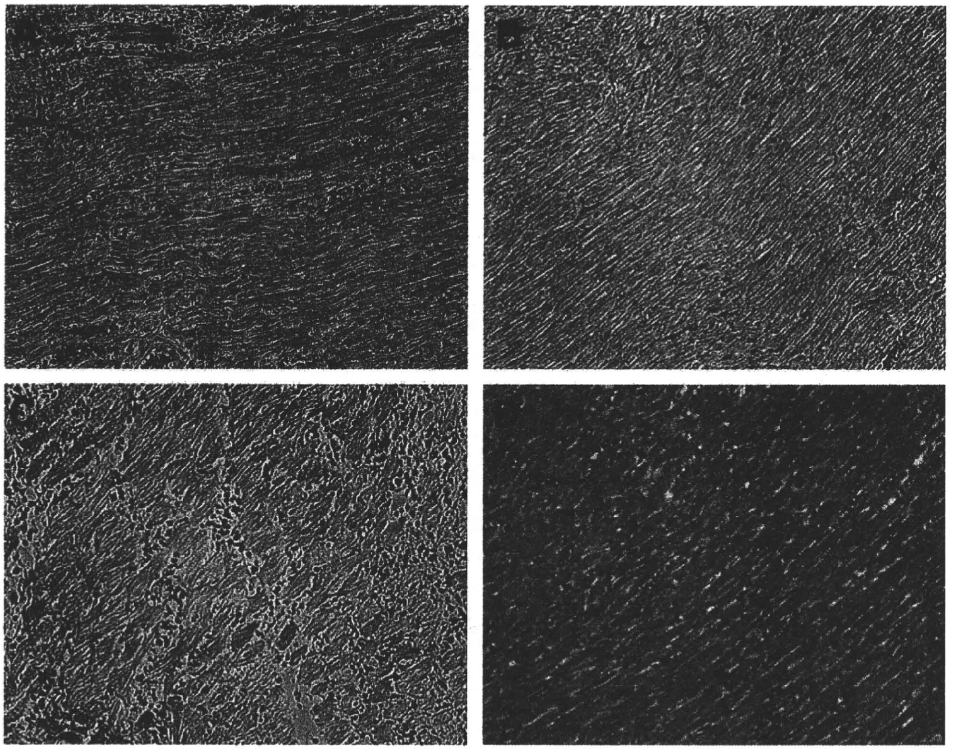

FIGURE 1. Histological analysis of cardiac allografts with hematoxylin-eosin staining. (A) Untreated allograft at day 3 after transplantation. (B) Allograft treated daily with SP600125 (40 mg/kg) at day 3. (C) Untreated allograft at day 5. (D) Allograft treated daily with SP600125 (40 mg/kg) at day 5. Note the marked mononuclear cell infiltration, interstitial hemorrhage, edema, and myocardial destruction in (C). These changes are significantly less in (D). Original magnification: ×200.

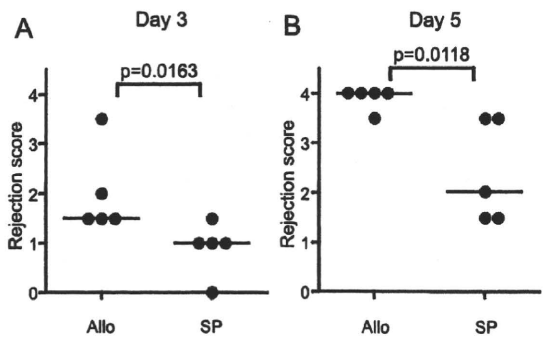

FIGURE 2. Effect of SP600125 on the histological cellular rejection of cardiac allografts. Rejection scores were determined according to the ISHLT histological grades of cellular rejection, and ranged from 0 (no rejection) to 4 (severe acute rejection) in untreated (Allo) and SP600125-treated allografts (40 mg/kg) (SP) at 3 days (A) or 5 days (B) after cardiac transplantation (n=5 in each group at each time point). Horizontal lines represent medians.

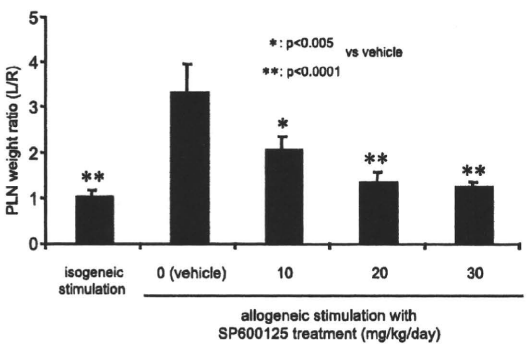

FIGURE 3. Effect of SP600125 on the alloantigen-induced popliteal lymph node (PLN) hyperplasia. To induce local alloimmune reactions, BN or LEW splenocytes (2.5×10^6 cells) were injected subcutaneously into the left hind footpads of LEW rats on day 0. The rats (n=5–6) were subcutaneously treated with vehicle or SP600125 (10–30 mg/kg) from day 0 to day 3. On day 4, bilateral PLNs were weighed. An increase in the left PLN weight was expressed as the left PLN weight/the right PLN weight. Each column represents mean±SE.

popliteal lymph node was observed when the ipsilateral hind footpad was injected with isogeneic splenocytes. The weight of the left popliteal lymph node was almost equivalent to that of the right (weight ratio of the left to the right PLN: 1.1±0.1). In contrast, the left PLN weight increased in vehicle-treated rats 4 days after stimulation by allogeneic splenocytes (weight ratio 3.4±0.6, $P<0.0001$ vs. isogeneic control). In rats treated with SP600125, this enlargement was significantly inhibited in a dose-dependent manner (weight ratio: 2.1±0.3 for 10 mg/kg, 1.4±0.2 for 20 mg/kg and 1.3±0.1 for 30 mg/kg; $P=0.0037$, $P<0.0001$, and $P<0.0001$ vs. vehicle control, respectively). Observations from the PLN model could result from the anti-lymphoproliferative effects of SP600125.

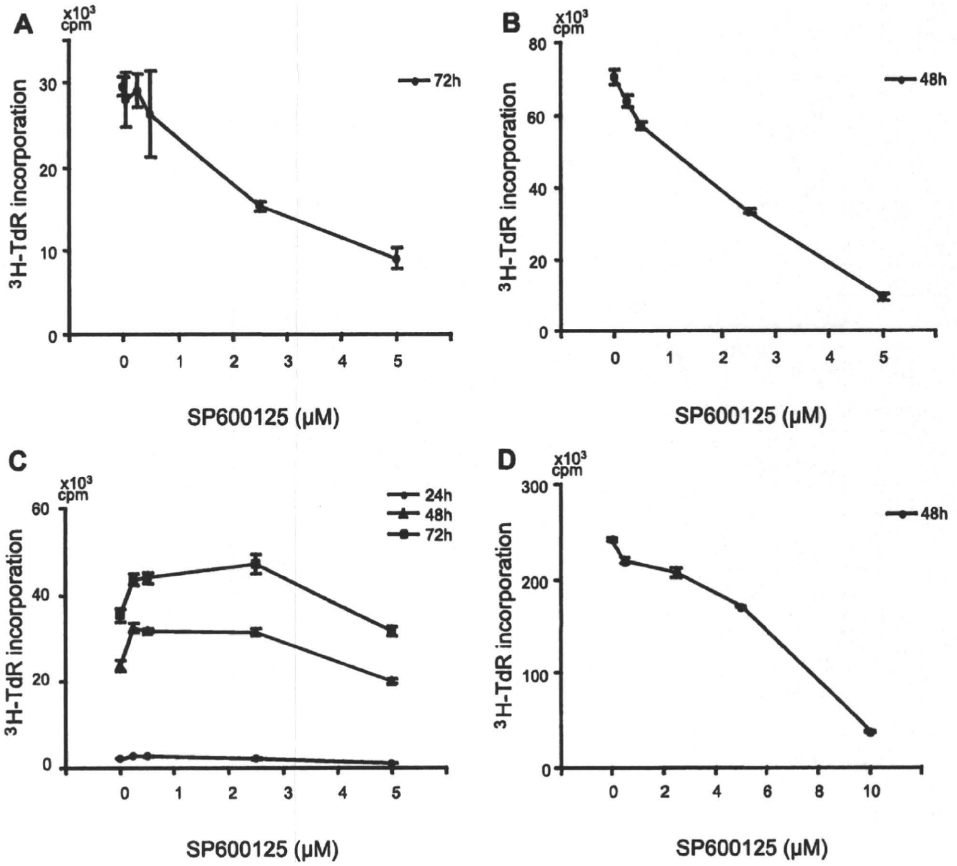

FIGURE 4. Effect of SP600125 on lymphocyte proliferation. Primary mesenteric lymphocytes (2.5×10^5 cells) from LEW rats were stimulated with irradiated BN splenocytes (2.5×10^5 cells; A), Con A (1 μg/mL; B), or LPS (25 μg/mL; C) for 24–72 h in the presence of vehicle or increasing concentrations of SP600125. OX52-positive mesenteric T-lymphocytes (1×10^5 cells, 97.1% CD3-positive) were unstimulated or stimulated with PMA (5 ng/mL) plus ionomycin (375 ng/mL) for 48 h in the presence of vehicle or SP600125 (D). Cell proliferation was determined by ^{3}H-TdR incorporation and quantified as cpm. The assays were performed in triplicates and data were expressed as mean±SE. MLR of isogeneic control was 4711±298 cpm (A). A value of unstimulated control was 2265±171 (B, C) and 180±18 cpm (D). Calculated IC_{50}: 2 μM (A), 1.5 μM (B), >5 μM (C), 5.7 μM (D). Data shown are representative of three independent experiments with similar results.

SP600125 Inhibits MLR and Mitogen-Induced T-lymphocyte Proliferation

Evidence of inhibition of the alloantigen-induced PLN enlargement by SP600125 in vivo led us to examine whether this compound could inhibit alloantigen-induced lymphocyte proliferation in MLR (Fig. 4A). When LEW mesenteric lymphocytes were incubated for 72 hr in the presence of irradiated BN splenocytes, robust-cell proliferation was detected using ^{3}H-TdR incorporation. As expected, addition of SP600125 to the cell culture at concentrations ranging from 0.05 to 5 μM caused concentration-dependent inhibition of lymphocyte proliferation with a calculated IC_{50} of 2 μM. When the lymphocytes were stimulated with T-cell mitogen Con A for 48 hr, SP600125 also effectively suppressed ^{3}H-TdR uptake in a similar fashion (calculated IC_{50}: 1.5 μM; Fig. 4B). In addition, we confirmed the antiproliferative effects of SP600125 in simultaneously performed experiments using the tetrazolium-based metabolical cell proliferation assay (data not shown). In contrast, SP100625 up to 5 μM failed to inhibit the lymphocyte proliferation induced by B-cell mitogen LPS (Fig. 4C). One might concern that the mesenteric lymph node included non-T-cell populations. Therefore OX52-positive lymphocytes were stimulated by PMA plus ionomycin to demonstrate a direct inhibitory effect of SP600125 on the T-cell proliferation. SP600125 still suppressed the induced cell proliferation (Fig. 4D).

