Development of Hyperfibrinogenemia in Spontaneously Hypertensive and Hyperlipidemic Rats: A Potentially Useful Animal Model as a Complication of Hypertension and Hyperlipidemia

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Abstract: According to current concepts, hypertension and hyperlipidemia cause vascular damage that leads to a hypercoagulative state. In this study, we investigated whether spontaneously hypertensive and hyperlipidemic rats (SHHR) can be a useful experimental model for complications in combined hypertension and hyperlipidemia, by comparing coagulative and fibrinolytic activities in SHHR with those in spontaneously hypertensive rats (SHR) and spontaneously hyperlipidemic rats (HLR). We measured coagulation and fibrinolysis markers in plasma and levels of fibrinogen and prothrombin mRNA in livers of eight-month-old male Wistar Kyoto rats (WKY), Sprague-Dawley rats (SD), SHR, HLR and SHHR. The plasma levels of fibrinogen in SHR, HLR and SHHR were significantly higher than those in WKY and SD, and were highest in SHHR. Higher plasma levels of antithrombin III and plasminogen were detected in increasing order in SHR, HLR and SHHR as compared to those in WKY and SD. Hepatic mRNA expressions of fibrinogen chains and prothrombin were enhanced in SHR, HLR and SHHR, resulting in increased plasma fibrinogen levels in SHHR. These results suggest that hypertension and hyperlipidemia can each cause hypercoagulation, with hyperlipidemia being a stronger factor than hypertension. Since a greater hypercoagulative state is a complication of combined hypertension and hyperlipidemia, the SHHR model is a good system for studying the early stage of atherosclerosis ensuing from hyperfibrinogenemia.

Key words: atherosclerosis, fibrinogen, hypercoagulation, mRNA

Introduction

Hypertension and hyperlipidemia, important risk factors for cardiovascular and cerebrovascular disease, are believed to cause vascular damage that progresses to a hypercoagulative state [14, 15, 30], characterized by...
elevated levels of fibrinogen, von Willebrand factor, plasminogen activator inhibitor-1, fibrin D-dimer, and factor VII activity [6, 22]. As shown by numerous prospective epidemiological studies and clinical observations, fibrinogen is a risk factor for cardiovascular disease and for hyperlipidemia and hypertension [5, 8, 17, 18]. Increased plasma fibrinogen levels are associated with and enhance major atherogenic cardiovascular risk factors, such as age, systolic blood pressure, total and high-density lipoprotein cholesterol, diabetes mellitus, angina pectoris, smoking and familial acute myocardial infarction [17]. In addition, the probability of developing cardiovascular disease increases when these risk factors are combined. Clinical studies have demonstrated that improving lipid metabolism and lowering blood pressure are the most effective means of preventing cardiovascular events in hypertensive patients with dyslipidemia [10, 24, 31]. In today’s environment, with an increasing occurrence of life-style related diseases, an appropriate animal model is essential for the development of prophylactic and therapeutic agents against atherosclerosis, to save the lives of patients and improve their quality of life.

Reports of studies using animal models of hypertension and hyperlipidemia are scant, because a suitable animal model is lacking and the few strains with heritable hyperlipidemia are associated with atherogenesis [4, 29]. We have developed a new model of combined hypertension and hyperlipidemia by crossbreeding spontaneously hypertensive rats (SHR) originated from Wistar Kyoto rats (WKY) with spontaneously hyperlipidemic rats (HLR) originated from Sprague-Dawley rats (SD). A new strain of spontaneously hypertensive and hyperlipidemic rats (SHHR) with endothelial degeneration and lipid deposits in the aorta was established at generation F10 through selective mating of brothers and sisters [21]. SHHR have a systolic blood pressure greater than 150 mmHg and a plasma cholesterol concentration greater than 150 mg/dl. Because the vascular disorder in female rats was found to be stronger than in male rats [21], we examined the characteristics of female rats in previous paper [2]. The enhancement of blood coagulation was observed in female SHHR, more than in female SD [2]. However, there has been no investigation of the coagulation and fibrinolytic systems in SHHR males.

