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Effects of Complement Suppression on Xenograft Survival in Hyperacute Rejection

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ABSTRACT

To examine the significance of complement in discordant cardiac xenograft rejection, morphological changes in the rejection reaction were investigated following administration of FUT-175 (FUT), an anticomplement reagent. Guinea pigs were the cardiac donors, and Wistar rats were the recipients. Four groups of rats were constituted as follows: Group 0 was the control group. FUT of 40 mg/kg was injected intraperitoneally in group I. It was followed by continuous intravenous infusion (20 and 40 mg/kg/hr) in groups II and III. In one series, the effects of FUT on complement suppression was examined. In the FUT groups of rats (groups I to III), the serum levels of CH50 and ACH50 were measured at 0, 1, 2 and 4 hr following injection of FUT. In the second series of rats with identical treatments, the graft heart beating time following cardiac transplantation was measured. Cardiac transplantation into untreated rats was also performed as a control (group 0). In another series, the graft hearts in the FUT groups were extracted after 15, 30, 60 and 90 min of coronary reperfusion for morphological examination with scanning electron microscopy.

The complement levels decreased significantly in the FUT-treated rats in a dose-dependent manner. Although the graft heart beating times in the FUT-treated groups were significantly longer than in group 0 (103, 106, and 112 min versus 14.7 min, $p<0.01$), there was no significant difference in the graft heart beating time or in the morphological changes among the three FUT groups. Our results suggest the presence of factors other than complements contribute to the cardiac xenograft rejection.

Key words: *Hyperacute rejection, Xenotransplantation, Complement*

The most critical problem facing clinical transplantation today is the short supply of organs of human origin[4,6,10,13]. This shortage of acceptable donors makes it more and more difficult to address the increasing number of patients for organ transplantations, and urges us to find solutions to this problem. Xenotransplantation could be one of these solutions, since the use of organs from nonhuman primates might help reduce the cost of such surgery, offer grafts of more appropriate sizes, and ensure greater virological safety in the future. However, organ transplantations between phylogenetically disparate species are invariably followed by hyperacute rejection (HAR)[6,9,10,14,26]. Many investigators have examined various interspecies combinations for xenotransplantation, and they generally agree that complement activation is an essential step in the development of HAR[9,12,27,29,34,35]. Some studies have emphasized the significance of the complement system, recognizing it as the most major factor in this rejection reaction[10,25–27,32,36]. However, we have hypothesized that the presence of factors other than complements might contribute to this rejection reaction, because the precise mechanisms underlying HAR have not been clearly evaluated. HAR is a rapid and violent phenomenon that leads inexorably to destruction of the xenografts, thus we have supposed that it contains many factors and cascades which mediate the large extent of the inflammatory reaction.

In spite of a large number of morphological studies on HAR, little has been reported on the morphological, especially, the ultrastructural changes in grafted organs. Many researchers have investigated the histopathology of HAR, using specimens obtained from xenografts in which their function was terminated. We believe that the rejection reaction consists of dysfunction of the endothelial cells leading to apotosis, and, consequently, the observation in detail of ultrastructural changes in the grafted organs is indispensable for the morphological investigation of HAR.

In this study, in order to examine the role of the

complement system in cardiac xenograft rejection, we modified the recipient complement system with FUT-175 (FUT), an anticomplement inhibitor, and then investigated the morphological changes during the rejection reaction in our guinea pig-to-rat heart transplantation model by means of scanning electron microscopy.

MATERIALS AND METHODS

Animals

Male outbred guinea pigs (200–270g), 8 to 10 weeks of age, and male inbred Wistar rats (250–300g), 10 to 12 weeks of age, were used as donors and recipients, respectively. The donor guinea pigs and recipient rats were paired based on a body weight ratio of 0.8–0.9. Both species were supplied by Hiroshima Experiments (Hiroshima, Japan) and were housed in cages under conventional conditions. The guinea pigs were provided with a diet of RC4 pellets (Oriental Yeast Co., Japan), and the Wistar rats were maintained on MF pellets (Oriental Yeast Co., Japan). All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the Institute of Laboratory Animal Resources and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institute of Health (NIH Publication No. 86–23, revised 1985).