SP600125 and CsA Additively Prolong Cardiac Allograft Survival

CsA is a widely used immunosuppressant in clinical transplantation and similarly acts downstream of T-cell receptor ligation. BN cardiac allografts were transplanted into LEW recipients to evaluate the pharmacological relationship between SP600125 and CsA (Table 2). The percentage of viable grafts within each treatment group on day 11 was used as the study endpoint. The minimum doses required for 100% graft survival were 0.5 mg/kg for CsA and 20 mg/kg for SP600125, respectively (Tables 1 and 2). When CsA at 0.25 mg/kg was used in combination with SP600125 at 10 mg/kg, this combination was equi-effective and yielded 100% graft survival, indicating additivism between the two drugs (0.25/0.5+10/20 =1) according to the algebraic method described by Berenbaum (*18*). Furthermore, the similar pharmacological interaction was observed when CsA at 0.5 mg/kg was

TABLE 2. Interaction of SP600125 with CsA on rat cardiac allograft survival

Treatment	n	Graft survival (days)	Median	*P* value
CsA 0.25 mg/kg	5	9, 9, 10, 10, 10	10	—
CsA 0.5 mg/kg	5	13, 13, 13, 14, 14	13	0.0031[b]
CsA 0.25 mg/kg+SP600125 10 mg/kg	5	12, 12, 12, 13, 14	12	0.0031[b]
CsA 0.5 mg/kg+SP600125 20 mg/kg	5	18, 19, 23, 24, >30[a]	23	0.0023[c]

[a] The allograft was sacrificed at the time points described. Graft survivals were compared using the log-rank test.
[b] Versus CsA 0.25 mg/kg.
[c] Versus CsA 0.5 mg/kg.

administered with SP600125 at 20 mg/kg as a higher dose-combination. This combination approximately doubled median allograft survival of each drug alone, and quite comparable to the treatment of SP600125 40 mg/kg (Table 1) in prolongation of allograft survival.

DISCUSSION

Here we confirmed the suppression of in vivo alloimmune responses by a specific inhibitor of JNK, SP600125. In the rat heart transplantation model, we observed that administration of SP600125 to LEW recipients significantly reduced infiltration of mononuclear cells into cardiac allografts from BN donors and preserved their histological structure, eventually resulting in significant prolongation of allograft survival without apparent adverse effects. Since we (*19*) and other investigators (*1, 2*) previously found that the majority of infiltrating cells in acute allograft rejection are CD4 and CD8 positive T-lymphocytes, and macrophages, it was reasonable to assume that the functions of these cells might be affected by SP600125. Therefore we first addressed in vivo antilymphoproliferative effects of SP600125 using the alloantigen-stimulated PLN hyperplasia assay, which is considered an in vivo model of MLR. MLR is routinely used to detect alloantigen-activated T-cell proliferation in vitro. In agreement with MLR, the induced PLN weight gain is known to be alloantigen-specific and reflects lymphocyte proliferation as evidenced by strong association of the PLN weight gain with in vivo [^{3}H]thymidine incorporation by the popliteal lymphocytes (*17*). Thus, our data demonstrating significant inhibition of the alloantigen-specific PLN hyperplasia by SP600125 could indicate its in vivo inhibitory effect on the T-cell proliferation. However, this inhibition may result from impairment of lymphocyte differentiation and recruitment rather than proliferation because the existing literature supports an effect of SP600125 on T-cell differentiation rather than activation as described in the Introduction. We and other investigators have shown that CsA and other immunosuppressive molecules inhibiting MLR also suppress the induced PLN hyperplasia with similar potency against the acute allograft rejection (*17, 20*). Like CsA, SP600125 dose-dependent activity in this assay correlated with its immunosuppressive activity to inhibit the cardiac allograft rejection.

Similarly to the case in the PLN hyperplasia assay, we confirmed the anti-lymphoproliferative effect of SP600125 in MLR. Since SP600125 did not affected the lymphocyte proliferation induced by B-cell-mitogen LPS, but inhibited T-cell-mitogen Con A-induced lymphocyte proliferation in a concentration-dependent manner, these observations indicate that the activity of SP600125 is not from general cytotoxicity, and is directed selectively to T-lymphocytes in this pathology. In fact, we further demonstrated that T-lymphocytes positively separated from mesenteric lymph node cells were still susceptible to the anti-proliferation by SP6000125. The IC_{50} values observed in these assays are consistent with reported values to suppress c-Jun phosphorylation and IL-2 expression in Jurkat T-cells, and human Th1 and Th2 cells (*12*). Furthermore, SP600125 was reported to inhibit CD25 (α chain of IL-2 receptor) expression in human CD4 positive T-cells cultured with anti-CD3 and anti-CD28 monoclonal antibodies under Th1 polarizing conditions (*12*). Similarly, inhibition of IL-2 production, CD25 expression and cell proliferation by SP600125 has been reported in CD3/CD28-activated CD8 positive T-cells (*9*). Thus, it is plausible that SP600125 inhibits both production of IL-2 and its receptor-mediated signals to suppress T-cell proliferation. However, it is interesting to note that CD4 positive T-cells deficient in both JNK1 and JNK2 were reported to produce more IL-2 and proliferate better than wild-type cells in vitro, and developed pronounced Th2 cytokine responses under Th1 polarizing conditions (*11*), thus indicating a defect in Th1 differentiation as in the JNK1 or JNK2 knockout mice (*7, 8*). On the other hand, another group reported IL-2 and proliferation defects in mixed populations of CD4 and CD8 positive T-cells from mice lacking JNK1 or JNK2 and restoration of the proliferation by exogenous IL-2 (*5, 6*). Further investigations are required to clarify additional mechanisms of action of SP600125. Nevertheless, taken together, our study demonstrates that genetically unmodified animals are susceptible in vivo to suppression of alloimmune responses by SP600125, and supports important roles for JNK in T-cell activation and proliferation in physiological condition.

The phosphatase, calcineurin regulates NF-AT activation may also contribute to JNK activation after T-cell stimulation (*21*), and this signaling pathway is sensitive to inhibition by a calcineurin inhibitor CsA (*22*). In this context, our observations showing the additive relation between SP600125 and CsA are completely consistent with mechanism. This interaction may be advantageous if SP600125 is adjunctively utilized with CsA to control the immune response. In addition, SP600125 is reported to suppress interleukin IL-6-induced production of vascular endothelial growth factor (VEGF) in cultured fibroblasts more potently than CsA. Thus this might represent a beneficial feature of this compound over CsA in the transplant setting since angiogeesis is a common complication of organ-transplant rejection and one of the primary responsible molecules for enhanced angiogenesis is VEGF (*23*).

SP600125 also inhibits JNK3, which is primarily localized to neuronal tissues and cardiomyocytes (*12*). Although we did not evaluate it closely in the current study, no neurological and hemodynamic impairment was observed in rats treated with SP600125 through all the study period. Apart from the main issue of immunosuppression by SP600125 in our study, SP600125 is consistently reported to be neuroprotective against brain ischemia/reperfusion (I/R) injury with reduction of neuronal apoptosis (*24–27*). However, the protective effect of SP600125 against I/R injury is not consistent in the cardiomyocyte or the heart (*28–30*). Similarly, SP600125 promotes apoptosis, fibrosis, left ventricular dilatation and dysfunction in the dilated cardiomyopthy hamster model (*31*), whereas this compound does inhibit not only β-adrenergic receptor-stimulated apoptosis in cardiomyocytes (*32*) but also angiotensin II-induced apoptosis in cardiomyoblasts (*33*). Further studies are required to elucidate the complete contribution of SP600125 to cardioprotection.

In conclusion, we have shown for the first time that a specific inhibitor of JNK, SP600125 suppressed T-cell proliferation, alloantigen-induced immune responses and prolongs allograft survival in rat models. We have also demonstrated that the pharmacological relationship between SP600125 and CsA is additive. Our data support the potential of JNK as a novel therapeutic target to control undesired T-cell-mediated immune responses in the patient of transplant rejection or autoimmune disease.

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PROTECTIVE MECHANISM OF β-SQAG9 LIPOSOME, A SULFONOGLYCOLIPID EXTRACTED FROM SEA URCHIN INTESTINES, AGAINST HEPATIC ISCHEMIA REPERFUSION INJURY

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ABSTRACT—We previously reported that β-SQAG9 liposome, a sulfonoglycolipid extracted from sea urchin intestines, had a protective effect against hepatic ischemia reperfusion (I/R) injury. In this study, we made a detailed investigation of this protective effect and its mechanism. Rats were pretreated either with β-SQAG9 liposome (treated group) or with phosphate-buffered saline solution (control group). Thereafter, they were subjected to partial hepatic I/R. The serum levels of aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase were measured, and histological damage was evaluated with hematoxylin and eosin staining. To investigate the protective mechanism of β-SQAG9 liposome on I/R injury, the serum levels and the tissue messenger RNA levels of TNF-α and IL-1β were measured, and polymorphonuclear neutrophil (PMN) infiltration was histologically evaluated by immunohistochemistry. Moreover, to investigate an interaction between β-SQAG9 liposome and L-selectin on PMNs, flow cytometric analysis and immunofluorescence were performed. β-SQAG9 liposome reduced the hepatic I/R injury. The pretreatment with β-SQAG9 liposome reduced the PMN infiltration into the liver parenchyma. On the other hand, there was no apparent difference in the serum levels and the tissue messenger RNA levels of the proinflammatory cytokines between the two groups. Thus, β-SQAG9 liposome might reduce the hepatic I/R injury by inhibition of the PMN infiltration into the liver parenchyma, which was independent of the regulation of cytokine production. Moreover, we demonstrated that β-SQAG9 liposome specifically bound to L-selectin on PMN cell surface, which mediated the PMN infiltration. β-SQAG9 liposome might competitively antagonize L-selectin on PMNs and suppress the subsequent PMN infiltration, resulting in the reduction in I/R injury.

KEYWORDS—Polymorphonuclear neutrophil, L-selectin, tumor necrosis factor α, interleukin 1β, liposome

INTRODUCTION

Hepatic warm ischemia reperfusion (I/R) injury remains a problem in various situations, including liver transplantation, hepatic resection, trauma, and hypotensive or hemorrhagic shock during and after recovery (1–3). Bleeding and subsequent blood transfusions remain the main cause of mortality and morbidity in liver resection (4). To minimize blood loss while dividing the liver parenchyma, interruption of hepatic blood inflow was a common procedure during liver surgery (5, 6). However, the period of hepatic ischemia associated with this technique and the resultant reperfusion can lead to liver injury and dysfunction. Thus, hepatic I/R injury has been actively investigated, and recently, protective strategies consisting of surgical interventions, pharmacological agents, and gene therapy have been reported (5, 7).

We previously reported that sulfonoglycolipids extracted from sea urchin intestines possessed immunosuppressive effects such as inhibition of human and rat MLR and delay of rat skin allograft rejection (8–10). Moreover, we reported that 1,2-di-*O*-acyl-3-*O*-(D-sulfoquinovosyl)-glyceride with two stearic acids (β-SQAG9) formed a liposome structure, improved to gain structural stability in the solution of original sulfonoglycolipids, which might bind to L-selectin (CD62L) molecules on lymphocyte *in vitro* (8–10). L-Selectin is known as one of the selectin family constituting adhesion molecules, and it mediates adhesion of polymorphonuclear neutrophils (PMNs) to endothelial cells. Infiltration of PMN contributes to the development of hepatic I/R injury (11, 12). This PMN infiltration is a multistep process, which involves rolling, firm adhesion, emigration of PMNs, and subsequent parenchymal cell damage in the microvasculature in hepatic I/R injury (13–15). Selectins play critical roles in the initial capture and support the rolling of PMNs on sinusoidal endothelium (15). Thus, selectins are one of the key mediators of I/R injury in several splanchnic organs, including the liver (11, 16). Therefore, we have focused on the relation between β-SQAG9 liposome and L-selectin and previously reported a preliminary investigation about the protective effect of β-SQAG9 liposome against hepatic I/R injury (17). This time, we made a detailed investigation of this protective effect and its mechanism.

MATERIALS AND METHODS

Animal protocols and hepatic I/R procedure

This study was performed in accordance with the guidelines for animal experimentation of Sapporo Medical University. Male LEW rats weighing 200 to

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300 g were purchased from Sankyo Labo Service (Sapporo, Japan) and were housed under specific pathogen-free conditions, maintained at a constant temperature with a 12-h light-dark cycle. The rats were fasted for 12 h before operation but were allowed to drink water *ad libitum*. They were anesthetized by the inhalation of diethyl ether (Sigma-Aldrich Co, St Louis, Mo) and intraperitoneal injection of 40 mg/kg sodium pentobarbital (Dainippon Pharma Co, Ltd, Osaka, Japan). All procedures were performed with the animals breathing spontaneously. After anesthetization, the liver was exposed by a midline incision. Left and median liver lobe ischemia (70% of liver mass) was induced by clamping the corresponding hepatic artery, portal vein, and bile duct with a microvascular clamp (Mera Co, Tokyo, Japan). This method of partial hepatic ischemia allows for portal decompression through the right and caudate lobes to prevent mesenteric venous congestion. After 30 min of ischemia, the ischemic liver was reperfused by removal of the microvascular clamp. Afterward, the abdominal wall was closed in standard fashion with 4-0 Ethilon (Johnson and Johnson Medical Pty, Ltd, Tokyo, Japan), and the animals were returned to their cages. When the rats were killed, they were newly anesthetized by the inhalation of diethyl either.