The present study was designed to investigate the coagulation and fibrinolytic activity, especially the plasma fibrinogen level and hepatic mRNA levels of fibrinogen chains, in male WKY, SD, SHR, HLR and SHHR. We also assessed which of hypertension or hyperlipidemia strongly contributed to the hypercoagulation in SHHR. Furthermore, we discuss the usefulness of male SHHR as a model of complication of hypertension and hyperlipidemia.

**Materials and Methods**

**Experimental animals and blood collection**

Eight-month-old male WKY, SD, SHR, HLR and SHHR were used in this study. SHR (model for hypertension) and WKY were from stock originally supplied by the late Dr. K. Okamoto (Kinki University, Higasiosaka, Japan), whereas HLR (model for hyperlipidemia) were derived from brother-sister matings of selected SD (CLEA Japan, Tokyo, Japan). Eight-week-old male HLR and female SHR were interbred. Selected brothers and sisters were inbred to obtain offspring with both high blood pressure (>150 mmHg) and high plasma cholesterol (>150 mg/dl), and SHHR were generated in the breeding colony by brother-sister mating [21]. All procedures were performed according to the guiding principles for the care and use of laboratory animals of the Japanese Pharmacological Society. Rats were housed in a semi-barrier system under controlled room temperature (23 ± 1°C), humidity (55 ± 5%), and twelve-h light/dark cycle (6:00 am to 6:00 pm light), with access to standard food (CE-2, CLEA Japan, Tokyo, Japan) and tap water *ad libitum*. When rats were 4 months old, systolic blood pressure was measured using the tail-cuff method (PS-100; Riken Kaihatsu, Tokyo, Japan) in conscious rats placed on a hot-plate (37°C). Systolic blood pressure was about 160 mmHg, 190 mmHg, 125 mmHg, 140 mmHg and 125 mmHg in SHHR, SHR, HLR, WKY and SD, respectively [21]. In the present experiments, we determined systolic blood pressure of SHHR before sacrifice, and confirmed that blood pressure was over 150 mmHg.

Blood specimens were taken under pentobarbital anesthesia (35 mg/kg intraperitoneally) from the inferior vena cava and were collected in a 3.2% sodium citrate solution (9:1, v/v). Coagulation and fibrinolysis were assayed on the citrated plasma supernatant.
Biochemical determination of plasma levels

Levels of total cholesterol (TC) and triglyceride (TG) in plasma were measured by colorimetry (Cholesterol-B Test and Triglyceride-G Test: Wako Pure Chemical, Tokyo, Japan). Plasma fibrinogen levels were measured as previously reported [28]. Briefly, citrated plasma was mixed with CaCl₂ and trans-4-(aminomethyl) cyclohexane-1-carboxylic acid (Sigma-Aldrich, St. Louis, MO, USA), and the mixture was incubated at 37°C. The non-clottable proteins were removed by centrifugation, and the protein content of the fibrin precipitate was determined. Plasma levels of antithrombin III (ATIII), α2-plasmin inhibitor (α2-PI), and plasminogen were determined by chromogenic assay with synthetic substrates (Daiichi Chemical Pharmacy, Tokyo, Japan). We used urokinase (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) as the plasminogen activator. Plasma interleukin-6 (IL-6) levels were measured by a sandwich enzyme-linked immunosorbent assay (BioSource Int. Camarillo, CA, USA).

