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ORIGINAL ARTICLE

Effect of tacrolimus and partial hepatectomy on transthyretin metabolism in rats

Manuel E. Zeledon R.,[1] Yukio Ando,[2] Katsuhiro Asonuma,[1] Masaaki Nakamura,[2] Xuguo Sun,[2] Mitsuharu Ueda,[2] Junko Fujii[3] and Yukihiro Inomata[1]

1 Department of Pediatric Surgery and Transplantation, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan
2 Department of Diagnostic Medicine, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan
3 Department of Pharmacy, Kumamoto University, Kumamoto, Japan

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Correspondence
Yukio Ando, Department of Diagnostic Medicine, Graduate School of Medical Sciences, Kumamoto University, Honjo 1-1-1, Kumamoto 860-8556, Japan. Tel.: +81 96 373 5686; fax: +81 96 373 5686; e-mail: yukio@kaiju.medic.kumamoto-u.ac.jp

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Summary

Liver transplantation, which serves as treatment of familial amyloidotic polyneuropathy (FAP), and domino liver transplantation, which utilizes resected livers from patients with FAP for treatment of liver diseases, may induce changes in transthyretin (TTR), a pathogenic FAP-related protein. To evaluate this possibility, we performed a 70% hepatectomy or administered tacrolimus to Dark Agouti (DA) rats for 7 days and then measured changes in liver TTR mRNA levels and changes in serum TTR concentrations. After hepatectomy, TTR mRNA levels decreased by 77%; at day 3, they returned to preoperative levels. Except for slightly elevated serum TTR concentrations 12 h after operation, serum TTR levels remained unchanged. Thus, partial hepatectomy did not influence serum TTR concentrations. After tacrolimus administration, TTR mRNA declined by 56% 12 h after the experiment started; however, after day 3, a rebound phenomenon occurred until day 7. Tacrolimus may facilitate serum TTR degradation, although production of TTR in the liver also increased. This finding – that TTR, the source of FAP-inducing amyloid, did not increase after transplantation – may help post-transplantation treatment of patients who have FAP and other liver diseases.

Introduction

Various proteins, including transthyretin (TTR), have been identified as amyloidogenic proteins related to familial amyloidotic polyneuropathy (FAP); of these proteins, amyloidogenic transthyretin (ATTR) is the most common throughout the world [1–3]. TTR-related FAP is a hereditary amyloidosis in which mutated amyloidogenic proteins accumulate in organs and tissues such as peripheral nerves, heart, kidney, gastrointestinal tract, and ocular tissues [4].

Familial amyloidotic polyneuropathy can cause a myriad of symptoms and signs including polyneuropathy, cardiac and renal dysfunction, gastrointestinal abnormalities, and ocular disorders. Although certain new therapeutic options exist, treatment of FAP is still limited [5]. At present, because TTR is predominantly synthesized by the liver, liver transplantation is the only established therapy capable of halting production of ATTR and symptoms of FAP [6].

The positive outcome of such transplantations has stimulated research and use of more complex procedures, such as sequential liver transplantation (or domino liver transplantation), in which a resected liver from a patient with FAP is transplanted into a patient with a severe liver disorder or cancer [7]. By the end of March 2005, approximately 908 liver transplantations for FAP and 355 domino liver transplantations have been performed worldwide (FAP World Transplant Register; retrieved from http://www.fapwtr.org/index.htm, June 2005). Also, because of the shortage of donor livers, living donor liver transplantation (LDLT), which utilizes partial liver grafts, has often been performed.

Because serum TTR has proved to be a reliable indicator of nutritional status [8,9], serum TTR concentrations

have served for monitoring patients with malnutrition or cancer. It is also well known that serum TTR concentrations decrease during inflammation and infection [10]. Nevertheless, TTR behavior in the circulation has not been thoroughly evaluated after liver transplantation in domino graft recipients as well as in FAP patients.

The behavior of TTR under various liver transplant-related situations, such as after hepatectomy and during administration of immunosuppressants such as tacrolimus, may aid understanding of TTR metabolism. With the increasing number of liver transplantations being performed for FAP patients, the growing number of sequential liver transplant recipients, and the increasing need for LDLT, the impact of tacrolimus use and liver regeneration on TTR concentrations and amyloid formation has become an important subject worthy of extensive study.

Therefore, to better understand TTR metabolism during and after liver transplantation, we examined the effect of tacrolimus administration or hepatectomy on TTR mRNA in the liver and on TTR serum concentrations in rats.

