOP) was sufficient to meet the target number of stem cells in all eight patients mobilized while on the CHOP-R regimen. This makes scheduled leukapheresis possible; one can decide the day of leukapheresis before starting the mobilization regimen. Furthermore, CHOP with rituximab has become one of the standard regimens for patients with B-cell NHL; so mobilization with CHOP-R will make leukapheresis feasible during lymphoma therapy, without any additional regimen for mobilization.

Our data show that $CD20^+$ cells and IgH rearrangement in PBSC collections were undetectable by FCM and Southern blot analysis, respectively, in all eight patients receiving CHOP-R therapy. However, two of eight PBSC collections were not free of lymphoma contamination determined by PCR. It is not clear whether these positive grafts contained viable or nonviable lymphoma cells undergoing apoptosis, because rituximab was administered only 2 days before the stem cell collection. In fact, IgH rearrangement of bone marrow and PB mononuclear cells became undetectable by PCR after transplantation in all patients on CHOP-R therapy, including those reinfused with a PCR-positive graft. Further studies are needed to clarify the optimum timing and ideal number of rituximab administrations before PBSC mobilization.

$CD34^+$ cell-positive selection can also eliminate tumor cells from graft collections.[6] However, loss of stem cells during selection procedures and a higher rate of infectious complications after transplantation are major problems with the method.[20] With *in vivo* purging with rituximab, stem cell loss cannot occur. It is not clear whether infectious complications increase or not after transplantation using a graft mobilized with rituximab.

Our study population was small and there are some differences in patient demographics. In particular, 3/4 of the CHOP-R group had bone marrow involvement by lymphoma cells compared to 1/8 in the CHOP group. The presence of bone marrow involvement is disadvantageous for mobilizing PBSC. However, sufficient PBSC were collected from all eight patients in the CHOP-R group, and they did not differ significantly from those collected in the CHOP group. Therefore, at least, rituximab does not seem to adversely affect PBSC mobilization.

In conclusion, CHOP-R therapy can be safely and effectively used in the mobilization phase of PBSC collection without excessive clinical toxicity or deleterious effects on PBSC mobilization kinetics or engraftment times, and hence scheduled leukapheresis is feasible. The efficacy of purging on eliminating contaminating lymphoma cells in the graft with CHOP-R therapy and improving the outcome of patients undergoing autologous transplantation are subjects of ongoing study.

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Chloride Channel Blockade Attenuates the Effect of Angiotensin II on Tubuloglomerular Feedback in WKY but not Spontaneously Hypertensive Rats

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Key Words
IAA-94 · Tubuloglomerular feedback · Micropuncture · Interstitial perfusion

Abstract
Recent studies have shown that calcium-dependent chloride channels may play a crucial role in the modulation of the vascular effects of angiotensin II (ANG II). Thus, alterations in the function of these channels may be responsible for the enhanced renal vasoconstrictor and tubuloglomerular feedback (TGF) response to ANG II in spontaneously hypertensive rats (SHR). We investigated the effect of the calcium-dependent chloride channel blocker IAA-94 on renal hemodynamics and TGF responses. The renal interstitium was perfused with control solution, with ANG II, and with both ANG II and IAA-94. In Wistar Kyoto rats (WKY), perfusion with ANG II significantly increased renal vascular resistance (RVR), but the effect was significantly attenuated by perfusion with ANG II/IAA-94. In SHR, ANG II caused a significant elevation of RVR that was not altered by the simultaneous infusion of IAA-94. Proximal tubular stop flow pressure (P_{sf}) was monitored during perfusion of peritubular capillaries with control solution, and subsequently with IAA-94, ANG II or both ANG II and IAA-94. TGF response magnitude of WKY rats was significantly augmented with ANG II, and this effect was suppressed by perfusion with ANG II /IAA-94. However, in SHR peritubular perfusion with ANG II/IAA-94 did not suppress the TGF response. We conclude that chloride channels susceptible to IAA-94 may play a significant role in modulating the effects of ANG II on renal hemodynamics, and that this modulation is absent in SHR.

Introduction

There is considerable evidence in support of the notion that chloride (Cl) efflux is a crucial event in the agonist-induced depolarization of vascular smooth muscle cells [1, 2]. For example, a patch clamp analysis in rabbit pulmonary artery smooth muscle cells has shown that a calcium-activated chloride current contributes importantly to regenerative depolarization [3], and that the Cl channel blocker niflumic acid reduced serotonin- and phenylephrine-induced depolarization and contraction in the rat [4].

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These studies confirm earlier experiments in freshly isolated smooth muscle cells from dissected renal interlobar and arcuate arteries showing the presence of calcium-activated Cl currents, and their participation in endothelin-induced depolarization [5]. Studies in cultured mesangial cells have revealed that calcium-dependent chloride channels mediate the depolarization and contraction in response to angiotensin II (ANG II) and vasopressin [6, 7]. Finally, in the isolated perfused hydronephrotic kidney and in the perfused juxtamedullary nephron preparation blockade of Cl channels with IAA-94 largely prevented the constrictor effect of ANG II in afferent arterioles without altering the response to an increase in perfusion pressure (myogenic response) or to KCl [8, 9].

The present studies were performed to assess whether a role of calcium-activated Cl channels in angiotensin II-induced renal vascular vasoconstriction can be demonstrated at the level of the intact kidney. Furthermore, since ANG II has been consistently shown to augment tubuloglomerular feedback (TGF) responses, it seems conceivable that calcium-activated Cl channels play a role in the local control of afferent arteriolar tone exerted by TGF [10]. Preliminary studies have in fact confirmed this notion [11].

Evidence in mesangial cells has shown that the increase in cytosolic calcium concentration as well as the associated contraction of this cell type that is normally seen with a reduction in medium Cl concentration is greatly attenuated in cells derived from spontaneously hypertensive rats (SHR) [12]. One interpretation of these observations would be that calcium-activated Cl channels function abnormally in SHR.

Thus, the specific aims of the present experiments were (a) to determine the effect of an intrarenal administration of the Cl channel blocker IAA-94 on basal and angiotensin II-dependent renal vascular resistance in Wistar Kyoto rats (WKY) and SHR, and (b) to determine the effect of the Cl channel blocker on basal and angiotensin II-enhanced TGF responses in WKY and SHR. Our results indicate that IAA-94 blocked the vasoconstrictor effect of angiotensin II at the level of the intact kidney and at the level of the juxtaglomerular apparatus in Wistar Kyoto rats. However, IAA-94 did not prevent the vasoconstrictor and TGF-enhancing actions of angiotensin II in SHR suggesting that the expression or regulation of calcium-activated Cl channels is abnormal in SHR.

Methods and Materials

Experiments were performed in male normotensive Wistar Kyoto control rats (WKY; WKY/Izm; Sankyo Labo, Tokyo, Japan) and in spontaneously hypertensive rats (SHR; SHR/Izm; Sankyo Labo) in the 15- to 19-week age range. Animals were allowed free access to tap water and conventional rat chow containing 0.2% NaCl (MF®; Oriental Kobo, Tokyo, Japan) until the experiment. Body weights at the time of the studies were between 240 and 320 g. Rats were anesthetized with an i.p. injection of thiobutabarbiturate sodium (Inactin®; Reseach Biochemicals, Natick, Mass., USA; 100 mg/kg), and placed on a feedback-controlled operating table maintaining body temperature at 37°C. After tracheostomy to allow free spontaneous breathing, a catheter (PE-50) was inserted from the right femoral artery into the abdominal aorta to monitor mean arterial pressure (MAP) by using a pressure transducer (Nihon Koden, Tokyo, Japan). MAP in the abdominal aorta was regarded as equivalent to renal perfusion pressure. The femoral vein was cannulated with two PE-50 catheters, one for constant infusion of isotonic (0.9%) saline at 1 ml/h × 100 g body weight throughout the experiment, and the other for the administration of drugs. The left kidney was exposed through a flank incision and placed in a Lucite cup mounted on the operating table. The ureter was cannulated with PE-50 tubing for free egress of urine. On completion of surgery, 1 ml of littermate donor rat plasma was infused intravenously, and a 30–60-min equilibration period was allowed before initiating measurements. Experiments were done only if MAP remained stable and was >120 mm Hg in WKY, and >170 mm Hg in SHR at the end of the equilibration period. All data were analyzed on a personal computer using MacLab software.

To measure renal blood flow (RBF), the left renal artery was separated from the renal vein and fitted with an electromagnetic flow probe connected to a flow meter (Nihon Koden). Both MAP and RBF signals were passed to an A/D converter to obtain simultaneous on-line recordings throughout the experiments (MacLab, Melbourne, Australia).

To avoid major systemic effects, substances were infused into the renal cortical interstitium using the method described by Mattson et al. [13]. A 28-gauge needle connected to an extended piece of PE-10 tubing was inserted into the cortex from the lateral border of the kidney for about 2 mm and fixed against the kidney surface with tissue glue. MAP and RBF were monitored during perfusion. Infusion solutions were prepared to contain ANG II (10^{-7}, 10^{-6}, or 10^{-5} *M*), or a mixture of ANG II in the same concentrations and IAA-94 (300 μ*M*). On the basis of previous studies we considered a concentration of 300 μ*M* IAA-94 in the peritubular perfusate sufficient to completely block Ca-activated Cl channels [13]. The area of perfusion was identified by injecting solutions colored with lissamine green. Renal perfusion rate in all experiments was 1 ml/h using a precision pump (CFV-320; Nihon Koden). All solutions were freshly prepared immediately before use. The control solution contained the IAA-94 solvent dimethyl sulfoxide (DMSO; Sigma®, St Louis, Mo., USA; 25 mg/ml) in Ringer's solution. R(+)-methylindazone indanyloxy acetic acid 94 (IAA-94; Research Biochemicals) was dissolved in DMSO solution. Angiotensin II (ANG II; Sigma®) was dissolved in control solution.