Drug

The anticomplement reagent, FUT-175 (6-amidino-2-naphthyl p-guanidinobenzoate dimethanesulfonate), was supplied by the Torii Co., Japan. The FUT was dissolved in distilled water at 10mg/ml just before administration.

Experimental procedures

Donors

The guinea pigs (GP) were placed in the supine position, and were injected intraperitoneally with 1.0ml/kg of sodium pentobarbital (Nembutal: Abbott Lab., U.S.A.). A median sternotomy was performed, and the right and left superior vena cava were ligated. 50IU/kg of heparin and cold cardioplegia (GIK solution : K^+ : 25mEq/liter ; $HCO3^-$: 4.4mEq/liter ; pH : 7.70–7.80 ; 350mOsm ; 4°C) were then injected via the inferior vena cava. The left atrium was ligated, and the pulmonary artery and ascending aorta were transected. The extracted heart was rinsed with the cold GIK solution, and then placed in a saline bath at 4°C.

Recipients

The Wistar rats (WR) were placed in the supine position, and were anesthetized with 1.0ml/kg of sodium pentobarbital. The left femoral artery and vein were then exposed, followed by an intravenous injection of heparin (50IU/kg). A 24 gauge polypropylene catheter (Terumo Co., Japan) was inserted into the femoral artery to monitor the arterial pressure. Another 24 gauge catheter was inserted into the femoral vein for infusion with Ringer's solution to maintain the mean arterial pressure. A midline neck incision was made, and the right jugular vein and the right common carotid artery were exposed. The WRs were maintained at 38.5–39.0°C rectal temperature during the experiment.

Experimental design

Experiment 1 : In order to examine the effect of FUT on complement suppression, three groups of rats were used as follows. The group I rats were injected intraperitoneally with FUT (40mg/kg). This was followed by continuous intravenous infusions of FUT (20mg/kg/hr and 40mg/kg/hr) in group II and group III, respectively. The continuous administration of FUT was performed via the femoral vein. Five WRs were then sacrificed and exsanguinated at 0, 1, 2 and 4 hr following the FUT-injection, and blood samples were collected. These samples were centrifuged at 2500 r.p.m. for 10 min at 4°C to obtain the serum, which was stored at –70°C until the assay. The serum levels of the total complement hemolytic titer (CH50) and alternative complement hemolytic titer (ACH50) were measured.

Experiment 2 : The GP hearts were heterotopically transplanted into a second series of rats receiving the identical treatment protocol (group I to III), and the beating times of the grafted hearts were measured.

Heterotopic cardiac transplantation was performed according to the modified cuff-technique described by Heron[15]. Briefly, the right jugular vein and right common carotid artery of the WR were dissected free, and they were connected to the pulmonary artery and aorta of the donor heart respectively, with end-to-end anastomosis. The grafted heart was reperfused at one hour after the intraperitoneal injection of FUT. The graft heart beating time was defined as the time taken until the cessation of visible or palpable cardiac contraction. Cardiac transplantation into untreated rats was also performed as a control (group 0).

Experiment 3 : Morphological changes in the endothelial cell surface of the grafted heart due to the rejection reaction were investigated in every group by means of scanning electron microscopy. The GP hearts which were transplanted into the untreated rats were extracted at 1, 3, 5, 10 min following the coronary reperfusion, and shortly before they stopped. In the FUT-groups, the grafted hearts were removed after 15, 30, 60 and 90 min of reperfusion. The xenografted hearts were rinsed with cold saline (4°C) immediately after harvest, and were fixed with 2.5% glutaraldehyde buffered with 0.1M cacodylate at 4°C.

Hemolytic complement determinants

The rat serum levels of CH50 were determined by the standard test of Mayer[22]. The ACH50 levels were assayed according to the method of Platts-

Mills and Ishizaka[28] using rabbit erythrocytes as the target cells. The levels of hemolytic activity in the FUT-treated rats were represented as a percentage of the corresponding value at time 0.