Materials and methods

Animals

Ninety adult male Dark Agouti (DA) rats, each weighing between 190 and 270 g, were purchased from the SLC Co. (Hamamatsu, Japan). The rats were kept in the Kumamoto University Animal Center and given free access to water and rodent chow. All experiments were performed according to the Principles of Laboratory Animal Care (NIH publication No. 86–23, revised 1985). Procedures were carried out with the animals under ether inhalation anesthesia.

Experimental procedure

Rats were divided into four groups. The rats in the first group ($n = 25$) underwent a 70% hepatectomy using the Higgins–Anderson technique [11]. Those in the second group ($n = 25$) were injected with tacrolimus at a dose of 2 mg/kg i.m. in the leg, with each dose being injected in the alternate leg. The third ($n = 15$) and fourth ($n = 20$) groups of rats were used as controls for the first and second groups. A sham operation was performed on the third group, and an i.m. injection of normal saline was used as a placebo in the fourth group. In the four groups, the rats used at the 0 time point were the same ($n = 5$).

Tacrolimus or normal saline was injected at 0.5, 1, 1.5, 2, 3, 4, 5, and 6 days after the initial dose. All rats were exsanguinated at five time points: 0.5, 1, 2, 3, and 7 days. After the rats were killed, blood and liver tissue samples were obtained. Liver tissues were immediately frozen in liquid nitrogen and stored in a −80 °C refrigerator.

Measurement of blood tacrolimus concentrations (trough levels) and serum analysis

Tacrolimus (Prograf®; Astelas, Osaka, Japan) was generously provided by the Astelas Co. Trough levels of whole blood tacrolimus concentrations were measured by means of the microparticulate enzyme immunoassay (MEIA) using the IMx analyzer from Abbott Laboratories (Abbott Park, IL, USA).

Serum samples from rats given tacrolimus (1.0 or 2.0 mg/kg) were analyzed at the Department of Laboratory Medicine at Kumamoto University Hospital. Serum samples from each time point were evaluated for aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, and blood urea nitrogen (BUN). Serum TTR concentrations were measured by using the enzyme-linked immunosorbent assay (ELISA) technique as described previously [12] and an antirat-TTR immunoglobulin, previously prepared by Tajiri *et al.* [13].

Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the liver of the rats by means of PURESCRIPT RNA Isolation Kit (Gentra, Minneapolis, MN, USA) and TURBO DNase treatment and reagents (Ambion, Austin, TX, USA). External standards, consisting of serial dilutions of rat TTR cDNA (107, 105, and 103 copies), were constructed by using RT-PCR. To evaluate rat TTR mRNA copies, upstream and downstream primer sequences were 5′-TATTTGCG TCTGAAGCTGG-3′ and 5′-CCTTCC-GTGAA-CTTCTCA TCT-3′, respectively. The hybridization probe sequences were 5′-TGTGGCCGTGAAAGTGT-3′-Flu and LC Red 640-5′-CAAAAGGACTGCAGACGGAAGCTGGGAGCCGT TTGCCTCTGGG-3′-P. The primers, hybridization probes, and rat GAPDH external standards (4×10^4, 4×10^3, and 4×10^2 copies) for evaluating rat GAPDH mRNA copies were obtained from Nihon Gene Research Laboratories (Sendai, Japan). The reaction mixture consisted of 3.25 mM $Mn(OAc)_2$, 0.3 μM each primer, 0.2 μM hybridization probes, 7.5 μl of RNA LightCycler RNA Master Hybridization probes mixture (Roche Molecular Biochemicals, Tokyo, Japan), 10-ng cDNA samples or external standards, and MilliQ water up to a final volume of 20 μl. The crossing point values of these standards were used to generate an external standard curve to allow accurate quantification. The ratio of rat TTR mRNA copies to rat GAPDH mRNA copies was estimated.

Statistical analysis

Statistical evaluation was performed with the JMP statistical analysis software (SAS Institute, Cary, NC, USA) by means of the paired *t*-test. A *P*-value of <0.05 was considered to be statistically significant.

Results

Effect of hepatectomy on TTR metabolism

Serum samples from rats having had a 70% hepatectomy showed that TTR concentrations remained stable except for a small but significant elevation ($P < 0.05$) at 12 h after the procedure. Serum TTR concentrations did not fall below the initial value at any time point (Fig. 1a). However, the TTR mRNA level immediately decreased and was lowest – 23% of the initial value – at 1 day after the surgery ($P < 0.05$). After this point, the TTR mRNA level began to increase, reached the original value at day 3, and remained unchanged until day 7 (Fig. 1b). The sham operation caused significantly reduced serum TTR concentrations from day 1 until day 7 after the operation ($P < 0.05$) (Fig. 2).