Reverse transcription-PCR analysis

Total RNA was extracted from the median lobe of the rat livers. Approximately 150 mg of liver tissue were excised from a similar site on the lobe of each rat to be assessed and homogenized on ice with Isogen (Nippon Gene, Tokyo, Japan). The yield of total RNA was between 2 and 6 mg/g of wet liver tissue. Sense and antisense primers for rat fibrinogen, prothrombin and IL-6 receptor genes were designed based on the published complete cDNA sequences. The sense-primer sequence of α-fibrinogen was TCT GAT GAC ACT CCA ACC TG and the antisense-primer was TCA CTG GCC TCA TCT GC. The sense-primer sequence of β-fibrinogen was CGT CAA CTG CAA CAT CCC G and the antisense-primer was CAG TAC CAC GAT CCC TTC C. The sense-primer sequence of γ-fibrinogen was CAT CCC ATA CGC ACT GAG A and the antisense-primer was CAT GGT GGT TTC CTT CAT GG. The sense-primer sequence of prothrombin was CAA GCA CTC CAG AAC CAG A and the antisense-primer was TCC TCG CTT GGT GTC ATT C. The sense-primer sequence of IL-6 receptor was CTC TCA AGC TAT CCT CTC AG and the antisense-primer was CAG AAG TAG AGA GAG GAT GG. The sense-primer sequence of β-actin was TTG TAA CCA ACT GGG ACG ATA TGG and the antisense-primer was GAT CTT GAT CTG CAT GGT GCT AGG. Reverse transcription mixtures (final volume 50 µl) contained 2.5 µg of total liver RNA, 125 U of murine leukemia virus (MuLV) reverse transcriptase (Perkin-Elmer, Branchburg, NJ, USA), 2.5 µM random hexamer, and dNTPs (1mM each) in buffer (50 mM KCl, 10 mM Tris-HCl, 3.5 mM MgCl₂, 50 U of RNase inhibitor, pH 8.3). A mixture of total RNA and random hexamer in H₂O was heated at 70°C for 10 min and chilled on ice. PCR buffer, dNTPs, MgCl₂, MuLV reverse transcriptase, and RNase inhibitor were then added and the complete mixture was incubated at 42°C for 15 min, heated to and maintained at 99°C for 5 min, and the reaction was stopped.

PCR reaction mixtures (total volume 25 µl) contained 5 µl of cDNA, 0.4 µM of each primer, 2 µl of each dNTP and 1 U of Taq polymerase (Ampli Taq, Sigma-Aldrich) in PCR buffer (40 mM KCl, 8 mM Tris-HCl, 0.5 M MgCl₂, pH 8.3). PCR amplification was performed in a Takara PCR thermal cycler (Takara Shuzo, Tokyo, Japan). Fibrinogen α- and β-chain cDNAs were amplified by 24 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 60 s. Fibrinogen γ-chain cDNA was amplified by 24 cycles of 94°C for 30 s, 53°C for 60 s and 72°C for 60 s. Prothrombin cDNA was amplified by 26 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s. IL-6 receptor cDNA was amplified by 33 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 60 s. As an internal standard, β-actin mRNA was amplified from a cDNA template under PCR settings used to amplify fibrinogen α- and β-chain cDNAs. A 10 µl volume from each reaction was resolved on 2% agarose gel electrophoresis, stained with ethidium bromide and photographed under ultraviolet light (Atto Corp., Tokyo, Japan). The photographed images were analyzed by computerized densitometric scanning using the image-processing program Scion Image. RNA levels were expressed as ratio of the band density of the target PCR product to the band density of amplified β-actin cDNA.

Statistical analysis

Data are presented as mean ± SEM. Pair-wise comparisons between groups were made by the Tukey test following one-way ANOVA for unpaired observations. Differences with P values of less than 0.05 were considered significant.
Results

Body weights and plasma lipids

Table 1 shows body weights (BW), liver weight to body weight (LW/BW) ratios, and plasma levels of TC and TG in WKY, SD, SHR, HLR, and SHHR. As can be seen, SD, HLR, and SHHR were heavier than WKY and SHR, and LW/BW ratios for SHR, HLR and SHHR were greater than those for WKY and SD. Plasma levels of TC were significantly elevated in HLR and SHHR as compared to those in WKY, SD and SHR. Plasma levels of TG in SD, SHR and SHHR were higher than those in WKY and SHR.

Coagulative and fibrinolytic activity

Figure 1 shows plasma levels of fibrinogen, ATIII, plasminogen and α₂PI for each group. Plasma fibrinogen levels were significantly higher in SHR, HLR and SHHR than in WKY and SD. They were highest in SHHR, and higher in HLR than in SHR. Plasma levels of ATIII and plasminogen increased according to the same trend, except that ATIII levels were not significantly increased in SHR. Plasma α₂PI levels were also highest in SHHR, but did not differ significantly in the other strains.

