Antibody to Thrombin Receptor Inhibits Neointimal Smooth Muscle Cell Accumulation Without Causing Inhibition of Platelet Aggregation or Altering Hemostatic Parameters After Angioplasty in Rat

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Abstract—An antibody was raised in rabbits against SFFLRNPSEDTFEQF peptide, which is an NH₂-terminal peptide of the thrombin-cleaved rat thrombin receptor. In vitro, the antibody inhibited rat smooth muscle cell proliferation but had no effect on rat platelet aggregation or clotting time. These data indicate that the antibody is a specific blocker of the thrombin receptor–signaling pathway in rat smooth muscle cells but does not work as a blocker in rat platelets, suggesting the existence of a second thrombin receptor in the platelets. Using an in vivo balloon catheter–induced injury model in rats, we examined the effect of the anti-rat thrombin receptor IgG on intimal smooth muscle cell accumulation 2 weeks after angioplasty. Analysis of the ratio of intimal to medial cross-sectional areas showed that injection of immune IgG resulted in 43.7% and 53.1% reduction ($P<0.01$) of neointimal smooth muscle cell accumulation compared with saline and nonimmune IgG treatment, respectively. Moreover, the injection of immune IgG caused a significant decrease of thrombin receptor mRNA expression and also 40.5% and 43.0% decreases ($P<0.01$) of the proliferating cell nuclear antigen (PCNA) index in the intima compared with the PCNA index after saline and nonimmune IgG treatment, respectively. The suppression of the PCNA index was also observed in the immune IgG–treated group at an early stage after angioplasty. These results suggest that thrombin receptor activation is involved in the proliferation and accumulation of neointimal smooth muscle cells induced by balloon injury. (Circ Res. 1998;82:980-987.)

Key Words: smooth muscle cell proliferation ■ thrombin ■ thrombin receptor ■ restenosis ■ angioplasty

Thrombin, a serine protease derived from its precursor, prothrombin, plays an important role in cellular responses.¹² Stimulation of vascular endothelial cells by thrombin leads to the release of several vasoactive substances, such as prostacyclin, platelet-activating factor, and PDGF, increases [Ca²⁺], and also induces translocation of the neutrophil-adhesive molecule (P-selectin) to the endothelial cell surface.³⁴ Thrombin also has a strong mitogenic effect on vascular SMCs.⁵ These diverse cellular responses to thrombin may orchestrate the hemostatic, inflammatory, and proliferative responses to vascular injury.⁶ However, thrombin may be especially important as a mediator of vascular lesion formation, since it is present after balloon catheter injury.¹⁰

The thrombin receptor has been cloned, and it has been proposed that thrombin binds to its receptor and cleaves it after Arg41 in the receptor’s NH₂-terminal portion, thereby exposing a new NH₂ terminus, which functions as a tethered ligand for the receptor.¹¹ The thrombin receptor can mediate the mitogenic effect of thrombin,⁵ and increased thrombin receptor expression was observed in neointimal cells throughout vascular lesion formation.¹² However, the role of the receptor activation in disease processes such as restenosis after balloon angioplasty and progression of atherosclerosis remains unclear owing to the lack of suitable blockers that can inhibit specifically the thrombin receptor–signaling pathway.

In the present study, we raised an antibody to SFFLRNPSEDTFEQF peptide, which is an NH₂-terminal peptide of thrombin-cleaved rat thrombin receptor, and showed that it is a specific blocker of the thrombin receptor–signaling pathway. As such, it should be a useful tool for defining the roles of the thrombin receptor in vivo. It has also been reported that synthetic peptides mimicking the new amino terminus that is unmasked when thrombin cleaves the receptor fail to activate rat or mouse platelets, although thrombin can evoke aggregation even of mouse platelets, in which the thrombin receptor is disrupted.¹⁴⁻¹⁶ These data suggest the existence of distinct thrombin receptor subtypes. Therefore, we also investigated the effect of the antibody to rat thrombin receptor on rat platelet aggregation in vitro.