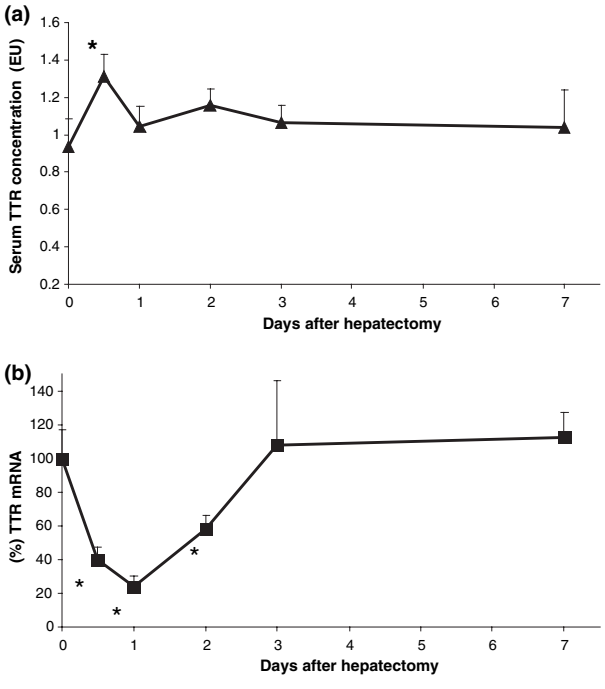

Figure 1 Effect of 70% hepatectomy on TTR metabolism. Dark Agouti (DA) rats ($n = 30$) underwent a 70% hepatectomy, and serum TTR concentrations (a) and TTR mRNA levels in the liver ($n = 18$) (b) were measured as described in the text. *$P < 0.05$ for each time point versus the 0 time point.

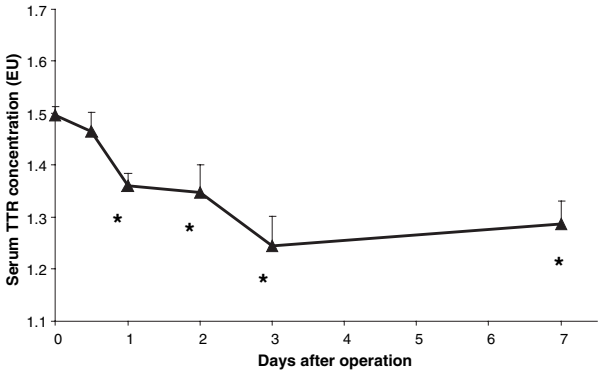

Figure 2 Effect of sham operation on serum transthyretin (TTR) concentrations. DA rats ($n = 25$) underwent a sham operation, after which the serum TTR concentration was measured by using ELISA. *$P < 0.05$ for each time point versus the 0 time point.

Effect of tacrolimus administration on TTR metabolism

In a preliminary experiment, we administered different doses of tacrolimus (1.0 or 2.0 mg/kg) to rats. We first used 1 mg/kg, but trough levels (levels in whole blood) increased slowly. In another study, a 2 mg/kg dose had been used with only slight signs of toxicity [14]; therefore, we chose 2 mg/kg as a nontoxic high dose. Trough levels of tacrolimus after the dose of 2.0 mg/kg showed a rapid increase; at day 2, levels had reached >20 ng/ml, and they remained between 20 and 30 ng/ml until day 7 (Fig. 3).

Serum samples of rats that had received tacrolimus (2 mg/kg) revealed no significant elevation in serum AST and ALT values (Fig. 4a). Serum creatinine and BUN did show the increased levels after 7 days of treatment (Fig. 4b), as has been reported in previous experiments [14]; because of the short duration of the experiment, however, renal function did not deteriorate significantly.