The second series of experiments was done to assess the effect of chloride channel blockade on stop-flow pressure (P_{sf}) without alterations in systemic conditions. Micropuncture procedures and measurements were started after the stabilization of blood pressure, usually within 15–30 min after the completion of each treatment.

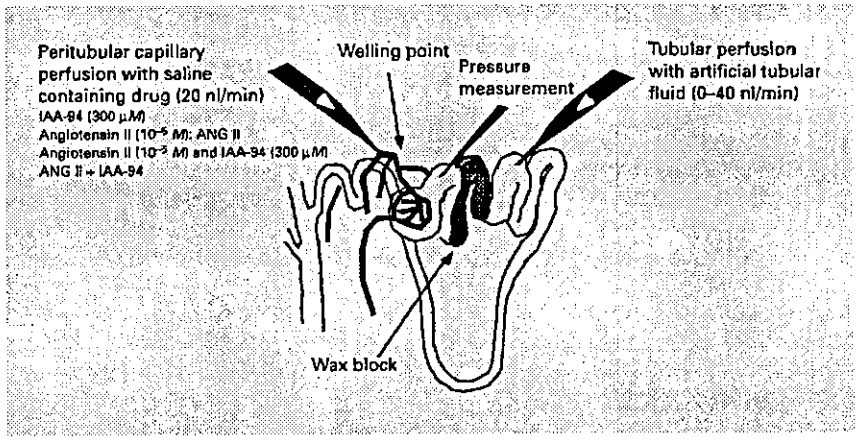

Fig. 1. Micropuncture procedure used to examine the effect of loop of Henle perfusion on P_{sf} during peritubular capillary perfusion from the welling point.

The animals were prepared as above. The left kidney was fixed in a Lucite cup with 2% agar, and the kidney surface was immersed in warm saline (37 °C). Tubules for study were identified by injecting a small amount of saline colored with lissamine green into a randomly chosen proximal tubular segment with a micropipette. The middle proximal segments were completely blocked by solid wax (Merck, Darmstadt, Germany) injected by a hydraulic pressure system (Effenberger, Attel, Germany). The most proximal segment upstream from the wax block was gently punctured with a micropipette (OD, 2 μm) filled with 2 *M* NaCl solution stained with lissamine green and mounted in a servo-null micropressure system (900A; WPI, Sarasota, Fla., USA) to P_{sf}. P_{sf} measured in an early proximal segment is a good index of P_{gc}, but the absolute value of P_{sf} decreases with distance from the glomerulus [14]. To avoid length-dependent variations in P_{sf}, we compared changes of P_{sf} in each nephron segment in different experimental conditions requiring repeated punctures of the early proximal segment. Segments revealing any leakage of tinted solution were discarded.

To determine the role of calcium-activated Cl channels without alterations in MAP, the effect of peritubular infusion of ANG II on TGF responses was determined in the absence and presence of the Cl channel blocker IAA-94. The welling point near the obstructed nephron was gently punctured with a pipette with a tip diameter of 8 μm attached to a microperfusion pump system (Effenberger). The perfusion pipette was filled with lissamine green colored control solution containing either ANG II (10^{-7} *M*), IAA-94 (300 μ*M*), or a mixture of ANG II (10^{-7} *M*) and IAA-94 (300 μ*M*). While providing a route for local application of agents, this technique is not well suited to determine the resulting concentration in the surrounding interstitial space [15]. We used the same concentrations of drugs, and fixed the capillary perfusion rate in all experiments at 20 nl/min, a rate reported to not obstruct normal peritubular blood flow [16]. A perfusion procedure was judged satisfactory if the interstitium surrounding the obstructed proximal segment was colored. P_{sf} was continuously monitored during the peritubular capillary perfusion. P_{sf} responses to a graded increase in loop perfusion rate (0, 10, 20, 30 and 40 nl/min) were determined during control, experimental and recovery in three groups of nephrons: control peritubular infusion followed by ANG II and recovery, control infusion followed by IAA-94 and recovery, and control infusion followed by ANG II/IAA-94 and recovery. The tubular perfusion solution was an artificial tubular fluid (ATF) containing 140 m*M* Na, 140 m*M* Cl, 4 m*M* K, 4 m*M* Ca, 8 m*M* HCO3, and 7.5 m*M* urea.

Statistical Analysis

All values are means ± SE. Analysis of variance (ANOVA) was performed to test for statistical significance of differences among the values observed in each treatment group. Data obtained from the same kidney or from a single nephron were compared with Student's paired t test. $p < 0.05$ was considered to have statistical significance.

Results

Renal Hemodynamic Study

The effect of renal interstitial infusion of ANG II at three different concentrations in the absence and presence of IAA-94 is depicted in figure 2 for the Wistar Kyoto control group of rats. While ANG II at 10^{-7} and 10^{-6} *M* had no detectable effects, interstitial infusion of ANG II at 10^{-5} *M* caused a significant elevation of mean arterial pressure (MAP) from 110 ± 3.8 to 126 ± 4.7 mm Hg. At the same time, renal blood flow (RBF) was decreased from 5.1 ± 0.2 to 4.5 ± 0.2 ml/min, and renal vascular resistance (RVR) was elevated from 22.7 ± 1.0 to 28.5 ± 12 mm Hg × min/ml. In contrast, MAP, RBF, and RVR did not change significantly when ANG II at 10^{-5} *M* was administered together with IAA-94 (300 μ*M*).

The results of similar experiments performed in SHR are summarized in figure 3. Like in WKY rats, the intrarenal administration of ANG II at 10^{-5} *M* caused significant alterations of MAP, RBF, and RVR. However, in contrast to the normotensive animals, the inclusion of IAA-94 in the infusion solution did not attenuate the vascular actions of ANG II.

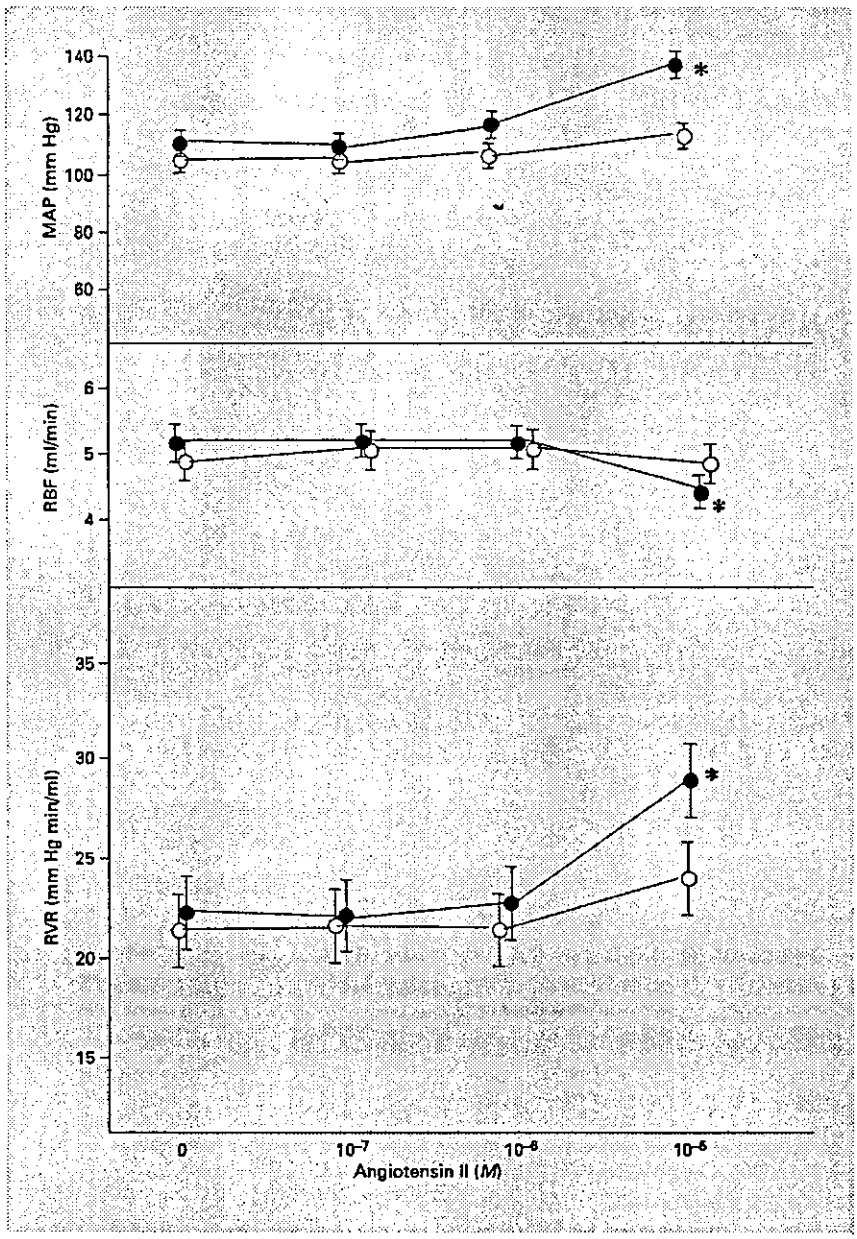

Fig. 2. Renal hemodynamics of WKY rats during renal interstitial infusion at 1 ml/h of control solution (●) or control solution containing IAA-94 (30 μ*M*; ○). Abscissa indicates the concentration of angiotensin II added to the perfusate. * $p < 0.05$ vs. baseline.