Animals were divided into 3 groups: the control group that was injected with phosphate-buffered saline solution (PBS; Dako Co, Carpinteria, Calif) and the groups treated with β-SQAG9 liposome at doses of 5 or 10 mg/kg. They were injected in the penile vein immediately before reperfusion. Rats were killed before the moment of ischemia and 1, 3, 6, 12, and 24 h after I/R. Afterward, hepatic tissues and blood samples were obtained.

Drug preparation

The 1,2-di-*O*-acyl-3-*O*-(-D-sulfoquinovosyl)-glyceride with two stearic acids (β-SQAG9) was synthesized as described in our previous report (8–10, 17).

Biochemical measurements

To measure the serum concentrations of markers of hepatic injury—aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH)—peripheral blood samples were taken from the penile vein immediately before ischemia and 1, 3, 6, 12, and 24 h after reperfusion. The blood samples were then centrifuged at 3000 rpm for 15 min at 4°C, and the serum supernatant was obtained for biochemical analyses.

Histological assessment

After drip infusion into the portal vein and washing of the vessels in the liver with cold isotonic lactated Ringer's solution (Otsuka Pharma Co, Ltd, Tokyo, Japan), hepatic tissues were obtained from the left lobe of the liver, which had been subjected to ischemia. Thereafter, they were fixed in 10% formalin neutral buffer solution (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and then embedded in paraffin. These sections were stained with hematoxylin and eosin (H&E) for light microscopic study.

Immunohistochemical staining

The ischemic hepatic tissues obtained from the left lobe of liver were cut into small pieces, covered with Tissue-Tek OCT Compound (Sakura Finetek, Torrance, Calif), and immediately snap frozen in liquid nitrogen. These sections were used in the immunohistochemical staining in the following manner. The hepatic tissue sections were incubated with Dako peroxidase blocking reagent, which suppresses nonspecific staining due to endogenous peroxidase and pseudoperoxidase activity. The sections were incubated with 10% goat serum for 10 min at room temperature. A mouse antirat HIS48 monoclonal antibody (mAb; diluted 1:500; Fujisawa Pharma Co, Ltd, Japan), which specifically recognizes rat granulocytes including PMNs (18–20), was applied in PBS (pH 7.4) containing 1% bovine serum albumin (Sigma Chemical Co, St Louis, Mo) for 90 min at room temperature. Dako LSAB2 System HRP (Dako Co), a refined avidin-biotin technique in which a biotylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules, was used followed by Dako AEC Substrate Chromogen (Dako Co) for the visualization of immunoreactive cells. Finally, nuclear counterstaining was performed with hematoxylin for 60 s at room temperature. Sections stained with HIS48 were used to investigate the degree of infiltrating PMNs. The PMNs were identified by the morphology, and the numbers of PMNs in each sample were counted in 40 high-power fields (HPF; original magnification ×400) of a light microscope.

Enzyme-linked immunosorbent assay

Serum tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) in peripheral blood were assayed by commercial enzyme-linked immunosorbent assay (ELISA) kits (BioSource International, Inc, Camarillo, Calif) according to the manufacturer's instruction. Each cytokine level was calculated from a standard curve generated from recombinant rat TNF-α or IL-1β.

TaqMan real-time polymerase chain reaction detection

For detection of proinflammatory cytokine expression, total RNA was extracted from 30 mg of hepatic tissue by RNeasy Mini Kit (Qiagen, Tokyo, Japan). First-strand complementary DNA was generated using SuperScript II Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, Calif) and then analyzed by TaqMan Expression assays with probe and primer sets TNF-α and IL-1β purchased from Applied Biosystems Japan, Ltd (Tokyo, Japan). The TaqMan probes contain 6-FAM reporter dyes linked to the 5 end of the probe and a nonfluorescent quencher at the 3 end of the probe. Simultaneously, housekeeping rodent glyceraldehyde-3-phosphate dehydrogenase control reagents were purchased from Applied Biosystems Japan, Ltd. The TaqMan probe carries a 5 VIC reporter dye and a 3 TAMRA quencher dye. TaqMan real-time polymerase chain reaction (PCR) assays were performed on triplicate complementary DNA samples in 96-well optical plates using an ABI PRISM 7000 Sequence Detection system (Applied Biosystems Japan, Ltd). Expression values for proinflammatory cytokines were compared with the expression in hepatic tissue of healthy rats. Relative quantification was carried out using the comparative cycle threshold method as described by the manufacturer.

Isolation of HIS48-positive PMNs using density gradient centrifugation and magnetic cell sorting

Peripheral blood of healthy control rats was obtained and suspended in PBS containing 2000 U of heparin (Novo heparin; Aventis Pharma, Ltd, Tokyo, Japan). The blood suspension was overlaid onto Lymphosepar II (Ficoll-Conray solution; specific gravity, 1.090 ± 0.001; IBL Co, Ltd, Tokyo, Japan (21)) and centrifuged at 3500 rpm at room temperature for 30 min. Subsequently, the lymphocyte fraction was collected and incubated with mouse antirat HIS48 mAb (1:100) at 4°C for 15 min. Afterward, this fraction was incubated with rat antimouse immunoglobulin M microbeads (1:10; Miltenyi Biotec, Inc, Auburn, Calif) at 4°C for 15 min. The magnetically labeled cell suspension was run through LS separation columns (Miltenyi Biotec, Inc). The HIS48-positive cell fraction collected was more than 98% pure as judged by flow cytometric analysis. The PMNs obtained by gradient centrifugation using Lymphosepar II were also used in the subsequent immunofluorescence.

Flow cytometric analysis

For flow cytometric analysis of peripheral blood PMN populations, the HIS48-positive cell fraction was incubated with PBS or anti-CD62L mAb or anti–β-actin mAb at 4°C for 1 h. Subsequently, calcein-SQAG, whose liposomes are composed of β-SQAG9, was internally capsuled with fluorescent calcein (9), pulsed to HIS48-positive cells, and incubated at 4°C for 1 h. The cells were then fixed with 1% formaldehyde in PBS. Flow cytometric analysis was performed using a FACSCalibur, and the data were analyzed using CELL Quest software (Becton Dickinson, Co, Ltd, Franklin Lakes, NJ).

Immunofluorescence in HIS48-positive cells

As with flow cytometric analysis, the lymphocyte fraction was incubated with mouse anti-HIS48 mAb (1:200) and with Alexa Fluor 594 F(ab)2 fragment of goat antimouse immunoglobulin G (H + L) diluted 1:200 at 4°C for 1 h, respectively. Subsequently, this fraction was incubated with PBS or anti-CD62L mAb or anti–β-actin mAb at 4°C for 1 h. Afterward, calcein-SQAG was pulsed to this fraction and incubated at 4°C for 1 h. The cells were then fixed with 1% formaldehyde in PBS and analyzed using a Radiance Confocal Scanning System (magnification ×660).

Statistical analysis

All data were expressed as means ± SD. Statistical analysis was performed with the unpaired Student *t* test or the Mann-Whitney test for comparison between 2 groups and by analysis of variance followed by the Scheffe F test among the 3 groups. A *P* value of less than 0.05 was considered significant.

RESULTS

β-SQAG9 liposome reduces the level of serum liver enzyme after I/R

To assess the damage to hepatic parenchyma after I/R, serum levels of AST, ALT, and LDH were measured before or 1, 3, 6, 12, and 24 h after I/R (Fig. 1, A–C). The levels of these serum liver enzymes were increased after I/R, peaked 3 h after I/R, and thereafter decreased gradually to almost normal levels

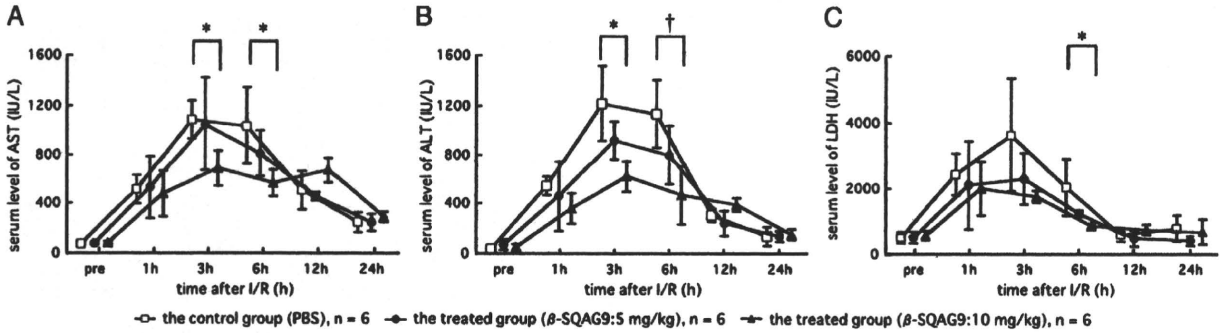

FIG. 1. **Serum levels of AST (A), ALT (B), and LDH (C) were measured before and 1, 3, 6, 12, and 24 h after I/R.** In the control group, the levels of these serum liver enzymes peaked 3 h after I/R. In the group treated with β-SQAG9 liposome (5 or 10 mg/kg), the levels 3 h after I/R were decreased dose dependently. Data were expressed as mean ± SD (n = 6 for each group). $^{*}P < 0.05$, $^{\dagger}P < 0.01$.

within 24 h in all groups. In the rats of the control group, the peak levels of these serum liver enzymes 3 h after I/R were 1085.3 ± 154.1, 1217.5 ± 360.3, and 3647.5 ± 1717.2 IU/L, respectively. In the groups treated with β-SQAG9 liposome (5 or 10 mg/kg), these levels 3 h after I/R were decreased dose dependently. At a dose of 10 mg/kg, the levels 3 h after I/R were reduced to 680.3 ± 147.6, 621.3 ± 127.7, and 1753.3 ± 181.0 IU/L, respectively. These levels at a dose of 10 mg/kg were significantly reduced in comparison with those in the control group at 3 or 6 h after I/R ($P < 0.05$). It was thus suggested that β-SQAG9 liposome might possess a protective effect against hepatic I/R injury. Moreover, the optimal dose of β-SQAG9 liposome might be 10 mg/kg. Therefore, in the subsequent assays described below, only the group with the 10-mg/kg dose and the control group were used.

β-SQAG9 liposome has no adverse effect at a dose of 10 mg/kg

To assess the adverse effect of β-SQAG9 liposome, serum levels of AST, ALT, and LDH were measured before and 1, 3, 6, 12, and 24 h after the injection with β-SQAG9 (10 mg/kg) into the healthy rats (Fig. 2, A–C). There was no significant difference in serum levels of the liver enzymes before and after the injection. In addition, all rats were alive without any physical symptoms during this experiment. Thus, β-SQAG9 liposome had no adverse effect at a dose of 10 mg/kg.

β-SQAG9 liposome has a protective effect on hepatic histological injury and a suppressive effect on PMN infiltration after I/R

The H&E staining of liver sections was observed before the moment of ischemia (Fig. 3A) and 1, 3, 6, and 24 h after I/R in the control group and the group treated with β-SQAG9 liposome at a dose of 10 mg/kg. The severest histological change was observed 3 h after I/R (Fig. 3, B and C), characterized by focal hepatocyte necrosis and PMN accumulation. This hepatocyte necrosis evidenced by nuclear degeneration and loss of distinct cellular borders was mainly accompanied by PMN accumulation. The degree of hepatocyte necrosis and PMN infiltration was markedly reduced in the group treated with β-SQAG9 liposome (Fig. 3C) in comparison with that in the control group (Fig. 3B).