Morphological examination

For the scanning electron microscopic examination in Experiment 3, small pieces of the intraventricular septum were resected from the xenografted hearts and then fixed overnight with 2.5% glutaraldehyde buffered with 0.1M cacodylate at 4°C, postfixed with 2% OsO4 in 0.1M cacodylate, and dehydrated in a graded ethanol series. The specimens were immersed in 2-methyl-2-propanol, critical point dried and sputter-coated with platinum. A high-resolution, field-emission SEM (Hitachi S800, Hitachi Co., Japan) was used for observation and photography.

The structural integrity of the endothelial surface of the coronary artery was evaluated by scanning electron microscopy at a magnification of 1000×. Ten microscopic fields were selected randomly, and were observed to obtain the "intimal injury score" of the left anterior descending branch of the left coronary artery. The score was defined as the number of fields which demonstrated severe injury to the endothelium, such as crater formation or denudation of the endothelial cells, in ten selected fields.

Statistical analysis

The statistical analysis of the differences between groups with respect to complement hemolytic titer, graft heart beating time, and intimal injury score was performed using a Wilcoxon-Mann-Whitney test. A P-value of less than 0.01 was considered to be statistically significant. The values are expressed as means ± SD.

RESULTS

Complement hemolytic titer

CH50

The complement hemolytic titer in the FUT-treated rats decreased immediately following the injection of FUT. The CH50 values at 1 hr after the FUT injection were as follows: 53.9±3.8% in group I, 44.6±3.9% in group II, and 18.1±2.2% in group III. The values in group I then showed a gradual recovery: 81.4±7.8% at 2 hr, and 82.9±17.9% at 4 hr after the injection. However, the CH50 values in groups II and III showed a statistically significant decline over time in a dose-dependent manner. The values at 2 hr and 4 hr following the FUT injection were as follows: 41.4±8.9% and 33.6±11.5% in group II, and 17.5±2.0% and 19.8±1.3% in group III (Fig. 1).

ACH50

In group I, the ACH50 values decreased gradually for 2 hours following the FUT-injection: 44.9±11.1% at 1 hr, and 22.9±5.2% at 2 hr. However, the values at 4 hours recovered to a similar level as the 1 hr value (44.2±9.8%). In group II, the ACH50 values showed a statistically significant decline in a dose-dependent manner. The values were as follows: 19.8±3.5% at 1 hr, 12.5±3.8% at 2 hr, and 10.8±1.1% at 4 hr following the FUT injection. The drop in the ACH50 values was the most marked in group III, as follows: 2.7±1.2% at 1 hr, 2.4±0.8% at 2 hr, and 3.9±3.9% at 4 hr. The ACH50 levels in group III were significantly lower than in group II, and the levels in group II were significantly lower than those in group I (Fig. 1).