Micropuncture Study

Table 1 summarizes the measurements of P_{sf} responses during peritubular infusion of ANG II, IAA-94, and their combination in Wistar-Kyoto normotensive and spontaneously hypertensive rats. Flow rate-dependent reductions of stop flow pressure in % of P_{sf} at zero loop flow are summarized in figure 4 for both strains of rats. MAP did not significantly change in any of the experimental groups. Similarly, P_{sf} responses were not significantly different during perfusion with the control solution (baseline phase in fig. 4). Peritubular perfusion with the IAA-94 containing solution did not measurably affect TGF responsiveness with P_{sf} falling 27.1 ± 1.9% with the control solution and 22.1 ± 4.7% with the IAA-94 solution. Peritubular perfusion with ANG II markedly enhanced the TGF response (50.8 ± 5.1%; $p < 0.05$ vs. corresponding

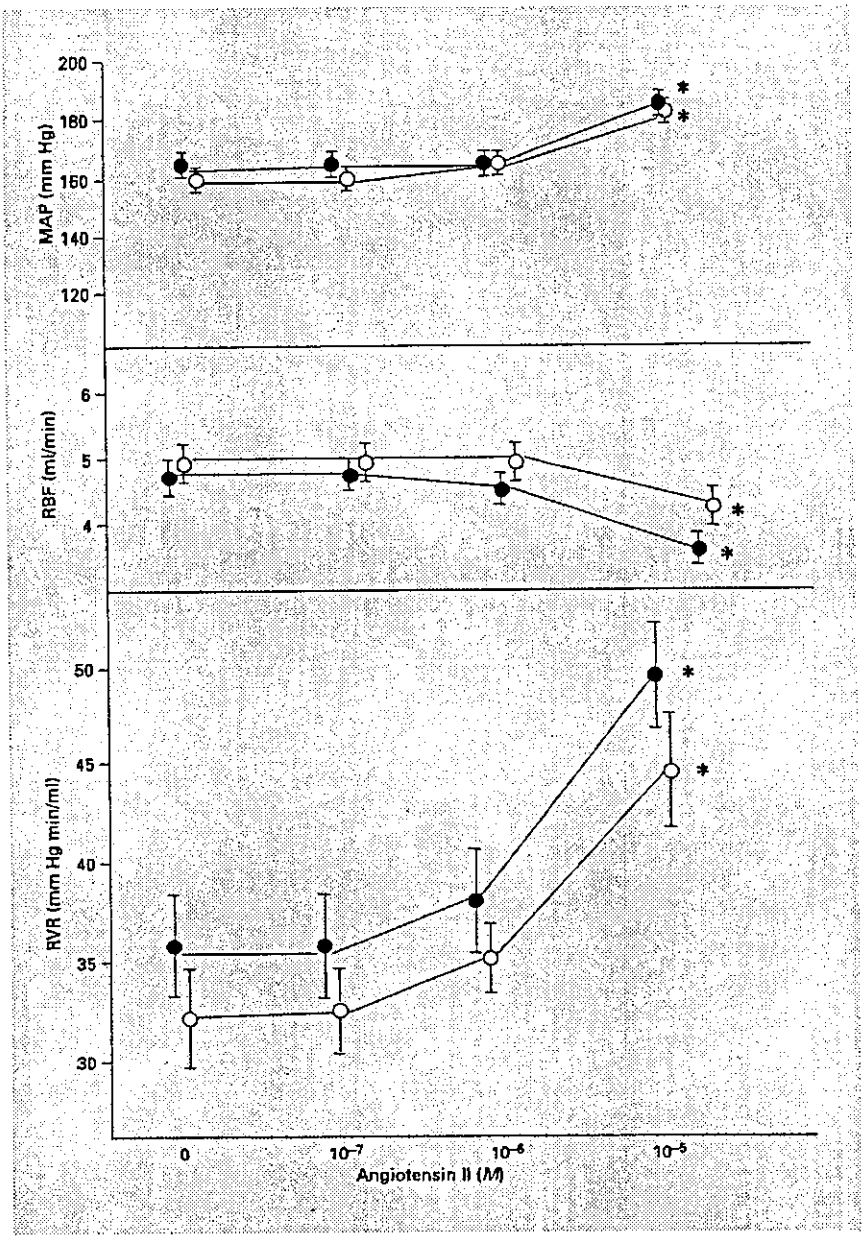

Fig. 3. Renal hemodynamics of spontaneously hypertensive rats during renal interstitial infusion at 1 ml/h of control solution (●) or control solution containing IAA-94 (30 μ*M*; ○). Abscissa indicates the concentration of angiotensin II added to the perfusate. * $p < 0.05$ vs. baseline.

baseline). This augmentation of the TGF response was fully prevented when ANG II was administered together with IAA-94 (26.4 ± 4.1%). Data obtained in SHR are included in table 3. TGF responses during perfusion with the control solution were similar to those observed in WKY. Again, peritubular perfusion with IAA-94 had no effect on TGF responsiveness, while infusion of ANG II caused a significant enhancement of TGF responses (45.5 ± 4.0%; $p < 0.05$ vs. corresponding baseline). However, in contrast to WKY, peritubular perfusion with ANG II in the presence of IAA-94 solution did not measurably suppress TGF response compared to infusion with ANG II alone (42.9 ± 4.7%; $p < 0.05$ vs. corresponding baseline).

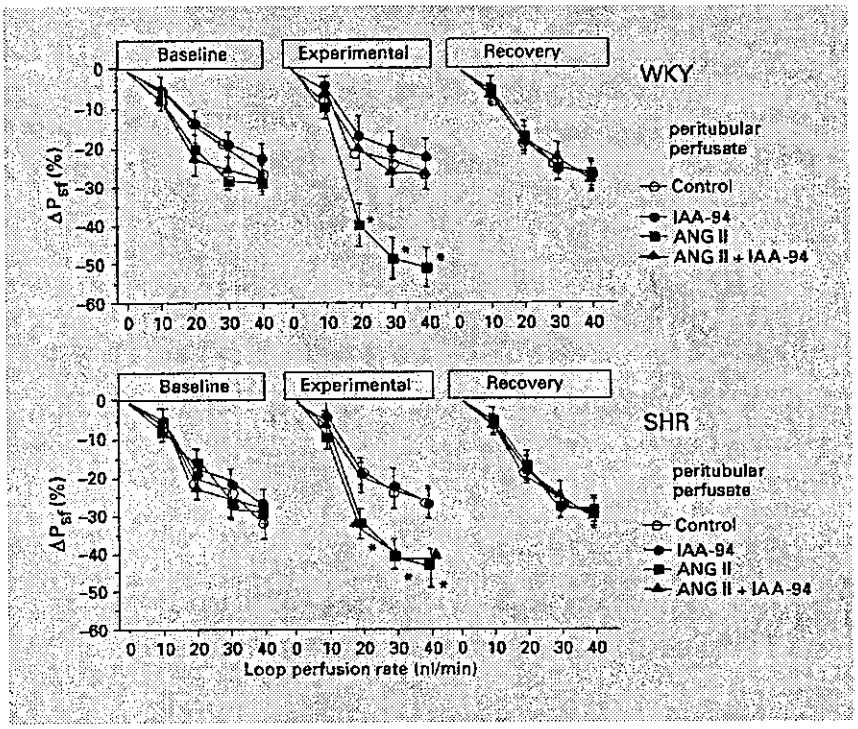

Fig. 4. Percent changes from the zero perfusion values (ΔP_{sf}) during perfusion of Henle's loop with artificial tubular fluid in WKY and SHR. In each nephron, P_{sf} responses were determined during peritubular capillary perfusion at 20 nl/min with control solution (baseline), a solution containing either IAA-94 (300 μM), angiotensin II (10^{-7} M), or IAA-94 and angiotensin II (experimental), and control solution (recovery). Abscissa indicates the rates of tubular perfusion.

Table 1. Effect of peritubular infusion of IAA-94 (300 μM), angiotensin II (ANG II, 10^{-7} M), and IAA-94 plus ANG II on stop flow pressure during loop perfusion at zero ($P_{sf,0}$) and 40 nl/min ($P_{sf,40}$) in WKY and SHR

	n	MAP mm Hg	$P_{sf,0}$ mm Hg	$P_{sf,40}$ mm Hg	$P_{sf,0}$ %
WKY					
Control	8	120.7±3.6	38.1±1.4	28.5±1.2	-27.1±1.9
IAA-94	6	119.0±3.2	36.3±1.7	26.7±2.2	-22.1±4.7
ANG II	8	119.0±3.5	38.2±2.9	19.2±2.7	-50.8±5.1*
ANG II + IAA-94	7	117.1±3.7	35.5±1.1	26.1±1.7	-26.4±4.1
SHR					
Control	8	171.8±4.4	40.1±2.9	29.7±2.1	-25.8±1.0
IAA-94	9	169.4±1.6	40.6±1.8	30.0±1.2	-25.9±2.7
ANG II	8	168.7±4.2	39.8±2.5	21.3±2.3	-45.5±4.0*
ANG II + IAA-94	9	167.4±3.6	42.4±3.2	23.2±0.8	-42.9±4.7*

Values are mean ± SE. The change of P_{sf} ($\%\Delta P_{sf,0}$) is given as the percent reduction of stop flow pressure caused by the 40-nl/min perfusion rate.

* $p < 0.05$ (for difference from control solution).