To evaluate PMN infiltration, immunohistochemical staining for HIS48 molecule, a PMN marker, was estimated (the arrows in Fig. 4, A–C indicate PMNs), and the number of infiltrated PMNs was counted (Fig. 4D). Infiltration of PMN into the liver parenchyma was increased to a peak 3 h after I/R and thereafter gradually decreased, as with the change of level in serum liver enzymes and hepatic histological injury after I/R. There were almost no PMNs before I/R (Fig. 4, A and D). The peak number of PMNs 3 h after I/R in the treated group (Fig. 4, C and D) was significantly reduced in comparison with the control group (Fig. 4, B and D). The peak number of PMNs 3 h after I/R was 136.5 ± 18.2 cells per 40 HPF in the control group. By contrast, the number had decreased to 59.3 ± 9.3 cells per 40 HPF in the group treated with β-SQAG9 liposome ($P < 0.01$ compared with the control group; Fig. 4D).

β-SQAG9 liposome had little effect on the dynamic state of proinflammatory cytokines

The serum concentrations of TNF-α and IL-1β were measured by ELISA (Fig. 5, A and B). Additionally, these proinflammatory cytokines' messenger RNA (mRNA) levels

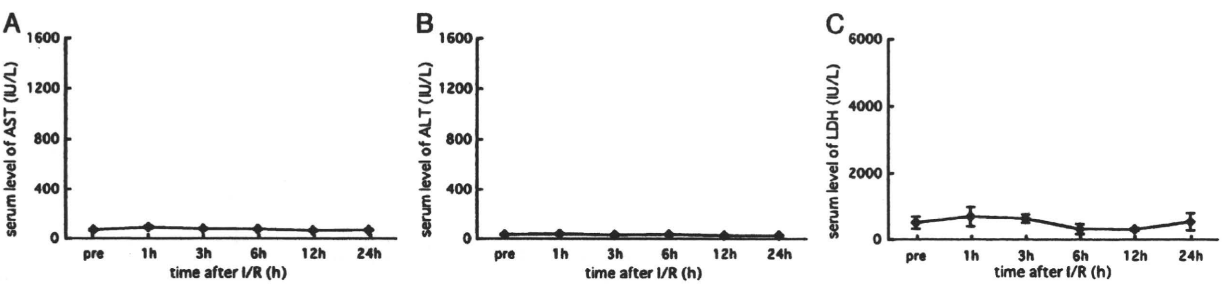

FIG. 2. **In the healthy rats, serum levels of AST (A), ALT (B), and LDH (C) were measured before and 1, 3, 6, 12, and 24 h after the administration of β-SQAG9 at a dose of 10 mg/kg.** There was no significant change in these serum levels before and after the injection. Data were expressed as mean ± SD (n = 6 for each group).

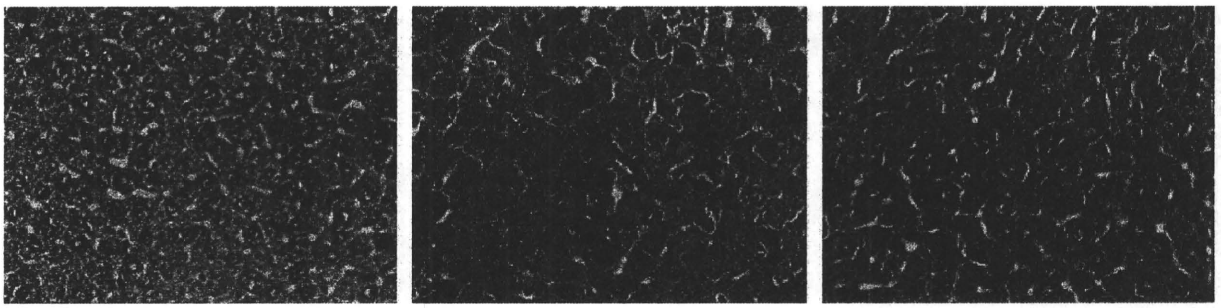

FIG. 3. **The H&E staining of liver sections was observed before the moment of ischemia (A) and after I/R (magnification ×400).** The PMN infiltration and focal necrosis peaked 3 h after I/R in the control group (B) and in the group treated with β-SQAG9 liposome at a dose of 10 mg/kg (C). Hepatocyte necrosis was mainly accompanied by PMN accumulation. The degree of hepatocyte necrosis and PMN infiltration was markedly more reduced in the treated group than that in the control group.

in the hepatic tissues were also assayed by TaqMan real-time PCR (Fig. 5, C and D). These data indicated similar time courses between the control group and the treated group. Thus, the administration of β-SQAG9 liposome had little affect on the dynamic state of proinflammatory cytokines.

β-SQAG9 liposome might specifically bind to CD62L molecules on HIS48-positive cells

To assess the direct binding activity of β-SQAG9 liposome to PMNs, calcein-SQAG, whose liposomes are composed of β-SQAG9, was internally capsuled with fluorescent calcein and pulsed to HIS48-positive cell fraction, which is comparable with PMNs, obtained from healthy rat peripheral blood (9). Subsequently, we estimated the number of HIS48-positive cells that bound with calcein-SQAG using flow cytometric analysis. The data indicated that calcein-SQAG bound to PMNs (Fig. 6A). Secondly, on the grounds that β-SQAG9 liposome possesses a binding ability to L-selectin on T cell (9), we investigated whether β-SQAG9 liposome would specifically bind to L-selectin on PMN by blocking assay with anti-CD62L mAb, which recognized L-selectin. The calcein-SQAG binding to the HIS48-positive cell was decreased by pretreatment with anti-CD62L mAb (Fig. 6B), whereas little difference was observed by pretreatment with anti–β-actin mAb, which was a control mAb (Fig. 6C).

Moreover, to further confirm these data by visualization, the localization of β-SQAG9 liposome and HIS48 molecule in peripheral blood cells of healthy rats was observed using immunofluorescence. The optical images obtained by confocal laser microscopy revealed green-stained calcein-SQAG and phycoerythrin-red–stained HIS48 molecule. This immunofluorescence disclosed calcein-SQAG to be localized on the HIS48-positive cell surface and not in the cellular cytoplasm (Fig. 6D). In addition, calcein-SQAG did not localize on HIS48-positive cell pretreated with anti-CD62L mAb (Fig. 6E) but did localize on HIS48-positive cells pretreated with anti–β-actin mAb (Fig. 6F).

DISCUSSION

We previously reported that β-SQAG9 liposome, which had improved solubility and stability in the solution of original sulfonolipid extracted from sea urchin intestines,

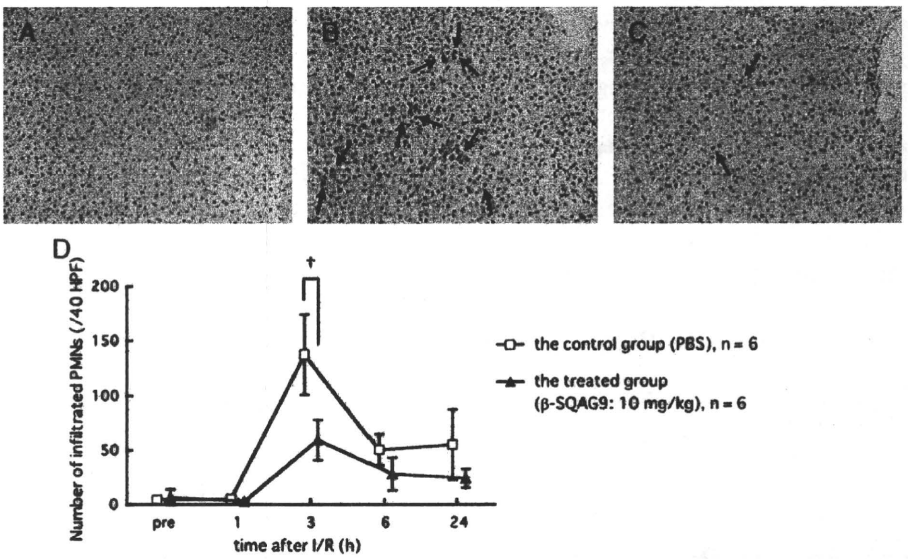

FIG. 4. **Immunohistochemical staining for HIS48 molecule as a PMN marker was assessed (magnification ×200).** The arrows indicate PMNs. There was almost no PMN before I/R (A). The number of PMNs 3 h after I/R in the treated group (C) was significantly reduced in comparison with the control group (B). The number of infiltrated PMNs was counted before the moment of ischemia and 1, 3, 6, and 24 h after I/R in 40 HPF (×400) with a light microscope (D). Infiltrated PMNs in the liver parenchyma were increased and peaked 3 h after I/R and thereafter gradually decreased. The peak number of PMNs in the treated group was significantly reduced in comparison with that in the control group. Data are expressed as mean ± SD (n = 6 for each group). $^{\dagger}P < 0.01$.

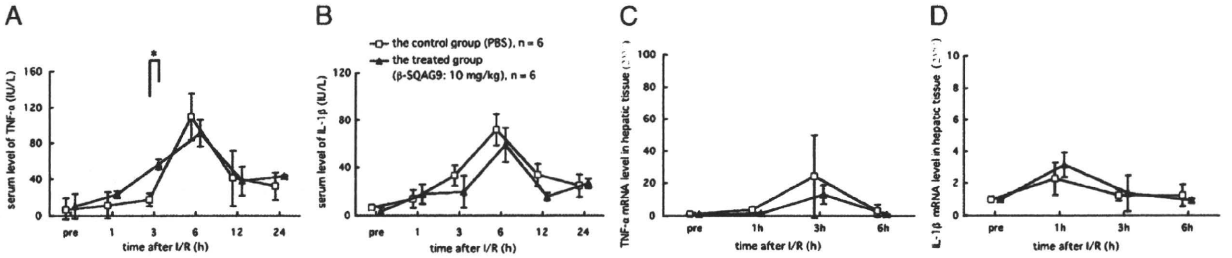

FIG. 5. **The serum levels of TNF-α (A) and IL-1β (B) were measured by ELISA.** The mean serum levels were increased to a peak level 6 h after I/R and thereafter gradually decreased in both TNF-α and IL-1β. There was almost no difference between the control group and the treated group. In addition, mRNA levels of TNF-α (C) and IL-1β (D) in the hepatic tissues were assayed by TaqMan real-time PCR. These mRNA levels indicated that there was almost no difference between the control group and the treated group. Data are expressed as mean ± SD (n = 6 for each group). $*P < 0.05$.

inhibited interaction between T cells and antigen-presenting cells and subsequently suppressed immunological reactions. Furthermore, β-SQAG9 liposome also possesses immunosuppressive effects in human and rat allogenic mixed lymphocyte reaction and prolongs skin allograft survival in rats (8–10). Because β-SQAG9 liposome has this anti-inflammatory property that might be mediated by L-selectin on T cell (9), we hypothesized that β-SQAG9 liposome might possess another anti-inflammatory effect. In this study, we investigated the protective effect and mechanism of β-SQAG9 liposome against rat hepatic I/R injury.

We used the rat experimental model of partial hepatic ischemia for 30 min followed by reperfusion. Progressive doses of β-SQAG9 liposome were given to rats to get an indication of the optimal dose to protect against hepatic I/R injury in this model. The protective effect by β-SQAG9 liposome was induced dose dependently at doses between 5 and 10 mg/kg. In addition, there was almost no change in the serum level of liver enzymes after the injection with β-SQAG9 at a dose of 10 mg/kg in the healthy rats, and all the rats were alive without any physical symptoms during this experiment. Therefore, the optically protective and safer dose of β-SQAG9 liposome was revealed to be 10 mg/kg. This drug safety was also reported in rat skin transplantation (8, 9).