Fibrinogen and prothrombin mRNA levels in rat liver

Figure 2 shows the intensity of PCR product bands with the number of PCR cycles for β-actin, prothrombin, α-, β- and γ-fibrinogen and IL-6 receptor cDNAs. PCR products were quantified by measuring fluorescence intensity of the relevant bands, which increased with each PCR cycle. Figure 3 shows the hepatic mRNA expression of α-, β- and γ-chains of fibrinogen in each rat group. The levels of β-fibrinogen mRNA were significantly elevated in SHR, HLR and SHHR as compared to WKY and SD, and were higher in SHHR than in SHR and HLR. Expression of γ-fibrinogen mRNA was also higher in SHR, HLR and SHHR than in WKY and SD, whereas α-fibrinogen mRNA expression was significantly elevated in HLR and SHHR as compared to WKY and SD, but not in SHR. Figure 4 shows that the hepatic mRNA expression of prothrombin was significantly higher in SHR, HLR and SHHR than in WKY and SD.

Plasma IL-6 level and hepatic expression of IL-6 receptor mRNA

Figure 5 shows plasma levels of IL-6 in WKY, SD, SHR, HLR and SHHR. Plasma IL-6 levels were significantly higher in HLR than in WKY, SHR and SHHR. Unlike plasma fibrinogen (Fig. 1), SHHR did not show a higher tendency for plasma IL-6. Figure 6 shows hepatic mRNA expression of IL-6 receptor in each rat group. The levels of IL-6 receptor mRNA in SHR and HLR were significantly higher than those in WKY and SHHR.

Discussion

In the present study of male rats, we demonstrated that plasma levels of fibrinogen were significantly higher in increasing order in SHR, HLR and SHHR than in WKY and SD. These plasma fibrinogen levels were accompanied by the enhancement of hepatic mRNA expression of fibrinogen chains, which are higher in SHHR than in SHR and HLR. These results suggest that hypertension and hyperlipidemia can each cause hyperfibrinogenemia, and that a greater hyperfibrinogenemic state was created by a complication of combined hypertension and hyperlipidemia.

Increases in plasma fibrinogen may contribute to the development of atherosclerosis and ischemic cardiovascular disease [8, 17, 18, 23]. Fibrinogen strongly affects hemostasis, blood rheology, platelet aggregation and endothelial function. Hyperfibrinogenemia is expected to reduce blood flow, predispose to thrombosis and enhance atherogenesis [9, 19]. However, the mechanism by which fibrinogen promotes atherosclerosis is still not fully understood. We previously reported that when fibrinogen levels were elevated in plasma, the expression of mRNA for each fibrinogen chain was significantly enhanced in the liver [25]. The three chains, α-, β-, and γ-fibrinogen are encoded by separate genes, all situated in a cluster of approximately 50 kb in the distal third of the long arm of chromosome 4 [16]. Therefore, we investigated the hepatic mRNA expression of α-, β- and γ-fibrinogen in this study using male rats, and found that an increased mRNA expression of these three chains correlated with elevated plasma fibrinogen levels in SHHR. In contrast, although α-fibrinogen mRNA expression was significantly higher in HLR and SHHR than in WKY
Table 1. Relevant physical and biochemical features of eight-month-old WKY, SD, SHR, HLR and SHHR

<table>
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<tr>
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<th>WKY</th>
<th>SD</th>
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<tr>
<td>BW (g)</td>
<td>427.8 ± 5.5</td>
<td>618.5 ± 27.9**</td>
<td>401 ± 6.8***††</td>
<td>535 ± 14.8***§§</td>
<td>521.2 ± 8.5***§§</td>
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<tr>
<td>LW/BW (×10⁻²)</td>
<td>3.2 ± 0.08</td>
<td>2.6 ± 0.08**</td>
<td>3.7 ± 0.07***††</td>
<td>3.6 ± 0.06***††</td>
<td>2.9 ± 0.08***§§</td>
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<tr>
<td>TC (mg/dl)</td>
<td>94.4 ± 4.1</td>
<td>55.4 ± 6.9**</td>
<td>54.1 ± 1.6**</td>
<td>168 ± 4.5***§§</td>
<td>148.8 ± 27.6***‡‡</td>
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<tr>
<td>TG (mg/dl)</td>
<td>44.8 ± 9.4</td>
<td>96.7 ± 3.8**</td>
<td>39.5 ± 4.6††</td>
<td>184 ± 24.0***‡‡</td>
<td>82.7 ± 5.0***‡‡</td>
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Values presented are mean ± SEM for 6 to 13 rats per group. *P < 0.05 and **P < 0.01 vs WKY. †P < 0.05 and ††P < 0.01 vs SD. §§P < 0.01 vs SHR. ‡‡P < 0.01 vs HLR. BW, body weight; LW/BW, liver weight/body weight ratio; TC, total cholesterol; TG, triglyceride.