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Selected Abbreviations and Acronyms

APTT = activated partial thromboplastin time
bFGF = basic fibroblast growth factor
EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
hydrochloride
PAR = protease-activated receptor
PCNA = proliferating-cell nuclear antigen
PDGF = platelet-derived growth factor
PT = prothrombin time
SMC = smooth muscle cell
WST-1 = 3-[4,5-di(3-methylphenyl)-2-(4-nitrophenyl)-2H-5-tetrazolol]-1,3-benzene disulfonate

Materials and Methods

Antibody Preparation

SFFLRNIPSEDTFEQF peptide, which was synthesized in an ABI Peptide Synthesizer (Perkin Elmer) and purified in our laboratories, was coupled to keyhole limpet hemocyanin using EDC as per the manual of the Immject Immunogen EDC conjugation kit (Pierce). New Zealand White rabbits (three) were injected subcutaneously at several sites on the back with a total of 200 μg of the peptide–carrier protein complex in complete Freund’s adjuvant. A booster of 100 μg of antigen in incomplete Freund’s adjuvant was administered 3 weeks later. Additional boosting was done twice more at intervals of 2 weeks. Rabbits were bled every 2 weeks, and the antibody titer was checked. These rabbits were maintained for 1 year without further immunization and then reimmunized in the same way as mentioned above. Ten days after the final boost, all blood was collected, left to stand at 4°C overnight, and centrifuged at 2000g for 30 minutes. The supernatant was heated at 56°C for 30 minutes. The resulting heat-inactivated antiserum was used for the preparation of IgG fraction. This IgG fraction was purified on an Affi-Gel protein A column using an Econo-Pac protein A kit (Bio-Rad) according to the manufacturer’s instructions. The pooled IgG fraction was dialyzed overnight against saline and concentrated by ultrafiltration (PM-10, Amicon Corp.). The resulting solution was used for determining the protein concentration by the method of Lowry et al. The IgG was sterilized by filtration through a 0.22-μm filter (Millipore) and stored at −20°C until use. Nonimmune rabbit IgG was prepared by the same method with serum from nontreated rabbits.

Cell Culture

SMCs were isolated from aorta of Sprague-Dawley rats by enzymatic digestion as described. The cells were cultured in DMEM (GIBCO/BRL) supplemented with 10% fetal calf serum (GIBCO/BRL), penicillin (100 U/mL), and streptomycin (100 μg/mL). Cells were harvested for passaging at subconfluence with a trypsin-EDTA solution. The data are mean values from two independent experiments.

[Ca2+]i Measurement

We basically performed [Ca2+]i measurement as per the method of Lum et al. Rat SMCs grown on glass coverslips were loaded with 5 μmol/L fura 2-AM and, at the same time, treated with nonimmune or immune IgG (0.145 mg/mL) for 60 minutes at 37°C. The cells were washed with HBSS (GIBCO/BRL) after loading and treatment and then placed in a perfusion chamber positioned on the stage of a Nikon Diaphot microscope, which was coupled to an intracellular Ca2+ analyzer (model FC-200, Mitsubishi Kasei). An optically isolated field of cells, containing 24 cells, was excited at wavelengths of 340 and 380 nm, and emitted light was collected at 510 nm with a photomultiplier. One minute after the excitation, 3 nmol/L thrombin (Sigma T 6759), 300 ng/mL PDGF-BB (Boehringer Mannheim), 300 nmol/mL angiotensin II (Peptide Institute), or 300 nmol/mL U46619 (Cayman Chemical Co) was added to the chamber without stirring, [Ca2+]i mobilization in each cell was monitored for 1 minute, and then 10 μmol/L ionomycin was added to evaluate the fluorescence of Ca2+-saturated fura 2. Background autofluorescence (in the absence of fura 2) determined at the beginning of each day’s experiment was subtracted automatically during data collection. The fluorescence ratio at excitation wavelengths of 340 and 380 nm (340/380) was calculated using the CA 1 program (Mitsubishi Kasei).

Four-tenths tracings showing the effect of each stimulant tested on the [Ca2+]i, response were used to determine the ratio of the cells responsive to the addition of each stimulant to the total cell number (24 cells). A value of 100% indicates that all 24 cells responded to the stimulant. This experiment was performed three times. The same results were obtained each time.