The serum levels of AST, ALT, and LDH indicated an increase after I/R, peaking 3 h after I/R for 30 min. The severest histological damage was also observed 3 h after I/R,

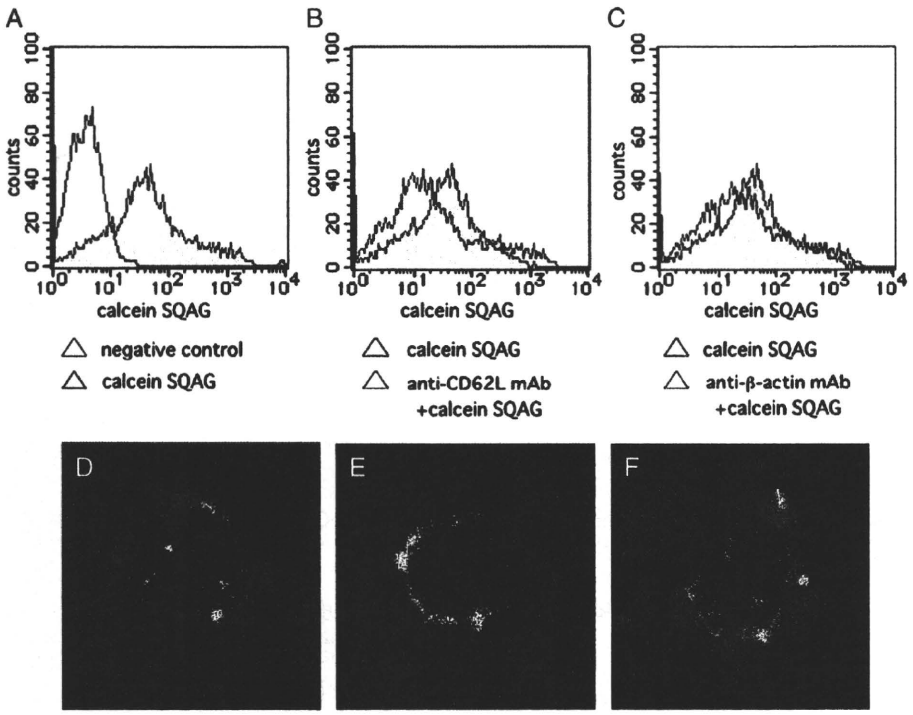

FIG. 6. **Calcein-SQAG, whose liposomes are composed of β-SQAG9, was internally capsuled with fluorescent calcein and pulsed to HIS48-positive cell fraction, which is comparable with PMNs, obtained from healthy rat peripheral blood.** Subsequently, we estimated the number of HIS48-positive cells that bound with calcein-SQAG using flow cytometric analysis. The data indicated that calcein-SQAG bound to PMNs (A). Secondly, blocking assay was done with anti-CD62L mAb. The calcein-SQAG binding to the HIS48-positive cell population was decreased by pretreatment with anti-CD62L mAb (B), whereas little difference was observed by pretreatment with anti-β-actin mAb, which was a control mAb (C). The optical images obtained by confocal laser microscopy revealed green-stained calcein-SQAG and phycoerythrin-red–stained HIS48 molecule (magnification ×660). This immunofluorescence revealed the localization of calcein-SQAG on the HIS48-positive cell surface, not in the cellular cytoplasm (D). Calcein-SQAG did not localize on HIS48-positive cells pretreated with anti-CD62L mAb (E) but localized on HIS48-positive cells pretreated with anti-β-actin mAb (F).

in sync with the change in serum levels of liver enzymes. In addition, we demonstrated by immunohistochemical staining that the count of PMNs infiltrated into the liver parenchyma also increased to a peak 3 h after I/R. By administration of β-SQAG9 liposome, the histological liver damage and the peak serum levels of liver enzymes were significantly reduced. The peak number of infiltrated PMNs was also reduced by β-SQAG9 liposome administration, although there was no apparent difference in the changes of serum levels or hepatic tissue mRNA levels of proinflammatory cytokines, such as TNF-α and IL-1β (22, 23), between the treated group and the control group. There are various reports on the mechanism of hepatic I/R injury, such as inflammatory cytokines production, PMN adhesion and infiltration, oxygen-derived free radicals generation, microcirculation impairment and intracellular Ca^{2+} increase, etc. (24–27). Considering the above reports and our data, the protective mechanism of β-SQAG9 liposome might not depend on the reduction in cytokine production but rather on the suppression of PMN infiltration into the liver parenchyma. Therefore, in this study, we focused on infiltrated PMN as a key mediator of I/R injury. In hepatic I/R, PMNs were recruited into the inflammatory site; they then infiltrated into the liver parenchyma and caused hepatic I/R injury. The selectin family of adhesion molecules is responsible for the initial attachment between PMNs and endothelium (7). In particular, L-selectin, which is constitutively expressed on neutrophils, mediates the intermittent adhesion process that precedes the extravascular infiltration of PMNs (28, 29). Some reports have stated that blocking L-selectin with mAb could decrease PMN migration and reduce hepatic I/R injury (7, 30). In this study, we demonstrated that β-SQAG9 liposome specifically bound to L-selectin on PMN using flow cytometric analysis and immunofluorescence by confocal laser microscopy. Furthermore, we demonstrated that β-SQAG9 liposome reduced the PMN infiltration into the liver parenchyma and ameliorated subsequent I/R injury. Taken together, these data led to the conclusion that β-SQAG9 liposome reduced hepatic I/R injury by the inhibition of the initial attachment between PMNs and sinusoidal endothelium, which L-selectin mediated.

In conclusion, β-SQAG9 liposome might competitively antagonize L-selectin on PMNs and suppress the subsequent PMN infiltration, resulting in the reduction in I/R injury. Thus, it is expected that this unique anti-inflammatory property of β-SQAG9 liposome could become one of the tactics of therapy against acute inflammation, such as I/R injury. β-SQAG9 liposome, which possesses both the immunosuppressive effect (8–10) and this anti-inflammatory property, might especially be helpful in liver transplantation or liver surgery. Moreover, it is reasonable to assume that the sea urchin intestine could be used as a medical source material, although it is recognized that, at present, it is regarded as an inedible waste material.

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Down-Regulation of HLA Class I Antigen is an Independent Prognostic Factor for Clear Cell Renal Cell Carcinoma

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Purpose: We determined the prognostic impact of human leukocyte antigen class I on the survival of patients with clear cell renal cell carcinoma.

Materials and Methods: Immunohistochemical staining for HLA class I was performed on specimens from 45 patients with clear cell renal cell carcinoma. We performed univariate and multivariate analyses of various factors affecting cause specific survival including HLA class I, Fuhrman grade, TNM stage and tumor size. Furthermore, we compared the survival of patients with HLA class I positive renal cell carcinoma to that of those with down-regulated HLA class I using the Kaplan-Meier method and log rank test.

Results: HLA class I was immunohistochemically down-regulated in 17 (37.8%) clear cell renal cell carcinomas. The down-regulation had no correlation with other clinicopathological parameters such as tumor size, perirenal fat invasion, tumor thrombus, TNM stage or nuclear grade. Univariate and multivariate analyses revealed that HLA class I expression, tumor grade and TNM stage were significant factors influencing the disease specific survival of patients with renal cell carcinoma. Patients with HLA class I positive renal cell carcinoma had longer recurrence-free survival than those with down-regulated expression at 5-year followup (95.5% and 61.1%, respectively).

Conclusions: Our data demonstrate that down-regulation of HLA class I on tumor cells is an independent prognostic factor for clear cell renal cell carcinoma. This finding suggests that HLA class I restricted cytotoxic T lymphocytes have an important role in the suppression of renal cell carcinoma.

Key Words: carcinoma, renal cell; histocompatibility antigens class I; prognosis; survival

Metastatic progression will develop after curative surgery in more than 30% of patients with RCC. Therefore, prognostic classification of RCC has been used in an effort to facilitate appropriate counseling of patients and to guide decisions pertaining to surveillance and adjunctive therapy.[1–3] Many prognostic factors have been identified in RCC, and the TNM stage, nuclear grade, sarcomatoid differentiation and tumor size are generally accepted for patients treated surgically.[4,5] However, because of its inherent resistance to chemotherapy and radiotherapy, no satisfactory treatment options exist for patients with advanced RCC at present,[6] and the response rate to immunotherapy using interferon-α and/or interleukin-2 is also unsatisfactory (less than 20%).[7] Therefore, it is important to determine immunological prognostic factors for the survival of patients with RCC.

Human leukocyte antigen class I has a critical role in recognition and lysis of tumor cells by CTLs, and defects in antigen presentation could allow the tumor to escape from CTLs.[8] Although the abnormalities of HLA class I and antigen processing molecules in RCC have been efficiently investigated,[9] there has been no study evaluating the relationship between down-regulation of HLA class I in RCC tissues and prognosis. In this study we assessed the influence of down-regulation of HLA class I on the survival of patients with RCC.

MATERIALS AND METHODS

Patients

We reviewed the clinical pathology archives of 138 consecutive patients who underwent radical or partial nephrectomy and were diagnosed as having clear cell RCC at the Sapporo Medical University Hospital, Sapporo, Japan from May 1991 to August 1998. Patients whose medical records were incomplete were excluded. We selected 45 of the patients based on the availability of sufficient material for immunohistochemistry. Informed consent was obtained from the patients to use the surgical specimens remaining after pathological diagnosis for the investigational study, which was approved by the Institutional Review Board for Clinical Research at our university. All hematoxylin and eosin stained slides were

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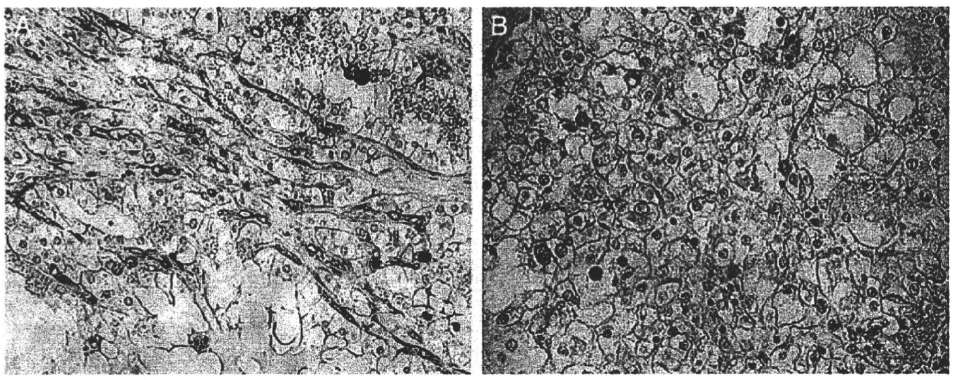

FIG. 1. Representative pictures of immunohistochemical staining with mAb reacting to HLA class I (EMR8-5) in RCC. *A*, in tumor cells staining is seen in cytoplasm but not in cell membrane, which demonstrates down-regulation of HLA class I (score 1). *B*, cell membranes of tumor cells are completely stained (score 2).

reviewed, and all of these specimens showed clear cell RCC. Median patient age at operation of the 27 male patients and 18 female patients was 61 years (range 24 to 80). Median followup was 63 months (range 3 to 117). All hematoxylin and eosin stained slides were reviewed, and clinical stage was assigned using the 2002 TNM classification of malignant tumors. There were 22 cases of stage I, 9 cases of stage II, 7 cases of stage III and 7 cases of stage IV disease. Fuhrman grade distribution was 15 cases with G1-2, 22 cases with G3 and 8 cases with G4. Median tumor diameter was 5.5 cm (range 1.2 to 18). No patients with stage I, II or III RCC underwent immunotherapy before recurrence.

Immunohistochemistry and Scoring

Immunohistochemical staining with the monoclonal anti-pan HLA class I antibody EMR8-5, established at our laboratory, was performed as previously described.[10] Human tonsil sections were used as positive controls for HLA class I. Negative controls had the primary antibody replaced by buffer. All specimens were reviewed independently using light microscopy in at least 5 areas at ×400 magnification by investigators who were blinded to clinicopathological data (IH and TT). The membrane immunoreactivity level for HLA class I was categorized from undetectable to +2. A score of zero was defined as undetectable staining. A score of +1 was defined as faint, incomplete membrane staining in more than 20% of the tumor cells, or as moderate to complete staining in cytoplasm but negative membrane staining in the tumor cells (fig. 1, *A*). Finally, a score of +2 was defined as complete membrane staining in more than 80% of the tumor cells (fig. 1, *B*). HLA class I expression was then classified as down-regulated (scores 0 and 1) or positive (score 2).