Discussion

The current findings demonstrate that the vasoconstrictor action of ANG II in the kidney depends in part on an activation of calcium-dependent Cl channels. The infusion of ANG II into the renal interstitium through an implanted catheter caused an increase in renal vascular resistance that was blocked by co-infusion of the Cl channel blocker IAA-94. Since the infusion of ANG II also caused an increase in mean arterial blood pressure, we cannot exclude the possibility that the increase in renal vascular resistance is the reflection of an autoregulatory

response. However, it has been shown previously that the autoregulatory response of afferent arterioles is not affected by Cl channel blockade [9]. Thus, it is likely that the absence of a vasoconstrictor effect of ANG II during IAA-94 administration indicates a requirement for functional calcium-activated Cl channels in the constrictor response to angiotensin II. These studies in the intact kidney in vivo complement earlier experiments at the cellular level that have clearly demonstrated the existence of calcium-activated Cl channels in renal vascular smooth muscle cells [17]. Furthermore, inhibitors of Cl channels such as IAA-94 or 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) have been shown to inhibit agonist-induced constrictor responses of afferent arterioles [9, 18, 19]. Activation of Cl channels appears to be the main mechanism by which angiotensin II depolarizes the cell membrane, a prerequisite for subsequent activation of voltage-dependent Ca channels.

A major new finding in the present study is the observation that Cl channel blockade does not to a major extent alter TGF responses. This is somewhat unexpected since there is good evidence to indicate that the TGF response to an elevation of loop perfusion rate above normal is an example of an agonist-induced vasoconstriction of afferent arterioles. The nature of the agonist is not entirely clear, but there is substantial experimental support for a role of adenosine leading to activation of A1 adenosine receptors (A1AR) in this response [20–22]. A1AR signal primarily through Gi proteins, but they are also coupled to activation of phospholipase C (PLC) causing an increase in cytosolic calcium concentrations of afferent arteriolar smooth muscle cells [23]. It is not clear why this does not appear to be associated with activation of Cl channels if these channels are in fact present in the TGF-sensitive segment of the afferent arteriole. It may be possible that the site of TGF information transfer in the juxtaglomerular apparatus (JGA) interstitium is not accessible to the inhibitor. Alternatively, it would seem conceivable that an increase in Cl concentration in the JGA interstitium and the resulting hyperpolarization compensates for the depolarizing effect of Cl channel activation. Regardless of the precise mechanism involved, one would have to postulate that the cell depolarization responsible for the activation of voltage-activated calcium channels, known to be critical for TGF responses, appears to be the result of a process distinct from Cl channel activation [16, 24]. Whether this process is an inhibition of potassium channels or some other membrane event remains to be determined [25].

In contrast to the absence of an effect of IAA-94 on basal TGF responsiveness, Cl channel inhibition completely prevented the enhancement of TGF responses by angiotensin II. It is well established that both systemic and peritubular application of angiotensin II augments the magnitude of TGF responses [26]. The current experiments demonstrate that this augmentation is associated with an activation of Cl channels suggesting that the consequences of this activation are critical for angiotensin-dependent enhancement of TGF responses. Our observation indicates that some of the membrane events associated with the basal TGF response and with its enhancement by angiotensin II appear to be distinctly different.

A surprising finding in our study is the unresponsiveness of angiotensin II-mediated vasoconstriction to IAA-94 in spontaneously hypertensive rats. Thus, while Cl channel activation is importantly involved in the constrictor actions of angiotensin in normotensive rats, opening of Cl channels does not seem to be necessary in SHR for angiotensin II to increase renal vascular resistance or to enhance TGF responses. The reason for this difference is unclear, but a number of membrane characteristics have been described that differ in SHR compared to normal rats. Okuda et al. [27] have observed that a lowering of extracellular Cl concentration caused attenuation of cell contraction and of the magnitude of calcium transients in response to angiotensin II in mesangial cells of normotensive rats. This effect of a low Cl concentration was absent in mesangial cells from SHR, apparently due to an inability to enhance prostaglandin E2 production. Furthermore, an increase in cytosolic calcium caused membrane depolarization and increased Cl currents in mesangial cells from WKY, but membrane hyperpolarization and an increase in potassium currents in mesangial cells from SHR [12]. Finally, insulin has been shown to attenuate the contractile and calcium responses of mesangial cells to angiotensin II, but this attenuation was absent in cells from SHR [28]. These studies in mesangial cells suggest some abnormality in the expression or function of chloride channels in SHR that may be consistent with the absence of an effect of IAA-94 in our studies. Nevertheless, since the constrictor action of angiotensin II in SHR was well maintained, one would have to assume that the initiation of smooth muscle cell activation has switched from Cl channel dependence to some other event that remains to be defined. It appears that afferent arterioles in SHR respond to angiotensin II in a way similar to efferent arterioles of normotensive animals where angiotensin II-induced constriction is also independent of Cl channel activation [8].

In conclusion, our results show that angiotensin II-induced renal vasoconstriction and enhancement of TGF responses is strongly attenuated by IAA-94 in normotensive rats suggesting that angiotensin actions include activation of Cl channels. In contrast, vasomotor and TGF response modification by angiotensin II in SHR does not require the participation of IAA-94 inhibitable Cl channels. Although the reasons for this conspicuous difference are unknown at present, it appears that angiotensin II induces smooth muscle cell activation by fundamentally different processes in WKY and SHR.

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ORIGINAL ARTICLE

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Diagnostic value of antiagalactosyl IgG antibodies in rheumatoid arthritis

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Abstract Our objective in this study was to explore the diagnostic value of antiagalactosyl IgG antibodies in rheumatoid arthritis (RA). The study comprised 266 Japanese patients with systemic autoimmune diseases, including 60 with RA. Human agalactosyl IgG was prepared enzymatically, and the serum levels of antiagalactosyl IgG antibodies were determined using a lectin enzyme immunoassay. Serum IgG and IgM rheumatoid factors (RF) were measured using laser nephelometry for IgM (LN-RF) and an enzyme-linked immunosorbent assay for IgG (IgG-RF). Antiagalactosyl IgG antibodies were significantly more common in patients with RA than in those without (78% vs. 18%, odds ratio (OR) 16.51, 95% confidence interval (CI) 8.12–33.58, $p < 0.0001$). Patients with RA also had a higher frequency of LN-RF than those without RA (75% vs. 28%, OR 7.81, 95% CI 3.91–15.58, $p < 0.001$). The specificity of antiagalactosyl IgG antibodies for RA was significantly higher than that of LN-RF (82% vs. 72%, $p < 0.0011$). There was a significant correlation between titers of antiagalactosyl IgG antibodies and C-reactive protein levels. Antiagalactosyl IgG antibodies are more specific markers for RA than conventional LN-RF, and may provide useful information for the diagnosis of RA.

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Abbreviations AKA: Antikeratin antibodies · RA: Rheumatoid arthritis · RF: Rheumatoid factor · SLE: Systemic lupus erythematosus

Introduction

Rheumatoid arthritis (RA) is a widely prevalent autoimmune disease characterized by destructive joint inflammation and the production of rheumatoid factor (RF). The diagnosis rests on clinical grounds and is usually based on the classification criteria proposed by the American College of Rheumatology (ACR) [1]. The only diagnostic test used in standard practice is the determination of serum RF. RF are polyclonal autoantibodies directed against epitopes in the Fc region of the IgG molecule [2, 3, 4] that can be detected by a variety of methods [5]. Agglutination tests such as the Rose–Waaler and the latex tests are widely used but their clinical value is limited because of low disease specificity [6]. Quantification of RF by nephelometry or enzyme-linked immunosorbent assays (ELISA) is achieving increasingly common usage because these methods are demonstrated to be reliable, accurate and sensitive for routine clinical use [7, 8]. In recent years several newly characterized antibodies have become promising candidates as diagnostic indicators of RA. Antikeratin antibodies (AKA) have been demonstrated to be highly specific for RA [9]. Anticyclic citrullinated peptide antibody (anti-CCP) is a novel RA-specific antibody that can be detected very early in the disease. It promises to be a key serological marker for the early diagnosis and prognosis of RA [10, 11].

Although RF is primarily associated with RA, these antibodies have low disease specificity and can be detected in sera from normal elderly people, healthy individuals, and patients with other autoimmune disorders or chronic infections [12].

In 1985, Parekh et al. [13] described RA as being associated with an altered glycosylation pattern of serum IgG, resulting in increased levels of oligosaccharides which lack terminal galactose residues. Interestingly, it has been shown that RF in patients with RA bind better to galactose-free IgG (agalactosyl IgG) than to galactosylated IgG [14]. Antibodies against agalactosyl IgG have been detected in patients with RA and the titers positively correlated with disease activity [15]. In this study we evaluated the presence of antiagalactosyl IgG antibodies in patients with a variety of autoimmune disorders and assessed the usefulness of this test in the laboratory diagnosis of RA.

Patients and methods

The study population comprised 266 patients with systemic autoimmune diseases visiting the Department of Internal Medicine II, Hokkaido University Hospital. All had a detailed clinical assessment at the time serum samples were drawn. Clinical records were carefully reviewed retrospectively and patients were grouped as follows: 1) patients with RA ($n=60$) fulfilling the ACR criteria for RA classification [1]; and 2) patients without RA ($n=206$), including 96 with systemic lupus erythematosus (SLE), 36 with primary Sjögren's syndrome, 19 with scleroderma and 55 with other autoimmune diseases. Patients in which RA overlapped with other diseases were included in the RA group. Table 1 shows the profile of the patients.