Fig. 1. Plasma levels of fibrinogen, ATIII, plasminogen and α₂PI in eight-month-old WKY, SD, SHR, HLR, and SHHR. ATIII, plasminogen, and α₂PI levels are presented as percentages of normal human plasma. Values are presented as mean ± SEM for 6 to 13 rats per group. *P < 0.05 and **P < 0.01 vs WKY; †P < 0.05 and ††P < 0.01 vs SD; §§P < 0.01 vs SHR; ‡P < 0.05 and ‡‡P < 0.01 vs HLR.
and SD, it did not increase in SHR, which may explain why plasma levels of fibrinogen are lower in SHR than in HLR and SHHR. In addition, we did not observe increases in plasma fibrinogen and hepatic mRNA expression of α-, β- and γ-fibrinogen and prothrombin in female SHR (data not shown). Therefore, hypertension in SHR is considered to be involved mildly in the hyperfibrinogenemia in SHHR compared with hyperlipidemia in HLR [1]. That is, from the data of mRNA expression for each chain, we can predict that parental genes of HLR contribute strongly to the increase in plasma fibrinogen in SHHR, compared with parental genes of SHR. However, hepatic expression of mRNA for β- and γ-fibrinogen in male SHR increased significantly compared to those in WKY and SD. These data mean that SHR shows sex differences regarding the hyperfibrinogenemia, the same as for blood pressure. Therefore, we predict the presence of some other genetic factors causing hyperfibrinogenemia in male SHR.

In this study, the increase in plasma fibrinogen concentration, correlating with an enhanced hepatic expression of mRNA for the α-, β- and γ-fibrinogen chains, was higher in SHHR than in SHR and HLR. These results suggest that a hypercoagulative state is a complication of hypertension and hyperlipidemia, as shown by the occurrence of hyperfibrinogenemia re-

Fig. 2. Increase in the intensity of PCR product bands with the number of PCR cycles. The electropherograms shown are each representative of four RT-PCR experiments with RNA from individual rats. A, β-actin; B, prothrombin; C, IL-6 receptor; D, α-chain of fibrinogen; E, β-chain of fibrinogen; and F, γ-chain of fibrinogen.
ANIMAL MODEL OF HYPERFIBRINOGENEMIA

Resulting from increased levels of mRNA for the component chains of fibrinogen in the liver. Indeed, the expression of the three fibrinogen chains is essential in fibrinogen assembly, which involves the formation of α-γ and β-γ dimers bound by disulfide bonds, to which a β- or α-chain is added to form α-β-γ half molecules; these dimerize via five disulfide bonds to form the intact fibrinogen molecule [11]. The hepatic mRNA expressions of α-, β- and γ-chains and prothrombin tended to be higher in male SHHR than those in female SHHR, so, the plasma fibrinogen level in male SHHR was significantly higher than that in female SHHR [2]. Other factors such as α2PI and ATIII increased by the same degree in male and female SHHR. Male SHHR showed more hypercoagulation than female SHHR. An increase in ATIII levels is expected to enhance fibrinolysis; however, ATIII instantly combines with thrombin to produce thrombin-ATIII complex (TAT), a marker of coagulation activation [3], in plasma. Plasma levels of TAT in male SHHR (8.3 ± 1.28 ng/ml) were over two-fold higher than in male WKY (3.5 ± 1.23 ng/ml). In addition, in SHHR, increased plasma levels of α2PI suggest that coagulation is upregulated. In the male rats, since there was a progressive increase in plasma

Fig. 3. Hepatic mRNA expression of fibrinogen α-chain (A), β-chain (B), and γ-chain (C) in eight-month-old WKY, SD, SHR, HLR and SHHR. Levels standardized to β-actin are shown as percentages of the standardized level in WKY. Values are presented as mean ± SEM for 6 to 13 rats per group. *P < 0.05 and **P < 0.01 vs. WKY; †P < 0.05 and ††P < 0.01 vs. SD; §§P < 0.01 vs. SHR.