Cell Proliferation Assay

Rat SMCs were plated at 3×10^4 per well into 96-well plates, cultured overnight, growth-arrested in serum-free DMEM without phenol red for 24 hours, and then stimulated with 0.3 nmol/L thrombin for 24 hours. Various concentrations of nonimmune or immune IgG were added to the cultures at 4 hours before stimulation by thrombin. Twenty-four hours after the stimulation, the cell number was measured by using a Cell Counting kit (Dojindo) based on a highly water-soluble formazan dye, WST-1. A solution of WST-1 was prepared at a concentration of 5 mmol/L in 20 mmol/L HEPES (pH 7.4) containing 1-methoxy-5-methylphenazinium methyl sulfate (0.2 mmol/L) and then sterilized by filtration through a 0.22-μm membrane filter. Ten microliters of this solution was added to each well 24 hours after the stimulation with thrombin and mixed well. The absorbance was measured at 450 nm against 650 nm after incubation of the cells for 1 hour at 37°C in a CO2 incubator.

Numbers of cells in the presence or absence of 0.3 nmol/L thrombin were represented as 100% or 0% on the vertical axis, respectively. Optical density at 450 nm against 650 nm was 1.05 or 0.31 in the presence or absence of thrombin, respectively.

Thrombin Clotting Time Assay

For thrombin clotting time assay, 100 μL of rat thrombin (20 U/mL, Sigma) and 100 μL of diethylbarbiturate acetate buffer, IgGs, or hirudin were incubated for 60 minutes at room temperature. After the addition of 100 μL of rat 10-fold–diluted plasma, the time until a clot formed was measured with a KC10 Koagulometer (Amelung). The data are mean values from two independent experiments.

Platelet Aggregation

Blood obtained from several rats was anticoagulated with 0.38% sodium citrate. Platelet-rich plasma was prepared by centrifugation at 800g for 10 minutes and was washed once with saline containing 0.1% EDTA. The resulting washed platelets were suspended in Tyrode’s buffer (pH 7.4) without Ca2+ at a concentration of 40×10⁴ platelets/μL and then used for aggregation assay. Two hundred microliters of the washed platelet suspension was preincubated with immune or nonimmune IgG dissolved in Dulbecco’s PBS for 60 minutes at room temperature and then stimulated with rat thrombin (0.5 U/mL) in microcuvettes (final, 250 μL) with continuous stirring (1000 rpm). Aggregation of platelets was monitored with an aggregometer (Mebanix). The area under the aggregation curve stimulated with thrombin in the absence of any IgG was represented as 100% on the vertical axis. The data are mean values from two independent experiments.

Determination of Rabbit IgG in Rat Serum

Sandwich ELISA was performed by using anti-rabbit IgG–coated microtiter plates (Boehringer Mannheim). For blocking of nonspecific binding, 200 μL of Block Ace (Yukijirushi Nyugyo) as a blocking agent was added to each well, and the plates were incubated for 1 hour at room temperature and then washed three times with the
washing buffer. Then 50 μL of anti-rabbit IgG–horseradish peroxidase conjugate (ZYMED, monoclonal antibody reagent) at a 1:2000 dilution was added to each well. After 1 hour of incubation, the plates were washed three times with the washing buffer, and 50 μL of 0.01% o-phenylenediamine dihydrochloride sodium (P 9187, Sigma) as a substrate for peroxidase was added to each well. The plates were incubated for 1 hour at room temperature and then examined with an immunoplate reader (Molecular Devices Corp) after the addition of 40 μL of 1N sulfonic acid to stop the enzyme reaction. The absorbance was measured at 490 nm with a microtiter plate photometer. Cross-reactivity to rat IgG was not observed in this system.

