Synergistic Effect of Triptolide and Tacrolimus on Rat Cardiac Allotransplantation

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SUMMARY

Recent studies have shown that triptolide inhibits T cell activation through mechanisms different from those of cyclosporine A and tacrolimus and we postulated that triptolide might have a synergistic effect with tacrolimus to enhance immunosuppression. Using a F344 donor-to-Lewis recipient rat combination, we investigated the immunosuppressive effects of triptolide alone or in combination with tacrolimus on the survival of cardiac allografts. Recipients were treated with placebo, triptolide, tacrolimus, and triptolide in combination with tacrolimus at different doses. The median survival time (MST) was 8 days for placebo; 9.5, 11, 14 and 19 days for triptolide monotherapy at doses of 0.04, 0.08, 0.16, and 0.32 mg/kg/day, respectively, and 11, 13.5, and 52 days for tacrolimus monotherapy at doses of 0.025, 0.05, and 0.1 mg/kg/day, respectively. Tacrolimus 0.025 mg/kg/day combined with triptolide 0.08 and 0.16 mg/kg/day prolonged the MST to 17.5 and 20 days, respectively; while tacrolimus 0.05 mg/kg/day combined with triptolide 0.04, 0.08, and 0.16 mg/kg/day prolonged the MST to 21, 23, and 23 days, respectively. These results suggest that triptolide is a moderately effective immunosuppressive agent. Triptolide combined with a subtherapeutic dose of tacrolimus produced a synergistic effect in prolonging rat cardiac allograft survival. (*Jpn Heart J* 2004; 45: 657-665)

Key words: Cardiac allografts, Immunosuppression, Triptolide, Tacrolimus, Combination therapy

DURING the past two decades, tremendous advances have been made in post-transplantation immunosuppressive therapy. Many new immunosuppressive drugs have been discovered and developed for clinical use in organ transplants. The high efficacy of immunosuppression of the calcineurin inhibitors, cyclosporine A (CsA) and tacrolimus, have contributed to improve the clinical outcome of

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organ transplantations. At this time, CsA-based or tacrolimus-based immunosuppression remains the cornerstone of immunosuppressive therapies.¹⁾

Tacrolimus is a macrolide isolated from *streptomyces tsukubaensis*. It was first demonstrated in 1984 to be immunologically effective in an *in vivo* rat heart allograft transplantation model.²⁾ Since then, the mechanisms of tacrolimus have been intensively investigated.³⁻⁵⁾ Like CsA, tacrolimus inhibits the T-lymphocyte immune response via blockade of the calcium-dependent signaling pathway.⁴⁾ Furthermore, tacrolimus inhibits lymphocyte activation *in vitro* 10 to 100 times more potently than CsA.⁶⁾ Tacrolimus is now being used as a primary immunosuppressant in clinical organ transplantations.^{5,7-9)} However, tacrolimus has dose-related side-effects, including nephrotoxicity, diabetogenesis, neurotoxicity, and it has also been shown to increase the incidence of infection and malignancies.^{7,9,10)} These adverse effects may restrict its clinical usage. The development of other immunosuppressive agents that act synergistically with tacrolimus, thereby permitting significant tacrolimus dose reduction, remains a compelling target in clinical organ transplantations.

Extracts of the Chinese herb *Tripterygium Wilfordii* Hook F (TWHF) exhibit potent immunosuppressive and anti-inflammatory properties and have been used extensively in China for the treatment of arthritis and other autoimmune diseases for many years.^{11,12)} Triptolide, a diterpenoid triepoxide compound purified from the root of TWHF, has been identified as one of the major components responsible for the immunosuppression of the herb.¹³⁾ The immunosuppressive activities of triptolide have been investigated both *in vitro*^{14,15)} and *in vivo*.^{16,17)} Qiu and co-workers demonstrated that triptolide inhibits both calcium-dependent and calcium-independent pathways and activates T cells via inhibition of interleukin-2 transcription at a site different from the targets of CsA or tacrolimus.¹⁸⁾ Based on these studies, we propose that triptolide might offer a new alternative for combined therapy with CsA or tacrolimus to enhance the therapeutic effects and to reduce the adverse effects of CsA or tacrolimus, which may allow tacrolimus to be used more effectively in clinical immunosuppression.