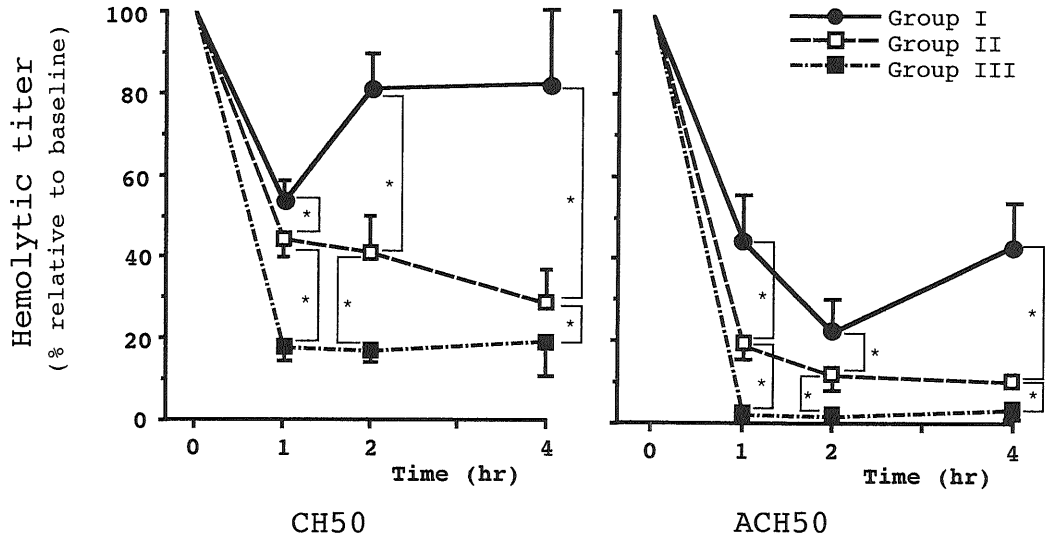

Fig. 1. Effects of FUT administration on rat serum complement.
The hemolytic activities of CH50 and ACH50 are plotted as percentages of the values in untreated rat serum. Complement levels in the FUT-treated rats show a statistically significant decline over time following FUT-injection. (*: $p<0.01$, Wilcoxon-Mann-Whitney test)

Table 1. Outcome of graft heart beating times

Group	No.	Treatment	Graft heart beating times		p-value*
			Individual results (min)	Mean±SD	
0 (control)	7	–	11, 11, 13, 14, 15, 19, 20	14.7 ± 3.6	
I	6	FUT: 40mg/kg (ip)	85, 104, 105, 105, 110, 110	106.0 ± 13.4	<0.01 vs. 0 NS vs. II NS vs. III
II	5	FUT: 40mg/kg (ip)+ 20mg/kg/hr (civ)	95, 102, 104, 108, 110	103.8± 5.8	<0.01 vs. 0 NS vs. I NS vs. III
III	5	FUT: 40mg/kg (ip)+ 40mg/kg/hr (civ)	94, 109, 111, 117, 129	112.0±12.7	<0.01 vs. 0 NS vs. I NS vs. III

ip: intraperitoneal injection; civ:continuous intravenous infusion
* Wilcoxon-Mann-Whitney test

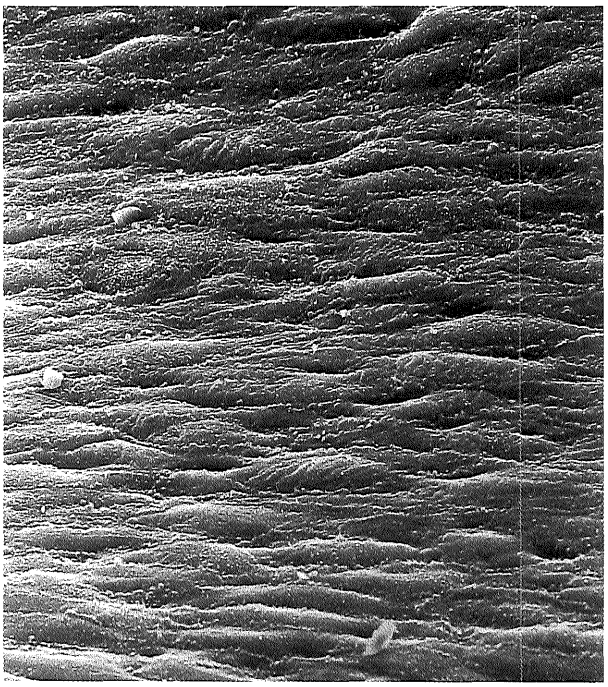

Fig. 2. Left anterior descending branch of the left coronary artery from a guinea pig heart before the reperfusion.
The endothelial cell borders are demarcated, with elevated central portions which accommodate the nuclei. (Scanning electron micrograph: Original magnifications: ×1000)

Graft heart beating times

The beating times for the four groups are shown in Table 1. The untreated rats rejected their GP hearts hyperacutely, with a mean beating time of 14.7±3.6 min. The beating times in the three FUT-treated groups were significantly longer than in the untreated group (106±13.4 min in group I, 103±5.8 min in group II, and 112±12.7 min in group III). There was no significant difference in graft beating time among the three FUT groups.