**Serum Levels of Immune IgG After a Single Injection Into Rat Tail Vein**

Three rats were administered immune IgG via the tail vein at a dose of 2 mg per rat, which was the same dose that the rats with balloon catheter–induced injury received. Blood (50 μL) was collected from the tail vein at several time points. Sera obtained from these samples were diluted 1:2000 with Dulbecco’s PBS containing 0.2% Tween 20 and then subjected to ELISA. Serum levels of rabbit IgG after a single dose were analyzed by using the two-compartment model to obtain pharmacokinetic parameters. On the basis of these parameters, we calculated the rabbit IgG concentration in rat serum after multiple (16) injections using the MULTI program.22

**Determination of PT, APTT, and Fibrinogen Contents**

A 300 μL aliquot of citrated plasma from each rat that had been killed was examined in a fully automatic coagulometer AMAX (Amelung). Reagents used for determining PT, APTT, and fibrinogen contents were thromboplastin D, APTT-LS, and Fibrinogen Assay Set, respectively, purchased from MC Medical Co, Ltd.

**Balloon Catheter–Induced Injury Model**

Male Sprague-Dawley rats (average weight, 334 g) were anesthetized with Nembutal (5 mg/100 g), and the left carotid artery of each animal was isolated by midline cervical incision. A 2F Fogarty catheter (Baxter) was introduced through the external carotid artery of each rat and advanced to the aortic arch, and then the balloon was inflated to produce moderate resistance to catheter movement and gradually withdrawn to the entry point.23 The entire procedure was repeated three times for each rat. Each animal received an injection of saline, nonimmune IgG (2 mg per rat), or immune IgG (2 mg per rat) via the tail vein daily for 16 days from 2 days before the operation. Fourteen days after the operation, animals were sedated with ether. All blood was drawn from the abdominal aorta by using a 50-μL syringe with a 22-gauge needle. Citrated plasma was obtained from 2 mL of blood anticoagulated with 0.38% sodium citrate for testing APTT, PT, or fibrinogen content. For determination of serum levels of rabbit IgG, the remaining blood was used for preparation of serum. The carotid arteries were removed immediately, rinsed in saline, dissected free of adherent fat and connective tissue, and fixed in 10% formalin in isotonic PBS (pH 7.4). The fixed carotid arteries were cut into five portions, which were embedded in paraffin for sectioning. The sections were stained by the elastica van Gieson method, and the intimal and medial cross-sectional areas were measured for 7 animals treated with saline, 7 animals treated with nonimmune IgG, and 8 animals treated with immune IgG. Measurements were made on five carotid sections per rat, and the intimal and medial areas were calculated for each rat using a digitizing pad (Wacom) connected to a computer.

**Northern Blotting**

Northern blot analysis was performed by using mRNA from carotid arteries of rats that had been treated with nonimmune or immune IgG at the indicated time points after balloon injury. The carotid arteries were removed, rinsed in saline, carefully dissected free of adherent fat and connective tissue, and then immersed in liquid nitrogen. Total cellular RNA was prepared by acid guanidium thiocyanate extraction after grinding the frozen arteries in a mill and homogenizing the ground arteries with a Polytron homogenizer (Hiijiriiseiko) and quantified by measuring the absorbance at 260 nm. Total RNA was size-fractionated by electrophoresis on 1% agarose gel. The subsequent procedure was performed according to the method of Schini-Kerth et al.24 Finally, the autoradiographs were analyzed by scanning densitometry. Thrombin receptor mRNA levels were normalized to the respective 18S ribosomal RNA levels and expressed as fold increases of the signal obtained with the carotid arteries 4 hours after angioplasty, because thrombin receptor mRNA was not detected in the uninjured arteries.

**Cell Proliferation In Vivo**

Cells that had been stimulated to enter the cell cycle within the intima and the media were identified by immunocytochemistry for PCNA. The PCNA index was defined as the number of PCNA-positive cells divided by the sum of PCNA-positive and -negative cells, expressed as a percentage. Sections (4 μm) were cut from each paraffin-embedded arterial segment and mounted on poly-L-lysine–coated glass slides. After dewaxing, they were taken through ethyl alcohol and immersed for 10 minutes in methanol mixed with 0.5% hydrogen peroxide to block endogenous peroxidase activity. Sections were incubated with a primary antibody to PCNA (PC10, DAKO) at a dilution of 1:1000 for 1 hour and then with a biotinylated anti-mouse IgG second antibody (Vector Laboratories) for 30 minutes. A solution of avidin-biotin complex (DAKO) in Tris-buffered saline was applied for 30 minutes, and diaminobenzidine mixed with hydrogen peroxide was finally added as a chromogen. Sections were subjected to a light hematoxylin counterstain such that positively stained nuclei appeared brown and negatively stained nuclei appeared blue.