To the best of our knowledge, the immunosuppressive efficacy of triptolide combined with tacrolimus has not been reported. Therefore, we investigated the effects of triptolide alone or in combination with tacrolimus on graft survival in a major histocompatibility complex (MHC)-compatible rat cardiac allograft model.

METHODS

Animals: Adult male inbred F344 (RT1 lvl) and Lewis (RT1 l) rats weighing between 200 and 250 g were used as donors and recipients, respectively. Both

were purchased from Charles River Japan, Ltd. (Atsugi, Japan). The rats were cared for in accordance with NIH regulations governing laboratory animals and housed in a specific pathogen-free room with controlled temperature and light/dark cycles in our animal facility.

Agents: Tacrolimus obtained from Fujisawa Pharmaceuticals (Osaka, Japan) was dissolved in distilled water to make a 1 mg/mL stock solution and stored in a freezer (-70°C). Final tacrolimus doses were diluted in saline to volumes of 0.1 mL containing treated doses, and it was administered to the animals intramuscularly. Triptolide obtained from Fujian Medical Science Research Institute (Fujian, China) was found to be 98% pure by reverse phase high-performance liquid chromatography (HPLC). A stock solution of triptolide was prepared by dissolving 10 mg of triptolide in 5 mL dimethyl sulfoxide (DMSO) and then stored at -70°C. Final triptolide doses were diluted in saline to a 1 mL volume containing treated doses, and it was administered to the animals intraperitoneally. The control animals received the vehicles of the two drugs at the same formulations.

Cardiac transplantation: The recipients were anesthetized with 40 mg/kg sodium pentobarbital intraperitoneally and supplemented with ether anesthesia. Heterotopic abdominal heart transplantation was performed by end-to-side anastomosis of the ascending aortic to the abdominal aorta and the pulmonary artery to the inferior vena cava, as described by Ono and Lindsey.¹⁹⁾ Cold ischemic time was less than 45 minutes. Cardiac graft activity was assessed daily by abdominal palpation. The time of rejection was defined as the last day of palpable contraction. This was confirmed by laparotomy if necessary.

Experimental groups: The transplanted rats were randomly assigned to one of the following groups for posttransplantation immunosuppressive treatment starting on the day of transplantation and continuing to the 13th posttransplantation day or the day of rejection: Group 1) saline and DMSO control ($n = 6$); Group 2) triptolide 0.04 mg/kg/day ($n = 6$); Group 3) triptolide 0.08 mg/kg/day ($n = 7$); Group 4) triptolide 0.16 mg/kg/day ($n = 6$); Group 5) triptolide 0.32 mg/kg/day ($n = 6$); Group 6) tacrolimus 0.025 mg/kg/day ($n = 6$); Group 7) tacrolimus 0.05 mg/kg/day ($n = 6$); Group 8) tacrolimus 0.1 mg/kg/day ($n = 6$); Group 9) triptolide 0.08 mg/kg/day + tacrolimus 0.025 mg/kg/day ($n = 6$); Group 10) triptolide 0.16 mg/kg/day + tacrolimus 0.025 mg/kg/day ($n = 7$); Group 11) triptolide 0.04 mg/kg/day + tacrolimus 0.05 mg/kg/day ($n = 6$); Group 12) triptolide 0.08 mg/kg/day + tacrolimus 0.05 mg/kg/day ($n = 9$); Group 13) triptolide 0.16 mg/kg/day + tacrolimus 0.05 mg/kg/day ($n = 9$).