Morphological examination

A scanning electronmicrograph of the coronary arterial endothelium from a donor heart before coronary reperfusion is shown in Fig. 2. The endothelial cell borders are well demarcated, with elevated central areas of the cells which accommodate the nuclei. Only minimal endothelial alteration can be seen, essentially consisting of a slight widening of the intercellular borders between the endothelial cells. Representative photomicrographs of the intimal alterations in the FUT-treated groups after coronary reperfusion for 15 min and 30 min, respectively, are shown in Fig. 3 and in Fig. 4. Marked separation and partial detachment of the endothelial cells from the underlying tissue and crater formation were observed more frequently in the specimens. The most severe alterations can be seen in Fig. 5, consisting of denudation of the endothelial cells and the deposition of formed blood elements. The underlying tissues have been exposed with platelet deposision. In contrast to these findings in the FUT-treated groups, much more progressive alterations of the endothelial cell surface could be demonstrated in group 0. Swelling and crater formation in the endothelial cells were observed at 3 min of reperfusion, followed by denudation or exposition of the underlying tissues at 10 min.

The intimal injury scores from the FUT-treated groups are shown in Fig. 6. The FUT groups showed minimal ultrastructural alteration within 30 min of reperfusion. The scores at 30 min of reperfusion were as follows: 3.0±1.0 in group I, 2.9±1.1 in group II, and 2.5±1.3 in group III. The ultrastructural deterioration of the integrity of endothelial cell surface was much less severe in the FUT groups than in group 0. After 60 min of reperfusion, the graft hearts showed severe microvascular injury characteristic of hyperacute vascular rejection, with destruction of the

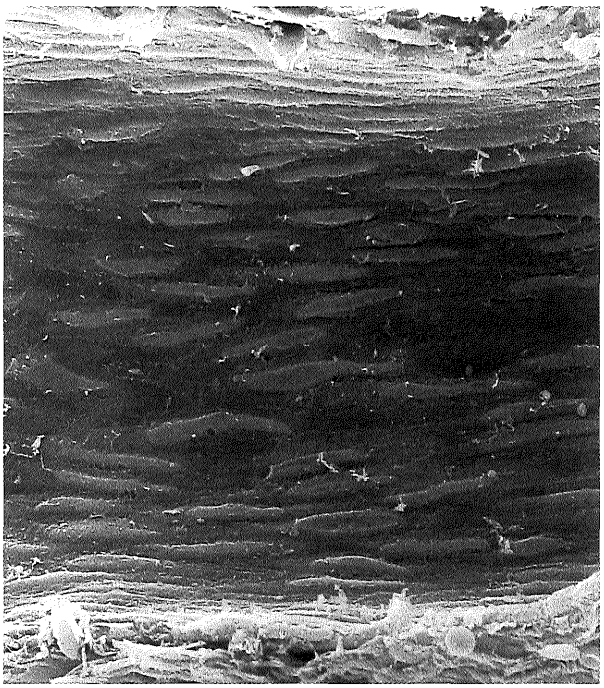

Fig. 3. The coronary endothelium from a grafted heart for 15 min of reperfusion in group III.
Swelling and partial detachment of the endothelial cells from the underlying tissue was observed. (Scanning electron micrograph: Original magnifications: ×1000)

Fig. 5. The most severe alteration was seen after coronary reperfusion for over 90 min, consisting of denudation of the endothelial cells, the deposition of formed blood elements and exposure of the underlying tissue. (Scanning electron micrograph: Original magnifications: ×1000)

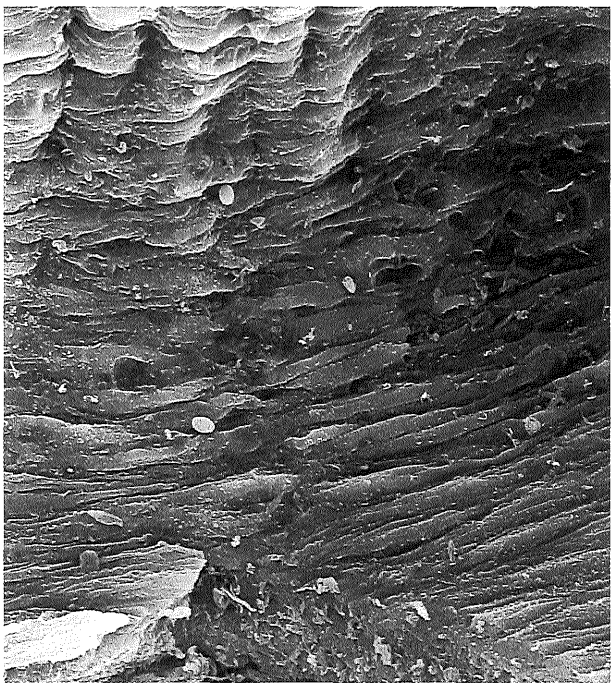

Fig. 4. Coronary endothelium of a grafted heart after the reperfusion for 30 min in group III.
Marked separation and partial detachment of the endothelial cells from the underlying tissue was observed with crater formation. (Scanning electron micrograph: Original magnifications: ×1000)