Cell counting was performed at high magnification using a video microscope that projected the image onto a visual display unit (Nikon).

Cells were considered positive for PCNA expression only in the presence of an intense brown staining of the nucleus. The number of positively stained nuclei was counted within the media or neointima in each section. The total cell population in the media or neointima was established by counting all the counterstained nuclei.

**Results**

The polyclonal antibody to rat thrombin receptor that we used was obtained by immunizing rabbits with the peptide described above and was confirmed to inhibit both intracellular signaling induced by thrombin and the mitogenic response to thrombin in vitro before being used in vivo (Figures 1 and 2).
The anti-rat thrombin receptor IgG (immune IgG) potently inhibited the increase of [Ca$^{2+}$], in rat SMCs stimulated with thrombin, whereas nonimmune IgG did not (Figure 1). When angiotensin II, U46619 (thromboxane A$_2$ agonist), or PDGF-BB was used as a stimulant instead of thrombin, the immune IgG had no effect on the increase of [Ca$^{2+}$]. (Figure 1). Furthermore, when the antibody was preincubated with the peptide used as an antigen, it could no longer block the aggregation of rat platelets and found that at concentrations up to 0.8 μmol/L, the immune IgG did not inhibit the aggregation of rat platelets stimulated by thrombin (Figure 3).

It was difficult to know what antibody level would be effective in vivo. Hence, we determined the serum levels of rabbit IgG after a single intravenous injection of 2 mg per rat (≈6 mg/kg) of immune IgG, which was the same dose used in the following in vivo experiment. This injection resulted in a serum concentration of 0.52 μmol/L at 1 hour, and the serum half-life of the IgG was 66 hours in the rat (Figure 4).

Serum levels of rabbit IgG after a single dose were analyzed by using the two-compartment model to obtain pharmacokinetic parameters. On the basis of these parameters, we calculated the rabbit IgG concentration in rat serum after multiple (16) injections. No significant difference was found between the calculated and the measured serum levels of rabbit IgG 24 hours after the final injection (C$_{\text{min}}$, as shown in Table 2), suggesting that rapid clearance of the injected rabbit IgG from blood circulation by generation of an autoantibody to the rabbit IgG does not occur. Hence, the serum concentration of IgG after several injections, namely the steady-state level of IgG, might be >0.89 μmol/L. On the basis of our in vitro study (Figure 2), cell proliferation should be inhibited by ≥80% in this range of immune IgG levels. Therefore, we chose to administer immune IgG at a dose of 2 mg per injection for each rat.

We then determined the effect of anti-rat thrombin receptor IgG on intimal SMC accumulation 2 weeks after angioplasty in rats treated with saline (n=7), nonimmune IgG (n=7), or immune IgG (n=8). Morphological analysis revealed that minimal intimal accumulation occurred in the immune IgG–treated group, whereas extensive SMC accumulation was observed in the other treatment groups (Figure 5). The measurements of arterial segments showed that administration of the antibody resulted in 35.8% and 46.8% reduction in the area of the neointima compared with measurements of

### Figure 2. Effect of antibody to thrombin receptor on cell proliferation in rat aortic SMCs. Various concentrations of nonimmune IgG (C) or immune IgG (●) were added to the cultures at 4 hours before stimulation with thrombin. Twenty-four hours after the stimulation, the cell number was measured. The number of cells in the presence or absence of 0.3 nmol/L thrombin was represented as 100% or 0% on the vertical axis, respectively. The data are mean±SEM from three independent experiments.