Measurement of antiagalactosyl IgG antibodies

The serum levels of antiagalactosyl IgG antibodies were determined by a lectin enzyme immunoassay (Eitest CARF, Eisai Co. Ltd, Tokyo, Japan) as previously described, with minor modifications using human agalactosyl IgG as antigen [16, 17]. Briefly, agalactosyl IgG was prepared from enzymatically treated oligosaccharides of human IgG. Human IgG was purified from human serum by ammonium sulfate precipitation, DE52 anion exchange chromatography, and a protein G coupled to agarose as an affinity column for chromatography (ImmunoPure immobilized Protein G, Pierce, Rockford, USA). Human IgG (10 mg) was subsequently treated with 1U neuraminidase (Roche Diagnostics KK, Japan) in 0.1 M acetate buffer for 24 h at 37°C, followed by treatment with 0.1U β-galactosidase (Seikagaku Corpotation, Japan) in 0.1 M citrate–phosphate buffer (pH 7.0) for 48 h at 37°C. Agalactosyl IgG was purified using protein G coupled to agarose as an affinity column for chromatography, and dialyzed against phosphate-buffered saline containing 0.02% sodium azide. Polystyrene plates (Eisai Co. Ltd, Japan) were coated with 100 µl of agalactosyl IgG (5 µg/ml) at 4°C overnight. After washing with Tris-buffered saline (TBS, 0.01 M, pH7.4), wells were blocked with 150 µl of TBS containing 0.05% bovine serum albumin (BSA, Oriental Yeast OC, Ltd, Japan) at 4°C overnight. One hundred microliters of serum samples diluted 201-fold in the standard dilution solution (0.25% BSA, 50 mM Tris-HCl, 0.15 M NaCl, 0.05% polyoxyethyleneoctylphenyl ether, 0.02% *p*-hydroxylbenzoic acid methyl, 0.5% 2-chloroacetamide, pH7.4) were added. After 60 min of incubation at room temperature (RT), the wells were washed three times and 100 µl of biotinylated *Ricinus communis* agglutinin 120 (RCA120, Seikagaku Corporation, Japan) was added. After incubation for 1 h at RT and washing three times, 100 µl of diluted streptavidin peroxidase (1:1000) (Oriental Yeast OC, Ltd, Japan) solution were added. After a further 1 h of incubation and three washes, 100 µl of chromogen substrate solution (chromogen: ABTS, 2'-azino(3-ethyl-benz-thiazoline-6-sulphonic acid, Wako Pure Chemical Industries Ltd, Japan; substrate hydrogen peroxide, Sankyo, Japan) were added. The reaction was halted with 2 mM sodium azide after 30 min of incubation, and the absorbance was read at 405 nm using an ELISA plate reader.

The levels (arbitrary unit (AU/ml)) of antiagalactosyl IgG antibodies in sera were measured, using a standard curve (3.125–50 AU/ml). A normal range was established using 125 healthy controls, with a cut-off of 12 AU/ml being the 95th percentile non-parametric setting.

Measurement of IgM and IgG RF

Serum IgM and IgG RF were measured using laser nephelometry (N-Latex RF kit, Dade Behring, Germany) for IgM (LN-RF), and ELISA (Eitest IgGRF, Eisai Co. Ltd) for IgG (IgG-RF). The normal cut-off value was defined as described above, being 16.3 International Units (IU) for LN-RF and 2 IU for IgG-RF.

Statistical analysis

Statistical evaluation was performed by Fisher' s exact test, the Mann–Whitney *U*-test or Spearman's rank correlation as appropriate. The relative risks were approximated by odds ratios (OR) with 95% confidence intervals (95% CI). The sensitivity and specificity were calculated by standard definitions. Receiver operating characteristic (ROC) curves were constructed to identify the most suitable cut-off values for each of the three assays investigated in this study. p values <0.05 were considered statistically significant.

Results

A positive titer of antiagalactosyl IgG antibodies was found in 47/60 (78%) of patients with RA but in only 37/206 (18%) of patients without RA (OR 16.51, 95% CI (8.12–33.58), $p<0.0001$). The presence of LN-RF was also significantly more frequent in patients with RA than in those without (75% vs. 28%, OR 7.81, 95% CI

Table 1 Profile of 266 patients with systemic autoimmune diseases

Diagnosis	Number of patients	Female	Age (years ± standard deviation)
Rheumatoid arthritis	60	52 (87%)	54 ± 13
Systemic lupus erythematosus	96	89 (93%)	38 ± 11
Primary Sjögren's syndrome	36	35 (97%)	57 ± 14
Scleroderma	19	19 (100%)	55 ± 15
Others autoimmune diseases[a]	55	53 (96%)	47 ± 16

[a]Includes patients with polymyositis ($n=5$), dermatomyositis ($n=8$), mixed connective tissue disease ($n=3$), vasculitis ($n=5$), primary antiphospholipid syndrome ($n=10$), Behçet's disease ($n=6$) and other rheumatic/autoimmune diseases ($n=18$)

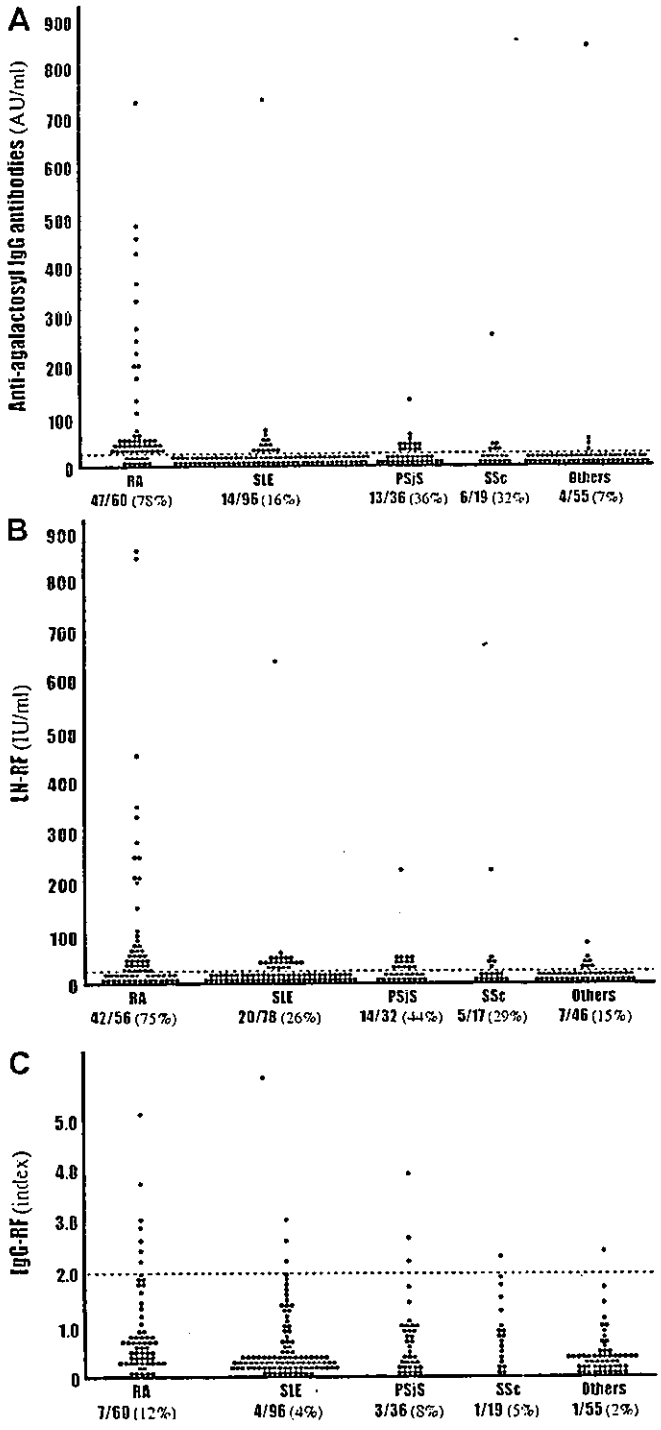

Fig. 1 Distribution of antiagalactosyl IgG antibodies, LN-RF and IgG-RF in patients with systemic autoimmune disorders. **A** Titers of antiagalactosyl IgG antibodies determined by lectin enzyme immunoassay were significantly higher in patients with rheumatoid arthritis (RA) than in those with systemic lupus erythematosus (SLE) ($p < 0.001$), primary Sjögren's syndrome (PSJS) ($p < 0.001$), scleroderma (Scc) ($p < 0.001$) and other autoimmune diseases ($p < 0.0001$). The dashed line indicates the cut-off for positivity. AU: arbitrary units. **B** Titers of IgM rheumatoid factor (LN-RF) measured by laser nephelometry were significantly higher in patients with RA than in those with SLE ($p < 0.0001$), PSJS ($p < 0.0001$), SSc ($p < 0.0001$) and other autoimmune diseases ($p = 0.0006$). IU: international units. **C** Titers of IgG-RF measured by ELISA. Only some patients were positive in all the groups

(3.91–15.58), $p < 0.0001$). No differences were found in the prevalence of IgG-RF between patients with or without RA (12% vs. 4%, OR 3.96, 95% CI (1.39–11.3), $p = 0.058$)

Titers of antiagalactosyl IgG antibodies and LN-RF were significantly higher in the group of patients with RA than in those without (Fig. 1).

Positivity for antiagalactosyl IgG antibodies was detected in 84 of the 266 patients. Among these, 65 were also positive for LN-RF and 10 for IgG-RF. Thirteen patients with RA did not have antiagalactosyl IgG antibodies, two of them had positive IgG-RF, and only one had positive LN-RF.

The presence of antiagalactosyl IgG antibodies was as sensitive as LN-RF but was significantly more specific for the diagnosis of RA (Table 2).