Evaluation of drug interaction: The following equation described by Berenbaum²⁰⁾ was used to evaluate the nature of the interaction between triptolide and tacrolimus:

$$\text{dose of A/Ae} + \text{dose of B/Be} = X.$$

A is the dosage of drug A in combination with B; A_e is the dosage of drug A required to produce an effect equal to that achieved by A in combination with B; B is the dosage of drug B in combination with A; B_e is the dosage of drug B required to produce an effect equal to that achieved by B in combination with A. $X < 1$ indicates synergy; $X = 1$ additivism; $X > 1$ antagonism. The equi-effective dosages of drug A (A_e) and drug B (B_e) were determined from the dose-effect curves (Figure 1).

Histology: Except for those used for survival count, a total of 16 cardiac allografts from groups 1, 4, 7, and 13 (4 in each group respectively) were harvested 5 days after transplantation for histological examination. At autopsy, the transplanted hearts were removed, fixed in 10% formalin solution, and embedded in paraffin. The specimen was sliced 4 μm thick and stained with hematoxylin-eosin. Rejection scoring was recorded blindly according to the classification of the International Society of Heart and Lung Transplantation (ISHLT).²¹⁾ 0: no rejection (grade 0); 1: focal, perivascular or interstitial infiltrate without necrosis (grade 1A); 2: diffuse but sparse infiltrate without necrosis (grade 1B); 3: one focus of aggressive infiltration and/or focal myocyte damage (grade 2); 4: multifocal aggressive infiltration and/or myocyte damage (grade 3A); 5: diffuse inflammatory process with necrosis (grade 3B); 6: diffuse, aggressive polymorphic infiltrate with edema, hemorrhage, vasculitis, and necrosis (grade 4). At sacrifice, the recipients' liver and kidney were also removed for histological assessment of drug toxicity.

Statistics: Survival data and ISHLT scores were analyzed by the Mann-Whitney U test using Statview 5.0 software. A *P* value less than 0.05 was considered significant.

RESULTS

1. Effect of triptolide or tacrolimus monotherapy on graft survival: The survival times of F344 heart grafts transplanted to Lewis rats are described in Table I. The median graft survival time (MST) of the control group was 8 days. Daily treatment with triptolide prolonged the MST in a dose-dependent fashion: triptolide 0.04 mg/kg/day had little effect on graft survival, with an MST of 9.5 days ($P = 0.5752$); triptolide 0.08 mg/kg/day improved the MST to 11 days ($P = 0.0453$), triptolide 0.16 mg/kg/day and 0.32 mg/kg/day improved it further to 14 days ($P = 0.0039$) and 19 days ($P = 0.0039$), respectively. Similarly, tacrolimus at doses of 0.025, 0.05, and 0.1 mg/kg/day prolonged the MST to 11 ($P = 0.0453$), 13.5 ($P = 0.0065$), and 52 days ($P = 0.0039$), respectively.

Table I. Effect of Triptolide and Tacrolimus on the Survival of F344 Heart Graft Transplanted to Lewis Rats