Statistical Analysis

We tested the relationships between HLA class I expression and the other clinicopathological parameters, ie the TNM stage, tumor diameter, tumor thrombus, perinephric fat invasion and Fuhrman grade, by logistic regression tests. Disease specific survival was assessed by the Kaplan-Meier method, and differences between 2 groups were compared using the log rank test. Univariate and multivariate regression analyses according to the Cox proportional hazards regression model, with disease specific survival as the dependent variable, were used to evaluate the down-regulation of HLA class I for potential independent prognostic factors. A value of $p < 0.05$ was considered to indicate statistical significance. The calculations were performed using JMP™ software.

RESULTS

Immunohistochemical study of HLA class I in cancer cells revealed that 1, 16 and 28 of the 45 cases had scores 0, 1 and 2, respectively. In other words, HLA class I was down-regulated in 17 (37.8%) of the clear cell RCCs. All normal cells of proximal convoluted tubules, the origin of clear cell RCC, and tumor infiltrating lymphocytes showed positive staining by EMR8-5. Logistic regression analysis revealed no relationship between HLA class I expression and tumor size ($p = 0.6286$), Fuhrman grade ($p = 0.6806$), perinephric fat invasion ($p = 0.8696$), tumor thrombus ($p = 0.7633$) or TNM stage ($p = 0.6869$).

The 5-year disease specific survival was 81.6% for all patients. Univariate analysis revealed that HLA class I expression, TNM stage and tumor grade were significant factors influencing disease specific survival of patients with RCC (see table). Multivariate analysis revealed that HLA class I expression was the only significant and independent factor that affected the disease specific survival (see table). The 5-year survivals were 95.5% and 61.1% in the HLA class I positive and down-regulated arms, respectively. Patients with HLA class I positive RCC had significantly longer disease specific survival than those with down-regulated expression (log rank $p = 0.0145$) (fig. 2).

Results of Cox regression analysis for disease specific survival

	Univariate Analysis		Multivariate Analysis	
	Risk Ratio Label (95% CI)	p Value	Risk Ratio Label (95% CI)	p Value
TNM stage	1.84 (1.25–2.90)	0.0014	1.74 (0.99–3.18)	0.0548
Fuhrman grade	2.32 (1.22–4.73)	0.0094	1.99 (0.62–6.98)	0.2501
Tumor size	1.08 (0.99–1.18)	0.0887	1.05 (0.86–1.25)	0.5946
HLA class I	0.13 (0.04–0.33)	<0.0001	0.21 (0.04–0.86)	0.0294

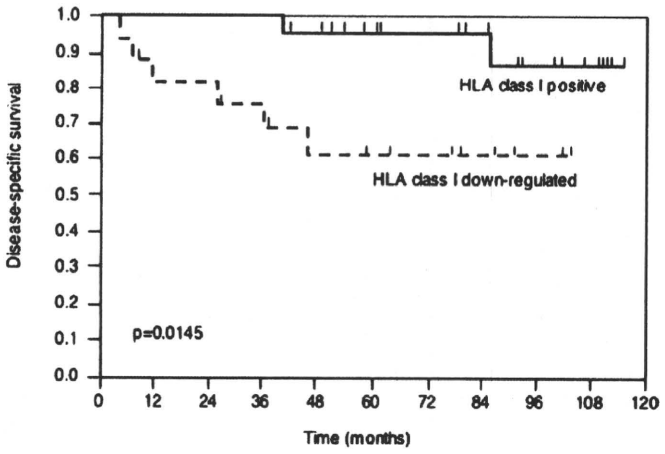

FIG. 2. Disease specific survival of patients with RCC stratified by whether HLA class I was down-regulated.

DISCUSSION

We used a novel antibody, EMR8-5, reactive with the heavy chains of all alleles of HLA-A, B and C in formalin fixed, paraffin embedded tissue sections. The use of this pan-HLA antibody revealed the incidence of HLA class I down-regulation in our series of RCC to be 38%. Marincola et al reviewed several small studies and reported that the down-regulation rate was 32%, a figure compatible with our results.[11] Meanwhile, Brasanac et al investigated HLA class I expression in 26 RCCs immunohistochemically, and reported that 15% of them showed a reduced presence.[12] They also showed that HLA class I down-regulation was associated with greater tumor diameter, and more frequent T3, T4 and M1 stages.[12] Atkins et al reported that 22% of clear cell RCC showed HLA class I down-regulation. [9] Thus, the frequency of HLA class I down-regulation in RCC is relatively low compared to other cancers.[11] In the present study the down-regulation was not associated with other clinicopathological parameters such as nuclear grade, TNM stage, tumor size, etc, suggesting that the antitumor effects of immunotherapy might not depend on grade, stage or tumor size in RCC.

To our knowledge this study is the first to report the importance of HLA class I down-regulation as a prognostic factor for patients with RCC. The results indicated that the natural history or biological activity of RCC had a close relation to immunological circumstances. The down-regulation of HLA class I molecules provides tumor cells with mechanisms to escape from T cell recognition and destruction.[13] Our results suggested that HLA class I down-regulated RCC cells might acquire such immunological escape. Unfortunately, however, we did not evaluate tumor specific CTLs in peripheral blood of the patients or in/around the tumor. Recently a new technique has been developed to detect pathogen specific T cells without knowledge of peptide specificity or HLA restriction elements.[14] Thus, a study of the relationships among HLA class I down-regulation, tumor specific CTLs and prognosis may be possible in the future. Abnormalities of HLA class I antigen can be caused by those of the HLA class I antigen processing machinery, TAP1, TAP2, tapasin, LMP2, LMP7 and LMP10/MECL-1, and the β2-microglobulin gene.[11,15] In RCC down-regulation or deficiencies of TAP1, tapasin, LMP2 and LMP7 were reported,[9,16] although there is a question why those abnormalities were also found in tissues normally expressing HLA class I. In any case further studies that clarify the molecular mechanisms of HLA class I down-regulation are needed. We suggest that the prognosis of patients with HLA class I down-regulated RCC may be improved if the key to the abnormality is identified.

Our results suggest that HLA class I expression in RCC tissues can provide useful prognostic information for patients, although this study has limitations in that it studied a small number of patients and lacked examples without indication for surgical treatment for RCC. In the next stage we will evaluate the relationship between HLA class I down-regulation in cancer tissues and the effects of cytokine therapies for advanced RCC.

CONCLUSIONS

Our data demonstrate that down-regulation of HLA class I expression on tumor cells is an independent prognostic factor in univariate and multivariate analyses of the survival of patients with clear cell RCC. This suggests that HLA class I restricted CTLs have an important role in the immune surveillance of patients with RCC.

ACKNOWLEDGMENTS

Emiri Nakazawa and Kumiko Shimozawa provided antibody EMR8-5. Yoshihiko Hirohashi and Eiji Sato provided technical assistance.

Abbreviations and Acronyms

CTLs	=	cytotoxic T lymphocytes
HLA	=	human leukocyte antigen
LMP	=	low molecular mass polypeptide
RCC	=	renal cell carcinoma
TAP	=	transporter associated with antigen processing

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EDITORIAL COMMENT

The management of renal cell carcinoma is changing with the introduction of anti-vascular endothelial growth factor therapy.[1–3] These improved treatments are the result of decades of research, demonstrating the importance of vascular endothelial growth factor in renal tumor development. With all this excitement it is important to recognize that the immune system has a prominent role in tumor formation, progression and treatment. Kitamura et al demonstrate that HLA class I antigen down-regulation is an independent prognostic factor for renal cell carcinoma. Does this reflect a more dedifferentiated tumor or an inability of the host immune system to recognize the tumor? The fact that the marker did not correlate with stage, grade or size would lend credence to the latter. Markers of tumor aggressiveness are only half the battle. Once we identify patients at high risk for progression we need to provide them with improved treatment options. What remains to be proven is if HLA status will identify patients who will respond better or worse to systemic therapies.

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REPLY BY AUTHORS

We agree that it is necessary to clarify the relationships between HLA class I status and clinical response to systemic therapies. The relationships between HLA class I status and, not only conventional cytokine therapies, but also new specific immunotherapies, molecular targeting therapies, etc need to be evaluated. Various vaccination studies report that the clinical responses or durable effects do not always correlate with the induction of CTLs specific for the cancer antigen.[1,2] We believe it is important to evaluate the relationships among clinical responses, cancer specific CTL induction and HLA class I status in such therapies.

Most candidates for systemic therapies have metastatic tumors. Unfortunately, however, our study included only primary RCCs. We need to know whether HLA I expression can be down-regulated in advanced RCC, even if future studies clarify that HLA I status is a predictive factor for the clinical response to immunotherapy. Investigation of the mechanisms of HLA class I down-regulation in RCC is important. Furthermore, if HLA I status is a key to predict clinical responses to systemic therapies, a study of how to up-regulate HLA class I will be one of our challenges.[3]

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Plasma Neuropeptides in Patients Undergoing Lumbar Discectomy

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Study Design. A prospective analysis of patients with lumbar disc herniation (LDH).

Objectives. To determine the role of neuropeptides as a biochemical signature of pain states in patients with LDH.

Summary of Background Data. Increases in the plasma level of neuropeptides have been reported in patients suffering from a variety of painful conditions. However, there is no such report on patients with LDH.

Methods. From a total of 27 patients with a single-level LDH, blood samples were collected before and 3 weeks after lumbar discectomy. Plasma levels of alpha calcitonin gene-related peptide (CGRP), galanin, neuropeptide Y, and substance P were determined by using enzyme-linked immunosorbent assay. The association or correlation between preoperative concentration of plasma neuropeptides and gender, patients' age, and VAS were analyzed statistically. Also, the concentration of plasma neuropeptides was compared before and after lumbar discectomy.

Results. Preoperative plasma levels of CGRP were correlated significantly with the extent of sciatica as determined by VAS. In addition, plasma levels of CGRP and galanin significantly decreased after lumbar discectomy in line with the disappearance of pain symptoms.

Conclusions. These findings indicate the role of plasma CGRP and possibly galanin as a systemic neurochemical signature of pain states in patients with LDH.

Key words: lumbar disc herniation, neuropeptide, discectomy, enzyme-linked immunosorbent assay (ELISA), calcitonin gene-related peptide (CGRP), galanin, pain assessment. **Spine 2007;32:E79–E84**

Lumbar disc herniation (LDH) is one of the most common spinal disorders. This condition has been well known to cause neurogenic radicular pain, nociceptive low back pain, and occasionally neuropathic pain symptoms such as allodynia. These diverse pain symptoms are a reflection of the unique pathology of LDH in both mechanical and anatomic aspects: 1) while peripheral nerve injuries are often caused by traumatic events outside of the body, LDH signifies internal trauma to the surrounding peripheral nerve terminals and nerve roots; 2) herniated nucleus pulposus contains inflammatory properties; and 3) in the majority of cases, the nerve root is damaged proximal to the dorsal root ganglion (DRG).[1,2] These clinical and pathologic characteristics of LDH distinguish it from other nociceptive and neuropathic pain conditions. Also, these features create difficulties in the development of animal models of LDH, which mimic human's nociceptive and neurogenic pain. Consequently, there is a much poorer understanding of molecular pathomechanisms of pain in LDH, when compared with peripheral neuropathic pain.[3,4]

Neuropeptides are multifunctional amino acids essential in all life forms from invertebrates to humans.[5] They act as transmitters and modulators for hormone secretion and pain signals.[3,5,6] The role of neuropeptides in pain signaling has been investigated mainly in peripheral neuropathic pain conditions[3,7] and headaches.[8] A few animal model studies[9–12] have investigated their role in the pathophysiology of pain associated with disc herniation. Besides their regulatory function in pain signaling, neuropeptides can also serve as the local and systemic neurochemical signature of pain states.[7] Increases in the plasma level of neuropeptides have been reported in patients with a variety of painful conditions, including burns,[13] fractures,[14] whiplash,[15] and headaches.[8] Nevertheless, such available information on LDH has been limited to the analysis of cerebrospinal fluid (CSF), the results of which showed no significant increases of neuropeptide levels in patients with this condition.[16,17]

Clinical evaluation of pain associated with LDH still depends on subjective measures such as the visual analogue scale (VAS). As a quantitative objective sensory test for patients with lumbar radiculopathy, we previously introduced current perception threshold evaluation.[18] The present study was designed to extend our quantitative approach to pain assessment in patients with LDH, and also to understand the role of plasma neuropeptides in the pathophysiology of LDH. Focused on these aims, we analyzed levels of alpha calcitonin gene-related peptide (CGRP), galanin, neuropeptide Y (NPY), and substance P (SP) in blood serum and CSF of patients who were elected for lumbar discectomy.