A highly significant correlation was found between the titers of antiagalactosyl IgG antibodies and LN-RF in patients with RA ($r = 0.918$, $p < 0.0001$) (Fig. 2). No correlation was observed between the titers of antiagalactosyl IgG antibodies and IgG-RF.

Titers of antiagalactosyl IgG antibodies significantly correlated with the serum C-reactive protein levels (CRP) ($r = 0.329$, $p = 0.029$).

ROC curves showed that the significance of antiagalactosyl IgG antibody assay is overall compatible with that of LN-RF assay (the area under the ROC curves (95% CI); 0.813 (0.742–0.884) vs. 0.816 (0.745–0.887), respectively) (Fig. 3).

Discussion

This study shows that antiagalactosyl IgG antibodies are more specific markers of RA than the conventional RF.

A number of reports have mentioned alterations in glycosylation of IgG molecules in patients with RA, but the biological significance of that sugar-chain change is not well understood. Decreased levels of galactosylation of serum IgG sugars have been noted in other rheumatic diseases [18, 19, 20, 21], but the presence of agalactosyl IgG is a characteristic feature of RA [14]. In RA, levels of agalactosyl IgG correlate with the severity of the disease [22].

Antiagalactosyl IgG antibodies have been detected in patients with autoimmune diseases [17, 23]. We had presumed that these antibodies would be rather specific for RA, as the autoimmune reaction between agalactosyl IgG and autoantibodies against them would not occur in patients with other autoimmune diseases lacking agalactosyl IgG. In fact, we found antiagalactosyl IgG antibodies in 78% of patients with RA, but only in 18% of patients without RA. Most patients with positive LN-RF and negative antiagalactosyl IgG antibodies belonged to the non-RA group, leading to

Table 2 Comparison of different tests for the diagnosis of rheumatoid arthritis

	Sensitivity (%)	Specificity (%)	OR (95% CI)	*p* values
Antiagalactosyl IgG Antibodies	78	82	16.51 (8.12–33.58)	< 0.001
LN-RF	75	72	7.81 (3.91–15.58)	< 0.001
IgG-RF	12	96	2.8 (1.08–8.12)	ns

LN-RF: IgM rheumatoid factor; IgG-RF: IgG rheumatoid factor; OR: Odds ratio; CI: confidence interval; ns: not significant

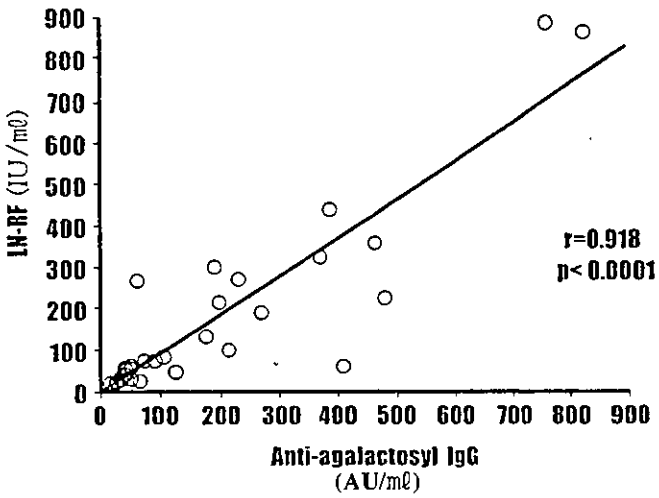

Fig. 2 Relationship between titers of antiagalactosyl IgG antibodies and LN-RF in patients with rheumatoid arthritis. The titers of antiagalactosyl IgG antibodies were determined by a lectin enzyme immunoassay and those of LN-RF by laser nephelometry in patients with RA. A highly significant correlation was found between them. AU: arbitrary units IU: international units

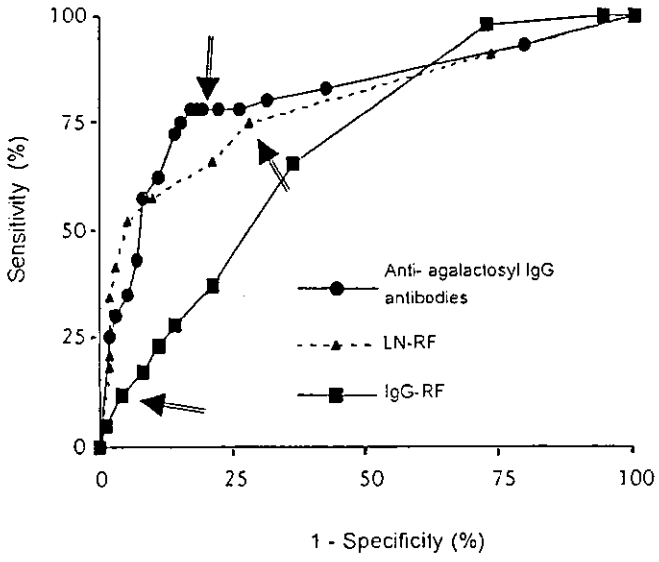

Fig. 3 Receiver operating characteristic (ROC curves) for antiagalactoysl IgG antibodies, LN-RF and IgG-RF. Sensitivity is represented on the *y* axis and 1-specificity on the *x* axis. Arrows show the cut-off value used for each assay

the high specificity of antiagalactosyl IgG antibodies for RA. Our data contrast with those reported previously [17] claiming a lack of specificity of antiagalactosyl IgG antibodies for RA. The discrepancy could be explained by the difference in the cut-off setting. In our study we raised the cut-off value to 12 AU using a nonparametric 95th percentile point that represents the double value of the one reported by Ichikawa et al. [17]. The cut-off setting is one of the most important factors for analysis, and the establishment of a higher cut-off value could be one way to improve the specificity of the test. Furthermore, to compare the specificity of antiagalactosyl IgG antibody and LN-RF assays, we established an alternative cut-off value for antiagalactosyl IgG antibody assay at 15 AU in which the sensitivity of the two assays was equal (75%). Using this cut-off value, we verified that the specificity of the antiagalactosyl antibody assay was significantly higher (85% vs. 72%). ROC curves approve the significance of the compatibility of antiagalactosyl IgG antibody and LN-RF assays overall.

We looked at the clinical values of the other assays that were already running in our laboratory and compared them with the antiagalactosyl IgG antibody test. The method used for the determination of antiagalactosyl IgG antibodies can detect all isotypes of the antiagalactosyl antibodies, as galactose moieties are common to the oligosaccharide chains on immunoglobulin molecules, and RCA120 can bind to the terminal galactose on all the isotypes of immunoglobulin. We found that antiagalactosyl IgG antibodies correlated highly with LN-RF, hence it may mainly detect the IgM isotype. In fact, IgG-RF was reported to be present in patients with RA [8, 24], but its prevalence was very low in our patients.

Some studies reported an age-related galactosylation of IgG, hence the levels of antiagalactosyl IgG might depend on the age of the patient [25, 26]. However, there was no statistically significant difference in age among our RA patients with or without antiagalactosyl IgG antibodies.

Titers of antiagalactosyl IgG antibodies correlated with CRP levels. In addition, serial determinations of antiagalactosyl IgG antibodies, LN-RF and CRP levels were performed in two patients with active RA before and after the initiation of methotrexate therapy. We found a clear reduction of both antibodies titers and CRP levels (data not shown).

Our data show that antiagalactosyl IgG antibodies are a useful tool for the diagnosis of RA, with a very high specificity.

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ORIGINAL ARTICLE

Synthetic selective inhibitors of coagulation factor Xa strongly inhibit thrombin generation without affecting initial thrombin forming time necessary for platelet activation in hemostasis

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Summary. DX-9065a and JTV-803, synthetic selective inhibitors of activated factor X (FXa), have recently been demonstrated as strongly effective antithrombotic agents in animal thrombosis models, yet with a low risk of bleeding. The aim of the present study was to elucidate these characteristics. Using a chromogenic assay with purified coagulation factors, 73.9% of thrombin generation was suppressed by the addition of DX-9065a (0.20 μM) and 75.7% by JTV-803 (0.18 μM). Inhibition by argatroban (0.19 μM) was less (36.0%) and initial thrombin forming time (T_{50}), the time required to generate 50% thrombin activity *in vitro*, which is considered important for platelet aggregation in hemostasis, was significantly prolonged by argatroban. In contrast, DX-9065a and JTV-803 had no apparent influence on T_{50}, suggesting that initial thrombin was formed immediately, as in the control. We also investigated platelet aggregation in defibrinated plasma induced by tissue factor, to clarify whether initial thrombin contributes to hemostasis. Aggregation was not affected by the addition of either FXa inhibitor, whereas it was significantly reduced by argatroban. Our results suggest that initial thrombin, which is formed despite the presence of a FXa inhibitor, can activate platelets. We concluded that DX-9065a and JTV-803 are able to inhibit thrombin generation significantly without affecting the formation of initial thrombin for platelet activation, which may contribute to hemostasis through the preservation of normal bleeding time.

Keywords: factor Xa inhibitor, platelet aggregation, thrombin inhibitor.

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The goal of most current antithrombotic strategies is to control the action or generation of thrombin, an enzyme in the coagulation system that causes fibrin clotting. For the treatment and prevention of thromboembolic disorders, heparin, warfarin, and direct thrombin inhibitors are widely employed as antithrombotic agents. However, they are well known to lead to a bleeding tendency as a complication of their use.