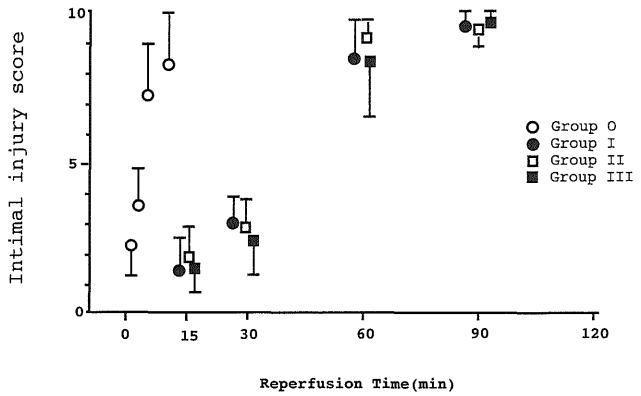

Fig. 6. Microscopic evaluation of the intimal changes in the four experimental groups.
The scores in the FUT groups were much less progressive than in the control group. However, there was no significant difference among the three FUT groups.

endothelial cells, as well as thrombocytic and erythrocytic thrombi. The scores at 60 min were as follows: 8.5±1.4 in group I, 9.2±0.7 in group II, and 8.2±1.7 in group III. The intimal injury scores of the graft hearts which were harvested after similar reperfusion periods showed no significant differences between the three FUT groups.

DISCUSSION

This report describes the effects of complement suppression during the HAR of xenografts in the guinea pig-to-rat model, and discusses the probable presence of factors other than complement which contribute to HAR.

Complement activation is believed to contribute to endothelial cell activation, the adhesion of leukocytes and platelets to the endothelial cells, and finally thrombosis and tissue necrosis[5,26,32]. Previous studies have reported that guinea pig hearts transplanted into rats were rejected by direct activation of the complement cascade via the alternative pathway in rat serum[10,25,27]. Recent progress in complement research has shown that the complement system can recognize and react with foreign materials without the mediation of antibodies[6,26,27]. Other studies also suggested that antibody binding is not a prerequisite for the triggering of the HAR in the guinea pig-to-rat xenograft model[12,19,27]. Complement activation, probably via the alternative pathway, occurs independently of antibody binding to the xenograft, and appears to be sufficient to initiate the HAR. In contrast, antibody depletion should not prevent complement activation within the xenograft. Those studies confirm that HAR can occur in the absence of natural antibody (Nab) binding to the endothelium of the xenografts in this guinea pig-to-rat xenotransplantation model through direct activation of the alternative complement pathway[9,13,25–27,29].

We and other investigators have examined various anticomplement drugs such as cobra venom factor, K76COOH, and FUT-175 in some xenograft models[10,20,25–27]. Current studies have shown that the HAR in xenografts occurs in some species combinations such as the guinea pig to rat or rabbit to newborn pig, in which little or no anti-donor Nab can be detected[10,19,33]. The guinea pig-to-rat model has been recognized as a well-established xenograft combination that results in a HAR. Using this model, many researchers have emphasized that the complement system can mediate HAR without the involvement of antibodies[18,24,26,32,36]. However, we have been resistant to this argument because the precise mechanism underlying HAR has not been clearly evaluated. In other words, several mechanisms including the complement system may jointly contribute to the development of HAR.