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■ Materials and Methods

Subjects. This study was approved under the institutional guidelines for the use of human subjects in research. Conditions for the evaluation of patients were those who had 1) sciatica with a VAS of 5 or more, 2) positive straight leg raising (SLR) test, 3) LDH at a single corresponding level, and 4) those who were admitted to the authors' hospitals to undergo lumbar discectomy. VAS was measured by using a horizontal line, 100 mm in length, with 11 vertical lines at 10-mm intervals. Patients were asked to mark a point that represented the level of their sciatica focusing on pain rather than discomfort. Exclusion criteria were patients with other concomitant painful conditions, those receiving diuretic drugs, and those who had residual pain 3 weeks after lumbar discectomy. Eligible patients gave their written consent to participate in the study, and blood samples were collected both before surgery and 3 weeks after lumbar discectomy (modified Love method).

Between April 2003 and March 2005, 29 patients enrolled on this program. Two patients were later excluded because of persistent pain 3 weeks after lumbar discectomy. From these 2 patients, postoperative blood samples were not collected. Accordingly, the remaining 27 patients represented the subject of the following analysis. There were 14 men and 13 women. The mean age was 44 years (range, 16–72 years). The level of disc herniation was L3–L4 in 3, L4–L5 in 10, and L5–S1 in 14 patients. The 3 patients with disc herniation at L3–L4 showed a positive SLR test. The VAS score for the entire group was 77 ± 17 mm (mean ± standard deviation). Their SLR results were 43° ± 19° (mean ± standard deviation).

Sample Collection and Processing. Blood samples were drawn from the peripheral veins in the forearm using a 22G needle (TERUMO NEEDLE, Terumo, Tokyo, Japan) between 6:00 AM and 7:00 AM. They were collected in tubes containing 3.8% sodium citrate (VENOJECT II, Terumo). The tubes were transferred to ice and centrifuged at 3000 rpm for 10 minutes. The plasma samples were snapped and stored frozen at −80 C until analysis. Samples were analyzed during the following 3 months.

Enzyme-Linked Immunosorbent Assay (ELISA). The concentration of CGRP, galanin, NPY, and SP in the corrected plasma samples was measured using commercially available ELISA kits. The kits used were, EIA 6009 (Cayman Chemical Co.) for CGRP, S-1210 (Peninsula Laboratories, St. Helens, UK) for galanin, S-1145 (Peninsula Laboratories) for NPY, and EIA 583751 (Cayman Chemical Company) for SP. Assays were performed according to the manufacturer's protocols. The peptides were incubated with biotinylated-labeled peptides. After washing, streptavidin-conjugated horseradish peroxidase (SA-HRP) was added. After again washing, tetramethyl benzidine dihydrochloride was allowed to react with bound HRP. Absorbance was read at 450 nm and all assays were performed in duplicates. The count bound figure was divided by the total counts and expressed as a percentage. In order to derive numerical values (pg/mL), these percentage numbers were then positioned onto the standard curve. The calculations were carried out by using Excel wordbook "EIADouble" downloaded from the web site of Cayman Chemical Company.

Statistical Analysis. Preoperative concentration of plasma neuropeptides between male and female patients was compared and analyzed using the unpaired Student *t* test. The correlation between preoperative concentration of plasma neuropeptides and 1) patients' age and 2) preoperative VAS was evaluated using the Pearson correlation method. The concentration of plasma neuropeptides in blood samples was compared before and again 3 weeks after surgery and analyzed statistically using paired Student *t* test and Wilcoxon signed-ranks test. In addition, factors that could potentially influence the postoperative changes of plasma neuropeptide levels were analyzed, including 1) gender, 2) use of nonsteroidal anti-inflammatory drugs (NSAIDs) at the time postoperative blood samples were drawn, and 3) duration of preoperative symptoms. Patients were divided into 2 groups: one showing postoperative decreases of plasma neuropeptide levels and the other without this decrease. Then data of gender and use of NSAIDs were analyzed in a 2 × 2 cross contingency table using the Fisher exact probability test. The correlation between postoperative changes of plasma neuropeptides and duration of preoperative symptoms was evaluated using Pearson correlation method. A probability of less than 0.05 is accepted as statistically significant.

■ Results

To determine the clinical significance of plasma neuropeptides in patients with LDH, we collected blood samples from the 27 patients with LDH before and after lumbar discectomy. We assessed the concentration of CGRP, galanin, NPY, and SP in these preoperative plasma samples and then analyzed the results with the demographic data of patients. As depicted in Table 1, the levels of neuropeptides did not differ significantly between male and female patients. Also, there were no significant correlations between the levels of plasma neuropeptides and the age of the patients with LDH (Figure 1).

We then analyzed the correlation between the levels of plasma neuropeptides and preoperative VAS (Figure 2). As shown, there was a significant correlation between the levels of plasma CGRP and VAS ($r = 0.39$, $P = 0.01$). In contrast, galanin, NPY, and SP failed to show such significant correlation with preoperative VAS. Subsequently, we compared the levels of plasma neuropeptides before and 3 weeks after lumbar discectomy (Table 2; Figure 3). With a VAS score of 0, all 27 patients reported a disappearance of pain after surgery, although 10 patients were taking NSAIDs at that time. As shown in Figure 3, following surgery the levels of CGRP and galanin decreased in 19 patients, respectively. Analysis of the 27 patients by paired Student *t* test revealed statistical

Table 1. Levels of Plasma Neuropeptides and Gender

Peptides	Male (pg/mL)	Female (pg/mL)	P†
CGRP	46.0 ± 44.0*	35.5 ± 31.6	0.25
Galanin	571 ± 347	685 ± 278	0.18
NPY	244 ± 99	277 ± 117	0.22
SP	10.9 ± 6.3	9.7 ± 6.2	0.31

*Values of neuropeptides are given as the mean ± SD.
†*P* values are determined by unpaired Student *t* test.

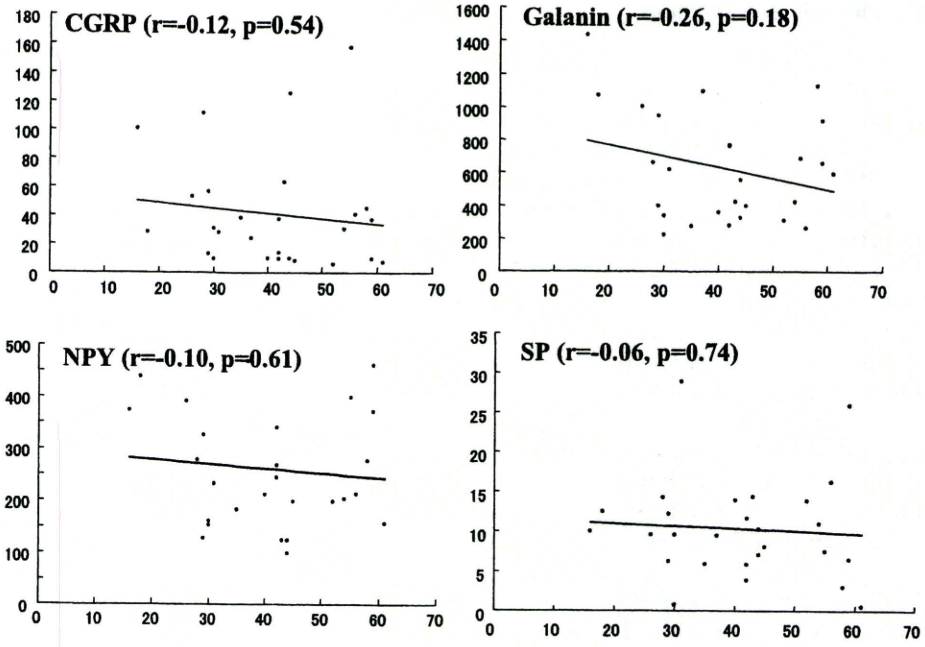

Figure 1. Correlation between patients' age and level of plasma neuropeptides. x-axis indicates the age of patients (years). y-axis indicates the concentration of neuropeptides (pg/mL). The concentration of CGRP, galanin, NPY, or SP in preoperative plasma samples was determined by ELISA and analyzed the correlation with patients' age by using Pearson correlation method. Values in parentheses in each neuropeptide indicate a correlation coefficient (r) and a probability (*P*). There were no significant correlations between the levels of plasma neuropeptides and the age of the patients with LDH.

significances in changes of CGRP and galanin levels (Table 2). In Wilcoxon signed-ranks test, the extent of decreases in CGRP and galanin levels was significantly larger than that of the increases (Table 2). With respect to NPY and SP, there were no such significant changes before and after surgery.

We subsequently analyzed factors that could potentially influence postoperative changes of CGRP and galanin. As depicted in Table 3, there was no significant association between gender and postoperative changes of CGRP ($P = 0.62$) or galanin ($P = 0.62$). Also, no significant relation was seen between the use of NSAIDs at the time postoperative blood samples were drawn and postoperative changes of CGRP ($P = 0.91$) and galanin ($P = 0.10$). In contrast, there was a significant correlation between duration of preoperative symptoms and the changes in the level of galanin (Figure 4). Patients with shorter duration of preoperative symptoms tended to show larger postoperative decreases of galanin. Such correlation was not seen in CGRP.

■ Discussion

In the present study, the authors examined plasma levels of CGRP, galanin, NPY, and SP in a group of 27 patients with LDH who were elected for lumbar discectomy and reported disappearance of pain after surgery. They discovered that there was no significant association or correlation between preoperative levels of these neuropeptides and gender or age. In contrast, preoperative plasma levels of CGRP were significantly correlated with the

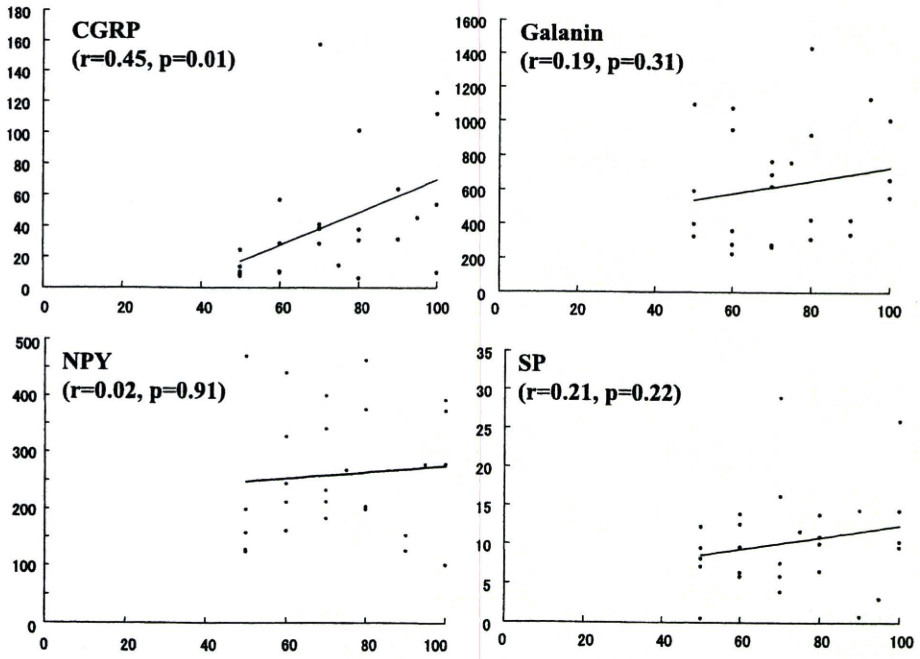

Figure 2. Correlation between preoperative VAS and level of plasma neuropeptides. x-axis indicates VAS score (mm). y-axis indicates the concentration of neuropeptides (pg/mL). The concentration of CGRP, galanin, NPY, or SP in preoperative plasma samples was determined by ELISA and analyzed the correlation with preoperative VAS score by using Pearson correlation method. Values in parentheses in each neuropeptide indicate a correlation coefficient (r) and a probability (*P*). There was a significant correlation between the levels of plasma CGRP and VAS score, whereas galanin, NPY, and SP failed to show such significant correlation.