Recently, a series of selective inhibitors of activated blood coagulation factor X (FXa) that act independently of antithrombin have been shown to be strongly effective antithrombotic agents in animal models. Of these, DX-9065a (2S-2-[4-{[(35S)-1-acetimidyl-3-pyrrolidinyl]oxy}phenyl]-3-[7-amidino-2-naphyl]propanoic acid hydrochloride pentahydrate) is a highly selective and competitive inhibitor of FXa [1,2], as its estimated dissociation constant (Ki) for FXa was reported to be 41 nM, while that for thrombin is > 2000 μM [2]. This synthetic inhibitor has been found to exert effective protection against experimental tumor-induced disseminated intravascular coagulation (DIC) in rats and suggested to improve the hypercoagulable state induced by the progress of a solid tumor [3]. Following DX-9065a, another synthetic FXa inhibitor, JTV-803 (4-[(2-amidino-1, 2, 3, 4-tetrahydroisoquinolin-7-yloxy) methyl]-1-(4-pyridinyl) piperidine-4-carboxylic acid monomethanesulfonate trihydrate), was developed. The Ki of JTV-803 for FXa was found to be 19 nM, with an effective dose ranging from approximately 0.1 to 0.5 μM [4]. This agent has been shown to inhibit thrombus formation in an arteriovenous shunt model [5], and was also demonstrated to be effective for treating both lipopolysaccharide-induced and tissue factor (TF)-induced DIC in rat models [6].

DX-9065a is considered to be a new type of antithrombotic agent with few hemorrhagic effects that does not prolong bleeding time [7–9], probably because it does not inhibit platelet activation [7], though its mechanism with platelet function has not been clarified. To investigate their antithrombotic and hemorrhagic properties, we studied the effects of the FXa inhibitors DX-9065a and JTV-803 on thrombin generation by prothrombinase, which consists of factor (F)Va, FXa, and

$CaCl_2$ (time 0). At 15-s intervals from time 0, a 50-μL aliquot of the mixture was added to 465 μL of buffer A containing 20 mM EDTA to stop the coagulation reaction. Next, 25 μL of 0.5 mM S-2238 were added, followed by incubation for 2 min at 37 °C. After adding 300 μL of a 50% acetate solution to stop the chromogenic reaction, thrombin activity in each sample was measured at 405 nm with an autoreader. Each experiment with each inhibitor was performed using triplicate samples and repeated 10 times. The results are expressed as the time taken to reach 50% of maximal thrombin activity, which was determined as maximal optical density at 405 nm (T_{50}). The thrombin forming time of each sample is expressed as a ratio of the T_{50} result of the sample to that of the control.

Effects of inhibitors on platelet aggregation induced by tissue factor

The effects of the antithrombotic agents on platelet aggregation, which was induced by thrombin formed in defibrinated plasma by TF, were investigated. Each sample, composed of defibrinated plasma (100 μL), washed platelets (40×10^4 platelets μL^{-1}, 80 μL), and 20 μL of either Tris buffer saline (0.02 M Tris–HCl pH 7.4, 0.15 M NaCl) or the inhibitor, was preincubated at 37 °C for 2 min, followed by the addition of 20 μL of TF to induce platelet aggregation. The antithrombotic agents used in this experiment were 0.07–0.71 μM (final concentration) of DX-9065a, 0.13–1.26 μM (final concentration) of JTV-803, and 0.13–0.50 μM (final concentration) of argatroban. As a source of TF, thromboplastin from rabbit brain samples concentrated four times with PT-reagent (Thrombocheck PT) was used as a trigger to generate thrombin in defibrinated plasma, which was considered the minimum concentration to cause aggregation of the platelets. Platelet aggregation was observed for 10 min using an aggregometer (Haema tracer 601; Niko Bioscience, Tokyo, Japan), with maximum aggregation expressed as a percentage. Absorbance of the mixture, composed of washed platelets, defibrinated plasma, and Tris buffer saline, was taken as 0% aggregation, and absorbance of the mixture without washed platelets was taken as 100% aggregation. Each experiment was repeated six times.

Statistical analysis

All data are expressed as mean ± SD. Grouped data were analyzed for significance by comparative analysis using a two-tailed Student's *t*-test. *P*-values < 0.05 were considered to be statistically significant.

Results

Coagulation assay

Each inhibitor (DX-9065a, JTV-803, and argatroban, in concentrations from 0.05 to 0.5 μM) prolonged PT in a concentration-dependent manner. Clotting times by the FXa inhibitors at concentrations more than 0.10 μM were slightly

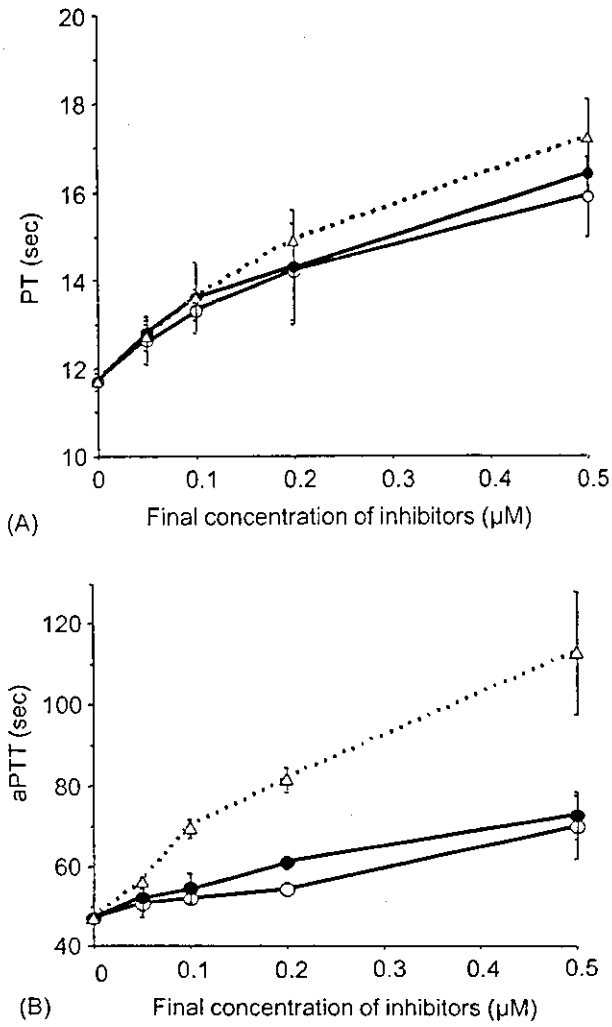

Fig. 1. Effects of synthetic inhibitors of activated factor X (FXa) on prothrombin time (PT) and activated partial thromboplastin time (APTT). (A) Clotting time in a PT assay was prolonged by the addition of DX-9065a (○) and JTV-803 (●) at concentrations from 0.05 to 0.5 μM, as well as by argatroban (Δ) at 0.05–0.5 μM. (B): FXa inhibitors (DX-9065a, ○; JTV-803, ●) at concentrations from 0.05 to 0.5 μM prolonged APTT in a concentration-dependent manner. Argatroban (Δ) at 0.1–0.5 μM prolonged APTT significantly more than the FXa inhibitors at the same concentrations.

prolonged compared with those by argatroban, though the differences were not statistically significant (Fig. 1A). Each FXa inhibitor also prolonged APTT in a concentration-dependent manner; however, argatroban at concentrations of 0.1–0.5 μM prolonged APTT significantly more than DX-9065a and JTV-803 at the same concentrations (Fig. 1B).

Influences of inhibitors on thrombin generation

The influence of each antithrombotic agent on thrombin generation was investigated using a chromogenic assay with purified human coagulation factors. As shown in Table 1, DX-9065a at final concentrations of 0.10–2.00 μM inhibited 61.6–95.1% of the thrombin activity generated in the control,

Table 1 Influence of antithrombotic agents on thrombin generation and initial thrombin forming time

	Inhibition of thrombin generation (%)	Initial thrombin forming time (T_{50} ratio)
Control	0 ± 5.6	1.00 ± 0.10
DX-9065a (μM)		
0.10	61.6 ± 1.2	1.05 ± 0.14
0.20	73.9 ± 0.6	1.08 ± 0.06
1.00	90.8 ± 3.6	1.12 ± 0.06
2.00	95.1 ± 1.4	1.35 ± 0.12*
JTV-803 (μM)		
0.04	47.9 ± 0.3	1.04 ± 0.09
0.18	75.7 ± 0.4	1.10 ± 0.04
0.36	85.0 ± 0.9	1.22 ± 0.10***
1.78	92.7 ± 1.6	1.46 ± 0.16*
LMWH (U mL^{-1})		
0.05	36.6 ± 1.2	1.04 ± 0.20
0.10	54.4 ± 0.2	1.07 ± 0.10
0.50	71.5 ± 2.2	1.46 ± 0.07*
1.00	80.3 ± 3.6	2.31 ± 0.14*
Argatroban (μM)		
0.04	19.7 ± 0.4	1.06 ± 0.07
0.19	36.0 ± 0.2	1.33 ± 0.04**
0.38	40.6 ± 0.3	1.45 ± 0.22*
1.88	74.6 ± 1.6	2.26 ± 0.14*

Mean ± SD. *P < 0.001 vs. control: **P < 0.002; ***P < 0.05. LMWH, Low-molecular-weight heparin.

JTV-803 at 0.04–1.78 μM inhibited 47.9–92.7% of the thrombin activity generated in the control, and LMWH at 0.05–1.00 U mL^{-1} inhibited 36.6–80.3% of the thrombin activity generated in the control. In contrast, argatroban at 0.04–1.88 μM inhibited 19.7–74.6% of thrombin activity generated in the control, which was significantly lower than the other inhibitors.