FUT-175 inhibits complement-mediated hemolysis via the classical pathway activation. This action is believed to be due to an inhibition of $C1\bar{r}$ and $C1\bar{s}$, based on the fact that FUT inhibited the esterolytic activity of $C1\bar{r}$ and $C1\bar{s}$ on synthetic substrates in an *in vitro* study[1,2,25]. The FUT inhibition of alternative pathway activated hemolysis has been shown to be attributable to its inhibition against $\bar{B}$, D, Bb and C3 convertase (C3bBb)[17,25]. The *in vivo* inhibitory effectiveness of FUT against complement-mediated hemolysis has also been studied. In the *in vivo* model of complement-mediated hemolysis via the classical pathway, FUT has been reported to be effective against hemolysis in a dose-dependent manner[25,27]. In contrast, in the *in vivo* complement-mediated hemolysis model via the alternative pathway, FUT was effective when administered intravenously. This is supported by the finding that inactivated dog serum, heated to 56°C for 30 min, induced no hemolysis[10,16]. It has also been reported to inhibit the rat alternative pathway completely, probably by direct inhibition of serine protease factor B in the guinea pig-to-rat model[1,17]. The present study suggests that FUT has a noticeable *in vivo* effect in various animal models in which the complement system plays an important role as a pathogenetic factor[16,17,25,27].

The histopathology of the HAR reaction is characterized by interstitial hemorrhage, edema, platelet and fibrin thrombi, and severe injury to the endothelial cells of both large and small vessels in the xenografts[5,8,11,30]. Prominent deposition of complement can also be demonstrated along the endothelial cell surfaces in the affected tissues using immunopathologic analysis[26,27]. A number of morphological studies on HAR have been reported by many investigators, and they have suggested that acute vascular xenograft rejection involves a severe dysfunction of the endothelial cells that may be caused by endothelial cell activation[18,20,25,27].

The untreated rats rejected their xenografts hyperacutely, and rapid and progressive injury to the endothelial cells was demonstrated immediately following coronary reperfusion in the grafted hearts. Morphologically, a widening of the intercellular junctions and alterations in the endothelial cells could be initially observed, followed by crater formation and denudation of the endothelium. On the surface of the endothelial cells, platelet deposition could be observed immediately, followed by erythrocytic and platelet thrombus formation with fibrin. Interstitial hemorrhage was observed after 1 min of reperfusion, but no cellular infiltration was observed until the cessation of xenograft beating. In contrast, the xenografts in the FUT-treated rats (groups I to III) showed no ultrastructural changes within 30 min, and extravascular infiltration of neutrophils or macrophages was observed after 1 hr of reperfusion. Furthermore, the graft heart beating times of the FUT groups were prolonged for approximately 2 hr, which was significantly longer than group O. Scanning electron microscopy of the grafted hearts revealed a delayed rejection reaction in the endothelial cells, and the intimal injury scores also reflected a delayed and gradual increase in HAR following the coronary reperfusion. An intraperitoneal injection of FUT was effective in immediately reducing the complement hemolytic

titers in the rat serum; however, the complement level dropped within one hour. In contrast, continuous intravenous infusion of FUT was beneficial in maintaining a reduced activity of the complements in the rat serum. The complement levels in the FUT-treated rats which were given an intraperitoneal injection followed by continuous intravenous infusion showed a statistically significant decline for over 4 hr. Although the reduced complement levels in the three FUT groups were significantly different in a dose-dependent manner, their graft heart beating times and intimal injury scores showed no significant differences. The complement reduction in the recipient rats effectively inhibited the initiation of HAR, but it was not effective in preventing the development of HAR.

FUT, a serin protease inhibitor, can have direct effects on the complement cascade, because complement consists of a series of serine proteases in the form of proenzymes that are activated by limited proteolysis[1,2,17,24]. It has been reported to inhibit not only the complement system but also other inflammatory reactions such as coagulation, fibrinolysis, and the kinin systems[5,16,18,23,25]. These anti-inflammatory effects could theoretically limit the extent of the pathological rejection reaction in the xenografts. Our results suggest that the mechanisms underlying HAR are not simple. Factors other than the complement system, such as thrombogenesis, neutrophils and vasoconstriction might also play a predominant role in the pathogenesis of hyperacute xenograft rejection. The relative importance of each of these factors appears to vary with the species combination used. We conclude that some of these factors plus several different mechanisms, alone or in combination, might contribute to the development of discordant cardiac xenograft rejection.

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