Group	Triptolide (mg/kg/day)	Tacrolimus (mg/kg/day)	n	Survival Day	MST (day)	P value	X value
1	0	0	6	9, 9, 8, 8, 8, 7	8		
2	0.04	0	6	12, 11, 11, 8, 7, 7	9.5	0.5752 (vs.1)	
3	0.08	0	7	16, 16, 14, 11, 10, 8, 8	11	0.0453 (vs.1)	
4	0.16	0	6	21, 15, 14, 14, 13, 12	14	0.0039 (vs.1)	
5	0.32	0	6	22, 21, 19, 19, 19, 18	19	0.0039 (vs.1)	
6	0	0.025	6	15, 14, 11, 11, 10, 7	11	0.0453 (vs.1)	
7	0	0.05	6	20, 15, 15, 12, 11, 9	13.5	0.0065 (vs.1)	
8	0	0.1	6	>100, >100, 54, 50, 30, 21	52	0.0039 (vs.1)	
9	0.08	0.0025	6	23, 19, 18, 17, 17, 14	17.5	0.0082 (vs.3) 0.0082 (vs.6)	0.71
10	0.16	0.025	7	21, 21, 20, 20, 20, 19, 13	20	0.1004 (vs.4) 0.0066 (vs.6)	0.82
11	0.04	0.05	6	26, 24, 21, 21, 17, 15	22	0.0027 (vs.2) 0.0163 (vs.7)	
12	0.08	0.05	9	31, 28, 26, 25, 23, 21, 20, 14, 11	23	0.0070 (vs.3) 0.0251 (vs.7)	0.85
13	0.16	0.05	9	>100, 29, 24, 23, 23, 21, 20, 19, 19	23	0.0056 (vs.4) 0.0039 (vs.7)	1.0

MST = median survival time; P value = calculated by Mann-Whitney U test; X value = calculated by Berenbaum equation.

2. Effect of triptolide in combination with tacrolimus on graft survival: The median graft survival times in recipients treated with triptolide in combination with tacrolimus were significantly longer than those in the monotherapy groups (Table I, Figure 1). Tacrolimus 0.025 mg/kg/day combined with triptolide at doses of 0.08 and 0.16 mg/kg/day extended the MST to 17.5 (X = 0.71 by Berenbaum equation) and 20 days (X = 0.82), respectively. Tacrolimus 0.05 mg/kg/day combined with triptolide at doses of 0.04, 0.08, and 0.16 mg/kg/day produced a similar extension, with MSTs of 21, 23 (X = 0.85), and 23 days (X = 1.0), respectively.

3. Histological evaluation: Histological examination of untreated control cardiac allografts removed 5 days after transplantation showed aggressive lymphocyte infiltration with congestion, edema, hemorrhage and severe myocyte necrosis (Figure 2A). Allografts treated with triptolide 0.16 mg/kg/day showed multifocal lymphocyte infiltration with mild to moderate myocyte damage (Figure 2B). Allografts treated with tacrolimus 0.05 mg/kg/day demonstrated marked reduction of lymphocyte infiltration with mild myocyte damage (Figure 2C). In contrast, the allografts treated with a combination of tacrolimus 0.05 mg/kg/day and triptolide 0.16 mg/kg/day showed only focal, perivascular lymphocyte infiltration without myocyte damage (Figure 2D). Drug toxicity studies demonstrated that there were no histological abnormalities observed in the liver or kidney of the recipients.

Figure 1. Dose-effect curve of triptolide (A) and dose-effect curves of tacrolimus alone and in combination with triptolide at doses of 0.08 and 0.16 mg/kg/day (B) to produce 100% cardiac allograft survival on posttransplantation day 18 (the 10-day prolongation of untreated cardiac allograft survival) are shown.

Figure 2. Representative histological photos of rat cardiac allograft removed 5 days after transplantation. **A:** Untreated control allografts showed aggressive lymphocyte infiltration with edema, hemorrhage, and severe myocyte necrosis. **B:** Allografts treated with triptolide 0.16 mg/kg/day demonstrated obvious infiltration by lymphocytes with mild to moderate myocyte damage. **C:** Allografts treated with tacrolimus 0.05 mg/kg/day showed a marked reduction in lymphocyte infiltration with mild myocyte damage. **D:** Allografts treated with combined triptolide 0.16 mg/kg/day and tacrolimus 0.05 mg/kg/day revealed mild, focal, perivascular lymphocyte infiltration without myocyte damage. (Hematoxylin and eosin staining. Original magnification $\times 100$).