### Figure 3. Effect of antibody to thrombin receptor on thrombin-induced platelet aggregation in rats. A suspension of washed platelets in Tyrode’s buffer (pH 7.4) without Ca$^{2+}$ was adjusted to 4.0×10$^7$/μL. Two hundred microliters of the washed platelet suspension was preincubated with immune (●) or nonimmune (▲) IgG dissolved in Dulbecco’s PBS for 60 minutes at room temperature and then stimulated with rat thrombin (0.5 U/mL) in microcuvettes (final, 250 μL) with continuous stirring (1000 rpm). Aggregation of platelets was monitored with an aggregometer (Mebanix). The area under the aggregation curve obtained with thrombin in the absence of any IgG was represented as 100% on the vertical axis. The data are mean values from two independent experiments.

### Table 1. Effect of Antibody to Thrombin Receptor on Thrombin Time

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Nonimmune IgG</th>
<th>Immune IgG</th>
<th>Hirudin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clotting, s</td>
<td>22.6</td>
<td>22.6</td>
<td>22.9</td>
</tr>
</tbody>
</table>

Rat thrombin preincubated with buffer, nonimmune or immune IgG, or hirudin for 60 minutes was added to rat plasma, and the time until a clot formed was measured. Values are means from two independent experiments.
segments from the saline-treated and nonimmune IgG–treated groups, respectively (P<0.01), without any apparent effect on medial SMCs (Figure 6). In the analysis of the ratios of intimal to medial cross-sectional areas, the injection of immune IgG caused 43.7% and 53.1% inhibition compared with saline and nonimmune IgG treatment, respectively. These observations provide direct evidence that thrombin receptor activation functions in vivo in the accumulation of SMCs that occurs after balloon catheter–induced injury.

We also assessed the effect of anti-rat thrombin receptor IgG on thrombin receptor expression in carotid arteries after angioplasty in rats treated with nonimmune or immune IgG. Thrombin receptor mRNA levels were measured by Northern analysis. A 3.0- or 2.4-fold increase in the level of thrombin receptor mRNA in nonimmune-treated or immune IgG–treated groups, respectively, was found 24 hours after angioplasty (Figure 7). Thereafter, the signal almost reached a plateau level at 7 days in the nonimmune IgG–treated group. At 1 day after angioplasty, the level of the receptor mRNA showed a 20% reduction in the immune IgG–treated group compared with the nonimmune IgG–treated group, but this was not statistically significant. The level was decreased significantly in the immune IgG–treated group 14 days after angioplasty.

To obtain a relative measure of the number of cells that had been stimulated to enter the cell cycle and proliferate, we studied the expression of the cyclin PCNA. PCNA is a DNA polymerase accessory protein that is essential for DNA synthesis and is therefore expressed maximally during the S phase of the cell cycle in all dividing cells.25,26 Immunocytochemical analysis revealed a small number of PCNA-positive cells in the intima in the immune IgG–treated group, whereas PCNA-positive cells were prominent in the other treatment groups. The injection of immune IgG caused 40.5% and 43.0% decreases (P<0.01) of the PCNA index in

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**Table 2. Pharmacokinetic Parameters of Rabbit IgG After Multiple (16) Intravenous Injections**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Calculated Value</th>
<th>Immune IgG</th>
<th>Nonimmune IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;, μmol/L</td>
<td>1.70±0.29 (3)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C&lt;sub&gt;min&lt;/sub&gt;, μmol/L</td>
<td>1.11±0.23 (3)</td>
<td>0.89±0.07 (8)</td>
<td>1.01±0.14 (7)</td>
</tr>
</tbody>
</table>

C<sub>max</sub> and C<sub>min</sub> represent serum levels of rabbit IgG just after and 24 hours after final injection, respectively; ND, not determined. Numbers in parentheses represent numbers of rats. Values are mean±SEM.
the intima 2 weeks after angioplasty compared with saline and nonimmune IgG treatment, respectively (Table 3). This result is well correlated with the observed ratios of intimal to medial cross-sectional areas. In the early stage (3 days) after angioplasty, ie, the first wave of SMC proliferation, the PCNA index in the media was also decreased significantly by continuous injection of immune IgG over 5 days.

As expected from the result of our in vitro study (Table 1), there were no significant differences in coagulation parameters such as PT, APTT, and fibrinogen content among the saline-treated, nonimmune IgG–treated, and immune IgG–treated groups (Table 4).