Influences of inhibitors on initial thrombin forming time

The influence of the antithrombotic agents on initial thrombin forming time (T_{50}), which was the time to generate 50% thrombin activity *in vitro*, was also investigated. The ratio of T_{50} with 0.10–1.00 μM of DX-9065a was from 1.05 to 1.12, which was not significantly different from the control (1.01 ± 0.10), whereas 2.00 μM of DX-9065a caused a significant increase in T_{50} ratio (1.35 ± 0.12, P < 0.001). When JTV-803 was added at concentrations of 0.04 and 0.18 μM, the T_{50} ratio was 1.04 ± 0.09 and 1.10 ± 0.04, respectively, which was not significantly different from the control, whereas JTV-803 at concentrations > 0.36 μM resulted in a significant increase of T_{50} ratio. LMWH at 0.10 U mL^{-1} with 0.5 U mL^{-1} antithrombin did not affect the T_{50} ratio (1.07 ± 0.10); however, the ratio was significantly increased by the addition of 0.50 and 1.00 U mL^{-1} of LMWH. In contrast, when argatroban was added at concentrations from 0.19 to 1.88 μM, the T_{50} ratio was significantly increased (T_{50} ratio 1.33 ± 0.04–2.26 ± 0.14, P < 0.002 vs. control) (Table 1).

Table 2 Effects of antithrombotic agents on tissue factor-induced platelet aggregation in defibrinated plasma

	Antithrombotic agents (μM)	Maximum aggregation (%)
Control		62.4 ± 24.0
DX-9065a	0.07	66.6 ± 7.8
	0.14	55.0 ± 21.3
	0.50	57.7 ± 4.3
	0.71	4.6 ± 5.2*
JTV-803	0.13	63.7 ± 8.5
	0.25	67.8 ± 8.1
	0.50	40.7 ± 30.6
	1.26	2.6 ± 4.8*
Argatroban	0.13	33.7 ± 35.1*
	0.26	11.8 ± 19.4*
	0.50	0.3 ± 0.6*

Mean ± SD. *P < 0.01 vs. control.

Effects of inhibitors on platelet aggregation induced by tissue factor

Next, platelet aggregation was induced by TF in order to determine whether thrombin formed by a minimum concentration of TF could activate platelets in the presence of each inhibitor. When TF was added to a sample without any inhibitor (control), maximum platelet aggregation within 10 min was 62.4 ± 24.0%. The addition of 0.13, 0.26, and 0.50 μM of argatroban significantly reduced maximum aggregation to 33.7 ± 35.1%, 11.8 ± 19.4%, and 0.3 ± 0.6%, respectively. In contrast, the addition of DX-9065a (0.07–0.50 μM) or JTV-803 (0.13–0.50 μM) did not have a significant effect on aggregation (DX-9065a, 66.6–57.7%; JTV-803, 63.7–40.7%) (Table 2).

An immediate aggregation of platelets was observed in the presence of 0.50 μM of DX-9065a and 0.50 μM of JTV-803, which was similar to the control. However, aggregation in the presence of 0.25 μM of argatroban was significantly delayed. Typical platelet aggregation patterns from these experiments are shown in Fig. 2.

Discussion

The generation of thrombin is a crucial step in the process of blood coagulation, and thrombosis results from a series of proteolytic activating reactions that are initiated via intrinsic and extrinsic pathways of blood coagulation cascades. FXa is a serine protease positioned at the convergence of those pathways, and DX-9065a and JTV-803 have been newly developed as synthetic inhibitors of FXa.

In the present study, the synthetic FXa inhibitors did not exert a serious influence on intrinsic coagulation, though argatroban, a selective thrombin inhibitor, significantly prolonged clotting time in APTT (Fig. 1B). The influence of these Xa inhibitors on PT and APTT may be different from our results if another reagent of APTT or PT is used. However, our findings suggested that a small amount of thrombin remained in the bloodstream after the FXa inhibitor was applied. Most of the existing FXa is inhibited when an FXa inhibitor is

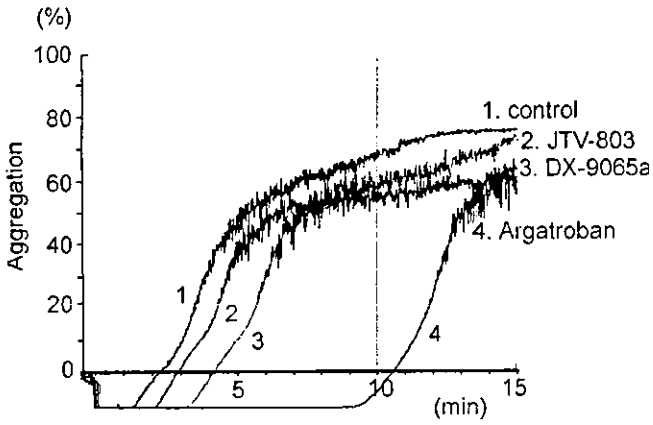

Fig. 2. Typical pattern of platelet aggregation in the presence of antithrombotic agents. The aggregation of washed platelets in defibrinated pool plasma was investigated by the addition of tissue factor, as described in Materials and methods. Immediate aggregation of platelets was observed in the presence of 0.50 μM of DX-9065a (line 3) and 0.50 μM of JTV-803 (line 2), similar to that of the control (line 1). In contrast, aggregation in the presence of 0.50 μM of argatroban (line 4) was delayed.

administered, but the small amount of FXa that is unaffected may bring about the generation of thrombin, which can consequently activate factor (F)XI independently of factor XII [13,14] and accelerate the intrinsic coagulation reaction. Further, there is a small amount of thrombin in blood that seems to be generated by the FXI that is activated automatically [13,14] and/or by factor VII activated through an unknown mechanism [15]. Thrombin can stimulate the intrinsic coagulation reaction, therefore the administration of a FXa inhibitor may exert a weak inhibitory influence on clotting time in APTT. On the other hand, when argatroban is added, it may completely inhibit not only the thrombin formed through coagulation cascades but also that remaining in blood. Thus, argatroban apparently prolongs clotting time in APTT, because no acceleration of the intrinsic coagulation pathway is caused by thrombin. In contrast, during administration of an FXa inhibitor, the small amount of thrombin generated by the remaining FXa may not directly affect the extrinsic coagulation pathway. Therefore, the present clotting time results in the PT assay following administration of the FXa inhibitors were not significantly different from those for argatroban.

The purpose of the present study was to investigate the efficiency of FXa inhibitors on thrombin generation. For analysis of whole thrombin generation, including endogenous thrombin potential, the method established by Hemker [16,17] is known to be reliable. However, we performed two different kinds of experiments to measure total thrombin formed for a definite term without influence by the innate inhibitor, antithrombin, and to determine precisely the time necessary for forming a definite amount of thrombin during initial thrombin generation, termed initial thrombin forming time.

We first established a thrombin generation assay using a chromogenic assay with prothrombinase consisting of purified human coagulation factors and phospholipid, which can measure the genuine inhibitory effect of an inhibitor on fibrin formation. Total thrombin generated for 10 min was significantly decreased by the presence of DX-9065a or JTV-803, as even low concentrations inhibited > 60% of the thrombin generated in their absence, while argatroban inhibited thrombin generation to a lesser degree than either of the FXa inhibitors. Therefore, we concluded that selective inhibitors of FXa were extremely effective for thrombosis, which was supported by our results of inhibiting FXa with LMWH, a coenzyme of antithrombin. The good effect of synthetic FXa inhibitors for prevention of thrombosis has also been shown in animal models with DIC [3,5,6].

To clarify whether immediate formation of thrombin actually occurred, we also investigated the influence of the antithrombotic agents on T_{50}, which represents initial thrombin forming time and is considered important for platelet aggregation in hemostasis, but not in the formation of fibrin clotting. This experiment was performed using defibrinated plasma, which contained an innate inhibitor both to thrombin and to FXa, antithrombin, and we detected a small amount of thrombin present under conditions similar to those seen *in vivo*. We consider that the formation of initial thrombin for platelet aggregation can be reflected in the determination of T_{50} and such an examination is useful for investigation of hemorrhage tendency, because the results correlate with coagulable potency, as shown by Ibbotson [12].

Argatroban significantly prolonged T_{50} in a concentration-dependent manner, probably because it immediately inhibited all of the initial formed thrombin. On the other hand, there was no significant difference in T_{50} results between the FXa inhibitors and the control (Table 1). Morishima [18] found that DX-9065a dose-dependently inhibited thrombus formation; however, it did not inhibit the elevation in plasma thrombin–antithrombin complex (TAT) levels in AV shunt model rats, whereas argatroban inhibited both thrombus formation and TAT elevation. Since the elevation of TAT indicates the formation of thrombin, those results suggested that thrombin may be formed during DX-9065a administration.

Our next area of investigation was whether the small amount of thrombin formed during administration of an FXa inhibitor could activate platelets, resulting in effective platelet aggregation for hemostasis. Accordingly, we tested the effects of the inhibitors on platelet aggregation induced by TF, because TF is an important factor for early hemostasis in humans. With the addition of argatroban, platelet aggregation in defibrinated plasma was remarkably delayed and the maximum percent of aggregation significantly decreased, compared with the control. On the other hand, the addition of either FXa inhibitor, even at a concentration able to inhibit thrombin generation, did not inhibit platelet aggregation, suggesting the presence of residual FXa that was able to form adequate thrombin to activate platelets despite the addition of an FXa inhibitor. These results support previous findings that the addition of a synthetic FXa inhibitor did not exert an influence on bleeding time. Yokoyama [19] reported that DX-9065a inhibited the formation of venous-type fibrin-rich thrombus by inactivating bound and soluble FXa without impairing platelet hemostatic function in