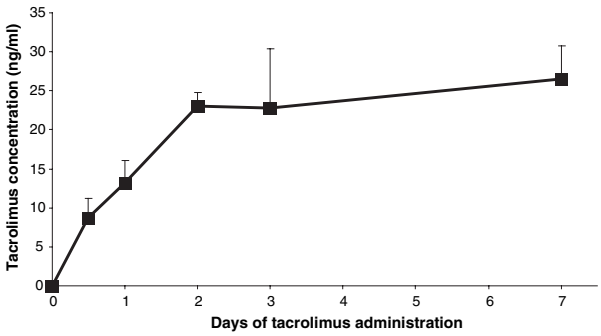

Figure 3 Trough levels of tacrolimus after the administration of 2 mg/kg. DA rats ($n = 30$) were given a 2 mg/kg dose of tacrolimus. As described in the text, trough levels were measured by using the microparticulate enzyme immunoassay (MEIA) technique.

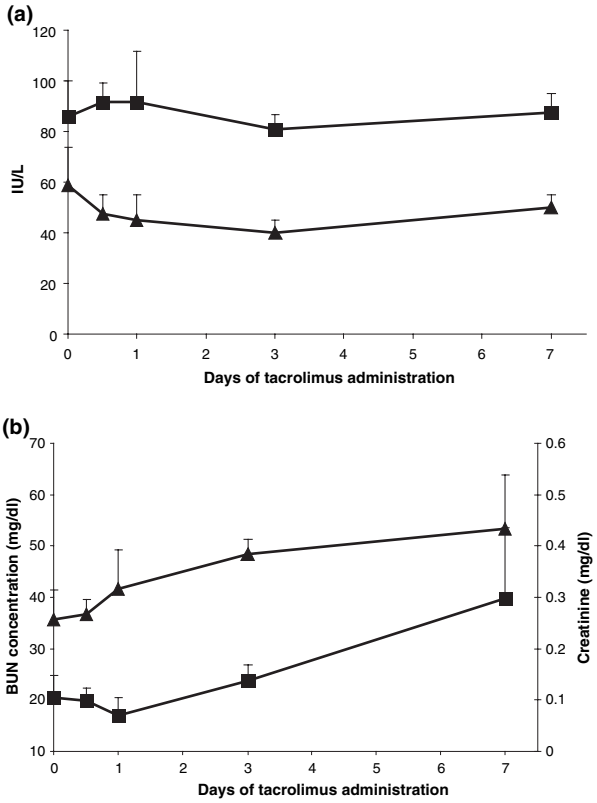

Figure 4 Aspartate aminotransferase (AST), alanine aminotransferase (ALT), serum creatinine, and blood urea nitrogen (BUN) levels after tacrolimus administration. Serum samples from rats that had been treated with tacrolimus at 2 mg/kg ($n = 15$) were evaluated as described in the text. (a) AST (closed squares) and ALT (closed triangles). (b) Creatinine (closed triangles) and BUN (closed squares).

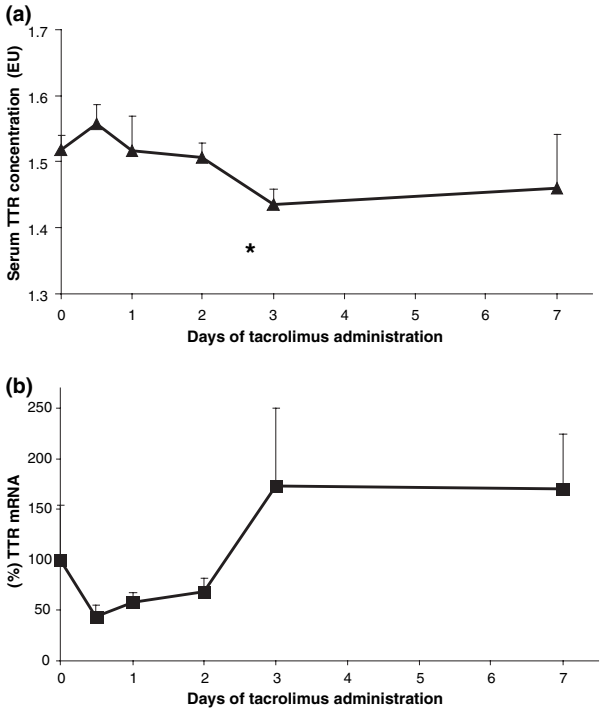

Figure 5 Effect of tacrolimus administration on TTR metabolism. After tacrolimus administration to DA rats ($n = 30$), serum TTR concentrations (a) and TTR mRNA levels in the liver ($n = 18$) (b) were measured as described in the text. $^*P < 0.05$ for each time point versus the 0 time point.