Discussion

To our knowledge, this is the first time that an antibody has been used to inhibit specifically the thrombin receptor signaling in rats with balloon catheter–induced injury. The antibody used in the present study did not inhibit either coagulation events in vitro and in vivo (Tables 1 and 4) or the aggregation of rat platelets stimulated by thrombin (Figure 3), suggesting that the antibody acted not by reducing the amount of thrombosis but by directly inhibiting SMC proliferation. This view is further supported by the observations that blockade of the thrombin receptor causes a decrease of the PCNA index, which is a marker of proliferative cells in vivo (Table 3) and also that upregulation of thrombin receptor expression in SMCs occurs very early (within several hours) after balloon catheter–induced injury and continues throughout neointimal development in the rat (Figure 7).12

On the basis of the hypothesis by Wilcox et al12 and other reports,24,27–29 we consider that in the early stage of vascular lesion formation, bFGF and both PDGF and serotonin released from aggregating platelets cause a proliferative response of SMCs and induce thrombin receptor expression, and then thrombin amplifies the bFGF action and also produces PDGF through an autocrine mechanism via thrombin receptor signaling. Thereafter, PDGF expressed by proliferating SMCs, in turn, maintains thrombin receptor expression throughout neointimal development. Furthermore, our data indicate that SMC proliferation in the media was inhibited by the injection of immune IgG 3 days after angioplasty (Table 3). Thus, the suppression of both the neointimal formation and the expression of thrombin receptor mRNA in injured carotid arteries at a later stage may be due to the inhibition of SMC proliferation at the early stage via the blocking of thrombin receptor signaling by the antibody. Our results suggest that thrombin receptor activation is involved in the accumulation of neointimal SMCs after angioplasty; thus, blockade of the thrombin receptor is an attractive candidate for an antirestenosis strategy, although

**Figure 6.** Intimal and medial cross-sectional areas and intimal/medial ratios of rat carotid arteries. A and B, Cross-sectional areas of the intimal and medial regions, respectively, of carotid arteries from rats treated with saline (S), nonimmune IgG (NI), and immune IgG (I). C, Ratio of intimal to medial areas for the same three groups of animals. All results are mean±SEM from 7 animals (S and NI) or 8 animals (I). The Duncan multiple range test was used for statistical analysis of the data. Values of P<0.05 were considered to be significant.

**Figure 7.** Time course of thrombin receptor (TR) expression in carotid arteries after balloon injury. Northern blot analysis was performed as described in "Materials and Methods." TR mRNA and 18S ribosomal RNA levels were quantified by densitometry. Changes in TR mRNA are expressed as a fold increase of the signal obtained with the carotid arteries 4 hours after angioplasty. The data are mean±SEM from three animals treated with nonimmune IgG (○) or immune IgG (●). *P<0.05 versus saline group 14 days after angioplasty (Student t test).

**Table 3.** Percentage of PCNA-immunoreactive SMCs in the Media or Neointima of Carotid Arteries After Balloon Injury

<table>
<thead>
<tr>
<th>Days After Injury</th>
<th>Area</th>
<th>Saline</th>
<th>Nonimmune IgG</th>
<th>Immune IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Media</td>
<td>20.5±2.1 (4)</td>
<td>22.3±2.5 (4)</td>
<td>13.8±1.8* (5)</td>
</tr>
<tr>
<td>14</td>
<td>Intima</td>
<td>11.6±0.9 (7)</td>
<td>12.1±0.8 (7)</td>
<td>6.9±0.7* (8)</td>
</tr>
</tbody>
</table>

Numbers in parentheses represent numbers of animals. Values are mean±SEM.

*P<0.01 vs saline or nonimmune IgG group (Duncan multiple range test).