Table II. ISHLT Scores of Allografts Removed 5 Days After Transplantation

Treatment	ISHLT Score	Mean $\pm$ SD	<i>P</i> value
Control (<i>n</i> = 4)	5, 5, 6, 4	5.0 $\pm$ 0.8	
Triptolide 0.16 mg/kg/day (<i>n</i> = 4)	3, 4, 3, 5	3.8 $\pm$ 1.0	0.11
Tacrolimus 0.05 mg/kg/day (<i>n</i> = 4)	3, 3, 2, 3	2.8 $\pm$ 0.50	0.021
Triptolide 0.16 mg/kg/day +	3, 2, 1, 1	1.8 $\pm$ 1.0	0.021
Tacrolimus 0.05 mg/kg/day (<i>n</i> = 4)			

ISHLT = International Society of Heart and Lung Transplantation; *P* value = calculated by Mann-Whitney U test.

The ISHLT scores of allografts removed 5 days after transplantation are shown in Table II. The ISHLT score decreased from 5.0 ± 0.8 in untreated control to 3.8 ± 1.0 for triptolide 0.16 mg/kg/day ($P = 0.11$), to 2.8 ± 0.5 for tacrolimus 0.05 mg/kg/day ($P = 0.021$), and to 1.8 ± 1.0 for triptolide 0.16 mg/kg/day in combination with tacrolimus 0.05 mg/kg/day ($P = 0.021$).

DISCUSSION

In this study, we evaluated the immunosuppressive efficacy of triptolide in prolonging the survival of allografts and in potentiating the immunosuppressive effects of tacrolimus with a MHC-compatible F344- to -Lewis rat cardiac allotransplantation model. The results showed that triptolide prolonged the cardiac allograft survival in a dose-dependent fashion. Using the equation of Berenbaum, we found that tacrolimus in combination with triptolide had a synergistic effect ($X = 0.71 \sim 1.0$). The histological studies revealed that the ISHLT score was significantly decreased by a combined treatment of triptolide 0.16 mg/kg/day and tacrolimus 0.05 mg/kg/day.

The Berenbaum equation was employed to evaluate the nature of the drug interaction between triptolide and tacrolimus. As recommended by Berenbaum, we choose a 10-day prolongation of untreated cardiac allograft survival (survival up to day 18 after transplantation in this study) as the equal specified effect of triptolide and tacrolimus. As shown in Figure 1, the dose of tacrolimus required to obtain 100% graft survival on posttransplantation day 18 can be reduced 5-fold when combined with 0.16 mg/kg/day triptolide. Tacrolimus has a relatively narrow therapeutic window (blood level of 5 to 15 ng/mL by TMX analysis).²² A high concentration of tacrolimus increases the risk of toxicities, whereas reduction of the dose sometimes leads to rejection. Therefore, therapy using tacrolimus in a subtherapeutic dose combined with another drug that acts synergistically is highly desired. The synergistic characteristics of triptolide in combination with tacrolimus may be of clinical significance.

It is well known that complete T lymphocyte activation requires antigen-mediated triggering of the T cell receptor/CD3 complex (signal 1) in conjunction with costimulatory signals provided by the CD28 molecule (signal 2).²³⁾ Tacrolimus inhibits T cell activation via the CD3 pathway, while the CD28 pathway remains unaffected.²⁴⁾ By contrast, triptolide inhibits T cell activation and triggers IL-2 gene expression through a CD28 costimulatory molecule resistant to CsA and tacrolimus.¹⁸⁾ The synergistic effect between triptolide and tacrolimus can be partially explained by their different mechanisms of action. With this novel mechanism of immunosuppression, triptolide may offer a new alternative for combined therapy with tacrolimus and broaden the therapeutic window of clinical immunosuppression.

In conclusion, triptolide is a moderately effective immunosuppressive agent that can act synergistically with tacrolimus to prolong rat cardiac allograft survival. Although further investigation is required to evaluate the mechanisms of interaction between these two drugs, the combination of triptolide with a subtherapeutic dose of tacrolimus might be an alternative strategy in organ transplantations.

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