Serum TTR concentrations of rats treated with tacrolimus (2 mg/kg) were stable for 2 days (Fig. 5a), when trough levels were <20–25 ng/ml. However, on day 3, serum TTR concentrations decreased slightly but significantly and remained at a somewhat similar level until day 7. The TTR mRNA value decreased immediately after the first tacrolimus administration to 40% of the initial level and remained low until day 2 (Fig. 5b). Between days 2 and day 3, TTR mRNA levels increased quickly to 170% and remained unchanged until day 7, although this change was not statistically significant. Samples from rats that had received saline injections showed no variation in serum TTR concentrations over time; TTR mRNA levels also showed no changes and remained stable until day 7 (data not shown).

Discussion

Liver transplantation for management of FAP and sequential liver transplantation using a resected liver from FAP patients are frequently performed. Nevertheless, these methods have resulted in several problems: for example, clinical manifestations of FAP, which may ultimately be related to the amyloidogenic protein TTR, have progressed in several patients even after liver transplantation [15,16]. Also, in one case, a recipient of an FAP liver started to manifest symptoms of FAP 8 years after the surgery [17]. Moreover, partial liver transplants obtained from living donors have often been used because of the shortage of donor livers. Thus, the effects of surgical procedures and immunosuppressants on TTR metabolism should be elucidated. In this report, we describe changes in TTR metabolism in rats after hepatectomy and after administration of the immunosuppressant tacrolimus, as evaluated by measuring TTR mRNA levels in the liver and serum TTR concentrations.

After a 70% hepatectomy, TTR mRNA levels decreased significantly for 2 days even though they returned to preoperative values at day 3 after the surgery. Studies on liver regeneration have found that in rats, after 70% hepatectomy, the liver regenerates quickly and attains its original size by 7–10 days [18]; nevertheless, during the

initial days, the liver mass is clearly reduced. Therefore, the net TTR production was significantly decreased, and as a result, serum TTR concentrations should have decreased. However, serum TTR concentrations did not decrease as expected. It should be noted that the liver is one of the major catabolic sites [19], as well as the major production site, of TTR. A comparably higher reduction in TTR catabolism by the liver may be the reason for the higher-than-expected serum TTR concentrations.

With regard to tacrolimus administration, a dose of 2 mg/kg caused an initial decrease in TTR mRNA levels in the liver, but only for 2 days after the first dose. Thereafter, levels increased to 170% of the original value at day 3 and remained elevated until day 7. At the same time, serum TTR concentrations decreased (significantly at day 3) and remained low until day 7.

Clinically, immunosuppressive therapy for liver transplantation mainly consists of tacrolimus or cyclosporine. Previous studies have found that after liver transplantation in FAP patients, serum TTR concentration may be modified. A study by Stangou *et al.* [20] found that the serum TTR levels of FAP patients receiving liver transplantation mostly decreased or remained constant in tacrolimus-treated patients. Other studies have also documented that FAP patients who have had transplants and recipients of FAP livers have shown lower serum wild-type TTR and mutated TTR levels than those in control subjects [21]. Serum TTR concentrations in rats after tacrolimus administration were quite consistent with those found in these patients. With regard to the TTR mRNA finding in the liver, protein mRNA levels are well known to be regulated by serum protein concentrations. We believe that the rebound phenomenon for the TTR mRNA finding, as shown in Fig. 5, may be the result of the reduced serum TTR concentrations.

The reason why serum TTR levels decreased after tacrolimus administration is not yet clear. Even though the main action of tacrolimus is by inhibition of interleukin 2, a powerful cytokine in the immune system response [22], tacrolimus also acts on interleukin 1-interleukin 6 pathways [23,24], which are important in the TTR production system. This phenomenon may be beneficial for recipients of an FAP liver because the level of TTR, the pathogenic protein of FAP, may decrease during tacrolimus administration. The first case of amyloid deposition documented in a patient 8 years after receiving an FAP liver was published recently [17]. In a personal communication (to Y. Ando), the authors reported that this patient was using tacrolimus as the main immunosuppressive therapy.

In summary, a large partial hepatectomy did not produce decreased or increased serum TTR concentrations. In contrast, tacrolimus administration did cause decreased serum TTR concentrations. In recipients of FAP livers, the variant ATTR produced by the donor liver appears in the blood circulation, and this ATTR may cause symptomatic FAP in the future, as demonstrated in one case [17]. Therefore, the effect of tacrolimus on the TTR serum concentration may help delay the onset of FAP. Investigations with a longer-time frame are required to obtain more precise information on the behavior of TTR in these two transplant-related situations.

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