**Table 4.** Effect of Antibody to Thrombin Receptor on PT, APTT, and Fibrinogen Concentration 14 Days After Angioplasty

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>PT, s</th>
<th>APTT, s</th>
<th>Fibrinogen, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>7</td>
<td>13.7±0.37</td>
<td>52.4±8.7</td>
<td>18.0±2.4</td>
</tr>
<tr>
<td>Nonimmune IgG</td>
<td>7</td>
<td>14.0±0.38</td>
<td>67.4±7.9</td>
<td>17.3±0.38</td>
</tr>
<tr>
<td>Immune IgG</td>
<td>8</td>
<td>13.7±0.29</td>
<td>62.3±11.3</td>
<td>17.4±0.58</td>
</tr>
</tbody>
</table>

n indicates the number of rats. Values are mean±SEM.
experimental and pathological studies have indicated that many factors may play a role in the restenosis process.

Gerdes et al. reported that a short-term treatment with hirudin (bolus of 1 mg/kg IV immediately before injury, followed by infusion of 1 mg · kg⁻¹ · h⁻¹ for 2 hours and an injection of 6 mg/kg SC) significantly reduced the neointimal area not only in the first injury but also in the second injury in rabbits. They also found that a 3-day infusion of hirudin after balloon injury resulted in a significant reduction of the neointimal area in rats, although short-term treatment with hirudin was ineffective in this species. They concluded that there was considerable interspecies variation in the time frame of susceptibility for reduction of neointimal growth by inhibition of thrombin after angioplasty and that the differences in responsiveness to hirudin treatment between rats and rabbits may be partly explained by differential activity of the coagulation system or by variable thrombogenicity of the injured vessel wall surface. There may also be a difference in responsiveness to thrombin between rat and rabbit SMCs, since we confirmed the inhibitory effect of the antibody to rat thrombin receptor on rat SMC proliferation at an early stage after angioplasty (Table 3). Hence, the discrepancy between the finding of Ragosta et al. and that hirudin failed to reduce SMC proliferation within the first 7 days after angioplasty in rabbits, and our result may be due to the species difference.

Cook et al. reported that a polyclonal antibody raised against a peptide derived from the thrombin-binding exosite region of the cloned human thrombin receptor prevents arterial thrombosis without altering hemostatic parameters or inhibiting the enzymatic activity of thrombin toward fibrinogen in the African green monkey. Human thrombin receptor expression was observed not only in platelets but also in endothelial cells and neointimal SMCs after angioplasty in the human vascular system. These observations imply that blockade of the human thrombin receptor may be a more effective approach to treatment than inhibition of the GP IIb/IIIa receptor.

Knockout of the gene encoding the thrombin receptor provided definitive evidence for a second platelet thrombin receptor in mouse platelets. Moreover, rat platelets do not undergo shape change and aggregation in response to thrombin receptor–derived activating peptides, although the platelets respond strongly to thrombin, suggesting that rat platelets may also have a second platelet thrombin receptor subtype, different from the cloned human thrombin receptor. As shown in Figure 3, our rat platelet aggregation data also favor the view that a second platelet thrombin receptor, different in type from the cloned human thrombin receptor, may be expressed on rat platelets. Recently, a second platelet thrombin receptor, designated PAR 3, distinct from the cloned human thrombin receptor (PAR 1), was cloned from rat platelets.

Herbert et al. reported that intimal hyperplasia following vascular injury was not inhibited by an antisense thrombin receptor oligodeoxynucleotide in rabbits. As they noted, this observation seemed inconsistent with the results obtained with recombinant hirudin administered as a daily subcutaneous injection, which confirmed the previous finding that specific inhibition of thrombin by recombinant hirudin significantly reduced myointimal thickening in rabbits.

In the present study, we have shown that an antibody to rat thrombin receptor cloned from rat SMCs inhibits neointimal SMC accumulation after angioplasty in rats. Taken together, the data imply that specific inhibition of the thrombin receptor–signaling pathway is much more effective as a means to prevent intimal thickening than inhibition of the expression of the receptor by using an antisense oligodeoxynucleotide. Blockade of the thrombin receptor–signaling pathway is thus an attractive approach for prevention of the proliferative response of SMCs to thrombin.

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References


Antibody to Thrombin Receptor Inhibits Neointimal Smooth Muscle Cell Accumulation Without Causing Inhibition of Platelet Aggregation or Altering Hemostatic Parameters After Angioplasty in Rat
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