Table 2. Levels of Plasma Neuropeptides Before and After Lumbar Discectomy

Peptides	Before Surgery (pg/mL)	After Surgery (pg/mL)	*P* Paired Student *t* Test	*P* Wilcoxon Test
CGRP	40.9 ± 39.6*	25.5 ± 29.5	0.001	0.006
Galanin	625 ± 326	515 ± 288	0.003	0.004
NPY	259 ± 111	264 ± 103	0.31	0.18
SP	10.3 ± 6.4	10.1 ± 4.6	0.39	0.32

*Values of neuropeptides are given as the mean ± SD.

Table 3. Influence of Gender and Use of NSAIDs on Postoperative Changes of CGRP and Galanin

Peptides	Gender		NSAID Use	
	Male (n = 14)	Female (n = 13)	Yes (n = 10)	No (n = 17)
CGRP				
Decrease (n = 19)	11	8	6	13
Increase (n = 8)	3	5	4	4
Galanin				
Decrease (n = 19)	10	9	9	10
Increase (n = 8)	4	4	1	7

No. of patients in each category was described in a 2 × 2 cross contingency table.

extent of sciatica as determined by VAS. In addition, plasma levels of CGRP and galanin significantly decreased after lumbar discectomy in line with the disappearance of pain symptoms. These findings indicate that CGRP and possibly galanin play a role in the pathogenesis of LDH, and also they can serve as systemic neurochemical signatures of pain states in patients with lumbar radiculopathy.

In the literature, plasma levels of CGRP and SP have been reported to be significantly higher in patients with tissue injuries than those in healthy controls. These include injuries of soft tissue,[19] skin (burns),[13] bone (fractures),[14] and ligament/joint capsule (whiplash).[15] Compared with these reports, changes in plasma levels of CGRP and galanin associated with lumbar discectomy in the present study were smaller. However, they showed statistical significance in paired Student *t* test and Wilcoxon signed-ranks test. These differences likely reflect the extent and area of the injury, which are smaller in LDH than in the injuries caused by external trauma. LDH serves as internal trauma to both nerve roots and nerve endings (nociceptors) in the posterior longitudinal ligament and the posterior parts of the anulus fibrosus.[20,21] Despite the relatively minor injuries caused by LDH, associated local inflammation may sufficiently sensitize the corresponding afferent neurons in the nerve root and the DRG. This in turn leads to radicular pain expressed by patients with a high VAS score.

It should also be mentioned that 8 of the 27 patients did not show decreases in plasma levels of CGRP and galanin after lumbar discectomy. In this regard, there was no significant association between postoperative changes of CGRP and galanin, and 1) gender or 2) the use of NSAIDs. In contrast, there was a significant correlation between duration of preoperative symptom and postoperative changes of galanin. Lack of decreases in plasma levels of galanin after lumbar surgery may be explained by the relatively long duration from onset to the surgery. However, it still remains to be established which factors influenced the plasma levels of CGRP in patients who underwent lumbar discectomy. In the present study, the authors excluded 2 patients who underwent discectomy and had persistent pain 3 weeks after surgery. They might also have not shown a postoperative decrease in the plasma levels of CGRP and galanin. Analyses of blood samples from these 2 patients and also

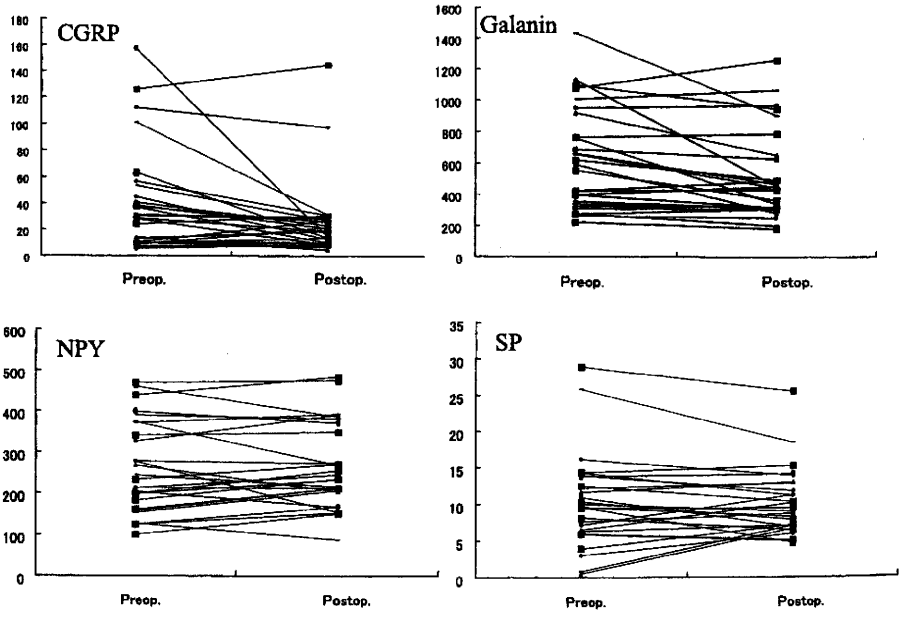

Figure 3. Plasma level of neuropeptides before and after lumbar discectomy. Plasma levels of CGRP, galanin, NPY, and SP in 27 patients were determined before and 3 weeks after lumbar discectomy. Lines are drawn from the preoperative value to the postoperative value in each patient. y-axis indicates the concentration of neuropeptides (pg/mL).

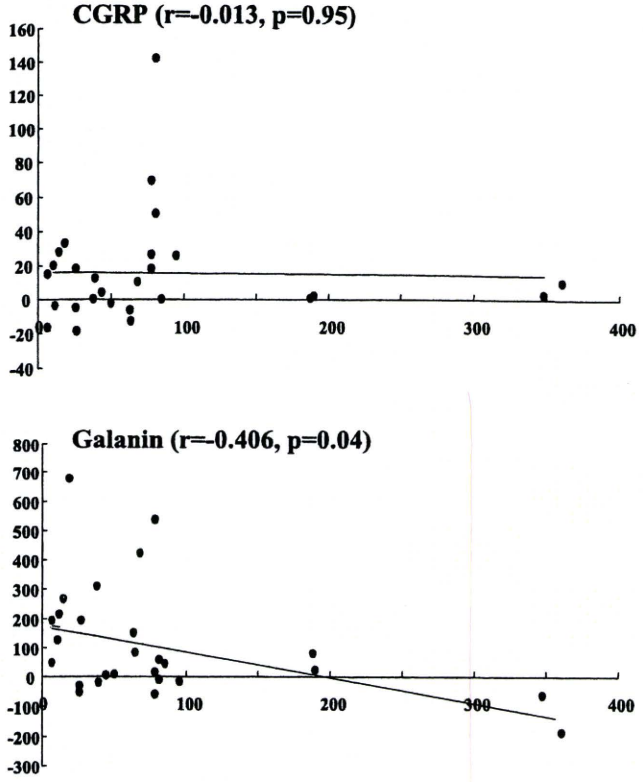

Figure 4. Correlation between duration of symptoms and postoperative changes of plasma CGRP and galanin. x-axis indicates the duration of symptoms from onset to lumbar discectomy (days). y-axis indicates the extent of postoperative changes of neuropeptides (pg/mL) calculated as preoperative concentration of the peptide − postoperative concentration. Negative values indicate that the level of plasma neuropeptide increased after surgery. The correlation between the duration of symptoms and postoperative changes of plasma neuropeptide levels was analyzed by the Pearson correlation method. Values in parentheses in each neuropeptide indicate a correlation coefficient (r) and a probability (*P*). There was a significant negative correlation between the extent of postoperative changes of plasma galanin levels and the duration of symptoms.

samples taken at an interval of 3 weeks from conservatively treated patients could provide insights into the magnitude of the changes in plasma neuropeptides.

Presently, little is known of the origin of neuropeptides in the peripheral blood. However, it is well established that CGRP and galanin are synthesized by DRG neurons, transported centrally and peripherally, and released at both terminals.[6,22,23] Centrally transported peptides may in part be released into the CSF. In this regard, Lindh *et al*[17] reported lower CSF levels of CGRP in patients with LDH than those in healthy individuals. The authors of the current study also found no significant correlation between plasma levels and CSF levels of CGRP, galanin, and NPY in 12 patients with LDH (Takeuchi *et al*, unpublished observation). These findings indicate that the plasma levels of neuropeptides in patients with LDH do not reflect centrally released neuropeptides.

Since a herniated disc sensitizes both nerve endings and nerve roots, peripherally transported neuropeptides are most likely released from both the nerve endings surrounding the herniated disc and the sensory nerve endings of the affected roots in the lower extremities. Indeed, CGRP and SP were detected immunohistochemically in the anulus fibrosus of rat intervertebral disc specimens[20] and human herniated lumbar disc specimens.[24] Notably, in the study of 12 herniated lumbar disc specimens by Ashton *et al*,[24] CGRP immunoreactivity was found not only in fine nerve fibers but also in the vicinity of blood vessels and nonperivascular locations. In contrast, SP immunoreactivity was faintly detected and confined to a few nerve fibers. These findings are consistent with the higher plasma levels of CGRP than SP in the current study.

It is generally known that neuropeptides released from the peripheral terminals exert their biochemical effects over various types of cells in its microenvironment.[6] Such biochemical events are termed as neurogenic inflammation. In LDH, they can take place at peripheral nerve terminals in the anulus fibrosus and the posterior longitudinal ligament injured by a herniated disc. While CGRP serves as a potent vasodilator and modulator of immune system activity,[6,25] galanin has trophic effects on sensory neurons.[26] It is likely that galanin released around the herniated nucleus pulposus may activate the regeneration of injured peripheral nerves in the anulus fibrosus and ligament tissues. Also, the CGRP stimulation of chemotaxis of immune cells leads to an infiltration of these cells into the herniated disc. As our group[27,28] and others[29,30] have demonstrated, such inflammatory infiltration plays a pivotal role in spontaneous regression of the herniated disc. Collectively, neurogenic inflammation associated with LDH occurs primarily to provide optimal microenvironments for healing and regeneration. However, this inflammatory response simultaneously facilitates sensitization of the afferent neurons in the affected nerve root.

There are several limitations to the present study. First, the present study includes a small number of patients. Therefore, the low participation of candidates with large changes of plasma neuropeptides after lumbar discectomy can determine the overall trend of our findings. The results might have been different if 2 patients who continued with pain after surgery were included. Second, only 4 representative neuropeptides were examined. Third, only one single time point was chosen to evaluate postoperative neuropeptide levels. And finally, it would also have been more practical to comparatively analyze plasma levels of neuropeptides and their local expressions in the herniated disc materials. Nevertheless, difficulties in the evaluation of pain states in animal models for LDH and the presence of species-dependent variations of neuropeptide expressions in injured nerves[23] highlight the importance in analysis of clinical materials in pain research.

■ Conclusion

This is the first study that shows the role of plasma CGRP and galanin as the biochemical signature of pain states in patients with LDH. Plasma levels of CGRP and