Fig. 4. Hepatic mRNA levels of the prothrombin (PT) in eight-month-old WKY, SD, SHR, HLR and SHHR. PT mRNA levels standardized to β-actin are shown as percentages of the standardized level in WKY. Values are presented as mean ± SEM for 6 to 13 rats per group. **P < 0.01 vs. WKY; †P < 0.05 and ††P < 0.01 vs. SD.
levels of ATIII and plasminogen in SHR, HLR and SHHR, as compared to those in WKY and SD, we think that balance of coagulation and fibrinolytic activity in each experimental group was maintained.

In our previous report, we reported endothelial lesions in female SHHR aorta at 9 months of age, visualized by Haematoxylin and Eosin (HE) staining [2], while Kumai et al. reported slight lipid deposition of the endothelium in male SHHR aorta at 8 months of age, visualized by HE and Sudan III staining [20]. They also examined histological changes of the aortic arch in male and female rats at 24 months of age, and found no apparent changes in aortas of SHR and HLR. On the other hand, lipid deposits and endothelial lesions were observed in the aorta of female SHHR [2], and at low frequencies in the aorta of male SHHR at 24 months of age [21]. We do not understand why vascular disorder in female SHHR appears more strongly than in male SHHR. However, we have demonstrated that some kinds of hyperlipidemic models induce hyperfibrinogenemia, using rabbits [12], rats [26, 28] and mice [27]. Therefore, one of reasons for this sex difference is thought to be due to the high levels of plasma triglyceride and cholesterol in female SHHR compared with their levels in male SHHR. These results suggest that the sustained high levels of plasma fibrinogen induced the pre-risky state of initial vascular injury, but it will be necessary to examine the time course changes of coagulation and fibrinolytic factors in SHHR.

IL-6 as a proinflammatory cytokine is supposed to be involved in various cardiovascular diseases [7], and it plays a pivotal role in stimulating the acute-phase response, which elevates the circulating concentrations of several plasma proteins including fibrinogen [32]. We previously reported that IL-6 and IL-6 receptor may partly regulate plasma fibrinogen increase in rats with Triton WR-1339-induced hyperlipidemia [26]. Accordingly, we also investigated the plasma levels of IL-6 and the expression of IL-6 receptor mRNA in livers of male WKY, SD, SHR, HLR and SHHR to evaluate the involvement of the IL-6 pathway in hyperfibrinogenemia. Plasma levels of IL-6 increased significantly in HLR, but tended to decrease in SHR and SHHR, whereas the expression of IL-6 receptor mRNA increased in SHR and HLR, but not in SHHR. These results suggest that the IL-6 pathway is not directly involved in the in-
crease in plasma fibrinogen in SHHR. Our previous report demonstrated that mice fed a high cholesterol diet had hyperfibrinogenemia associated with high collagen-induced platelet aggregation that was enhanced by adrenaline pretreatment and this enhancement was inhibited by the β2-adrenergic blocker, yohinbin [27]. In male SHHR, adrenaline and noradrenaline levels in plasma and the adrenal medulla were significantly higher than those in male SD and WKY [21]. These results suggest that in SHHR, high levels of adrenaline in plasma enhance platelet activity, stimulating blood coagulation including fibrinogen biosynthesis.

In conclusion, the results of the present study suggest that hypertension and hyperlipidemia each cause hypercoagulation, with hyperlipidemia being the stronger factor, and when combined, these two diseases synergize to further increase the hypercoagulative state. In addition, our study also indicates that SHHR are a good model system of complication of hypertension and hyperlipidemia which could be used for the investigation of changes of hyperfibrinogenemia before a clear arterial lesion develops.

References


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