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ORIGINAL ARTICLE

Toshihiro Kakefuda
Shin Enosawa
Xiao-Kang Li
Akihiko Tamura
Naoko Funeshima
Masaru Kanashiro
Hiroshi Amemiya
Masaki Kitajima
Seiichi Suzuki

Nitrosyl hemoglobin detected by near-infrared spectroscopy in rat liver allografts

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T. Kakefuda · S. Enosawa · X.-K. Li · A. Tamura · N. Funeshima · H. Amemiya · S. Suzuki (✉)
Department of Experimental Surgery & Bioengineering, National Children's Medical Research Center, 3–35–31 Taishido, Setagaya-ku, Tokyo 154–8509, Japan
e-mail: ssuzuki@nch.go.jp,
Tel.: + 81–3-3414–8121,
Fax: + 81–3-3411–7309

M. Kanashiro
Research Institute,
National Cardiovascular Center,
5–7-1 Fujishirodai, Suita,
Osaka 565–8565, Japan

M. Kitajima
Department of Surgery,
School of Medicine, Keio University,
Tokyo 160–8582, Japan

Abstract Near-infrared spectroscopy (NIRS) is a noninvasive biomeasurement system with rays in the near-infrared region that possess high permeability to biological tissues. NIRS was applied to liver allografts undergoing rejection in rats treated with deoxyspergualin (DSG) or tacrolimus (FK506). The nitrosyl hemoglobin (Hb) levels detected in the liver grafts increased 3 days and 5 days after grafting in both allogeneic and syngeneic transplantation. The levels on day 8 remained high in the allogeneic graft, but markedly decreased in the syngeneic graft. Although the serum levels of nitrite and nitrate were extremely low 8 days after grafting in allografted recipients treated with DSG or FK506, the nitrosyl-Hb level in DSG-treated graft was much higher than that in FK506-treated graft. There was no significant difference in survival time between DSG-treated and FK506-treated recipients. In conclusion, DSG and FK506 have a different effect on NO production in allografted liver with ongoing rejection, and circulating nitrite and /nitrate levels do not reflect the local levels of NO in the graft.

Key words Nitric oxide · Near-infrared spectroscopy · Nitrosyl hemoglobin

Introduction

Near-infrared spectroscopy (NIRS) is a noninvasive biomeasurement system with rays in the near-infrared region (wave length 700–1300 nm) that possess high permeability to biological tissues [8]. NIRS was originally developed for quick examination of the sugar content in fruits without any destructive procedures. However, the absorbance of biological materials is so subtle and complicated, especially in living tissues, that the application of NIRS has not been practicable for clinical diagnosis. Recent improvements in the procedure, including the multispectra analysis system, enable us to detect biological molecules such as hemoglobin, myoglobin, and mitochondrial cytochromes [5, 6, 8–10, 26, 30, 35]. The high permeability of NIRS in the body allows noninvasive monitoring of the oxygen sufficiency in living tissues [2, 13, 14, 22–25, 31, 32].

Nitric oxide (NO), which is known to relax vascular smooth muscles and inhibit platelet aggregation, is produced abundantly by macrophages, Kupffer cells, endothelial cells, and hepatocytes during the early phase of allograft rejection and graft-versus-host disease [19]. Daily measurements of serum nitrite and nitrate, the stable end products of NO, may provide an early marker of graft rejection in organ recipients treated with tacrolimus (FK506) or cyclosporine, since the serum level of nitrite and nitrate remains low in recipients treated

with these drugs [1, 15, 17, 18]. In contrast, NO inhibited in vitro mixed lymphocyte reaction and cytotoxic T-lymphocyte activity, suggesting that NO works as a down-regulator in immune response [19]. The role of NO, either in tissue destruction or protection, is thought to be dependent upon its concentration [19]. However, there is no reliable method to directly determine the tissue NO level because of its instability. Therefore, the question of whether an increased level of NO during acute rejection is advantageous or disadvantageous for graft survival is still controversial.

The stable end products of NO are nitrosyl-Hb, metahemoglobin, and nitrite and nitrate [27, 33]. Therefore, the concentrations of these stable end products, primarily nitrite and nitrate, are estimated by most researchers instead of a direct determination of NO. In addition, the development of NIRS allows us to analyze small changes in nitrosyl-Hb. The advantages of determining nitrosyl-Hb by NIRS are as follows: (1) more subtle changes are detectable than nitrite and nitrate determination, since NO has a high affinity to hemoglobin; (2) noninvasive and continuous analysis of nitrosyl-Hb is feasible directly in living organs; (3) the obtained data reflect regional real-time changes more precisely than the concentration of nitrite and nitrate in the circulation. In addition, the level of circulating nitrite and nitrate is affected by renal function [18], and therefore the measurement of these end products in peripheral blood is inaccurate in estimating local production of NO in allografted liver.

The present study was undertaken to investigate the usefulness of NIRS for detecting nitrosyl-Hb in liver allografts and the time course of the nitrosyl-Hb level in allografts undergoing rejection when treated with deoxyspergualin (DSG) or FK506. We also studied whether the circulating nitrite and nitrate levels can reflect the local production level of NO in the allograft.

Materials and methods

Liver transplantation

Ten- to fifteen-week-old male LEW (RT1l) rats and DA ($RT1^{av1}$) rats were used as recipients and donors, respectively. Orthotopic liver transplantation was performed as described elsewhere, using cuff anastomosis without hepatic artery reconstruction [12].

All experimental protocols were conducted in accordance with the policies of the Animal Ethics Committee of the National Children's Medical Research Center.

Immunosuppression

DSG and FK506 were supplied by Nippon Kayaku (Japan) and Fujisawa Pharmaceuticals (Japan). The drug administration was initiated at the onset of graft rejection (day 3 after grafting), when the rejection was histologically confirmed by observation, with clear lymphocyte infiltration around the portal duct. Thereafter, the cell infiltration spread over the periportal area to the liver parenchyma when untreated with drugs.

DSG was dissolved in saline (1 mg/ml) just prior to administration and given intraperitoneally to recipient rats at a dose of 5 mg/kg per day until day 14 postoperatively. FK506 was suspended in saline (1 mg/kg) and injected intramuscularly to recipients at a dose of 1 mg/kg per day until day 14 after grafting.

NIRS analysis

In vivo NIRS was carried out as described in our previous paper [22–25, 32]. In brief, the analyzing system, MCPD-1000 (Otsuka Electrical, Japan), consisted of a 150-W halogen lamp, emission and detection quartz optical fibers, a multichannel photodetector, and a personal computer (PC-9801FS; NEC, Japan). The rays reflected from the grafted liver were conveyed to the photodetector through the detection fiber bundle, which was placed 3 mm from the emission bundle. The photodetector scans wavelengths were between 300 and 1100 nm with 512 photodiodes.

The spectra were taken from the donor liver under three different conditions: before removal from donor rats, immediately after perfusion of the isolated liver with cold lactate-Ringer solution, and 3–8 days after grafting. The difference between the absorption spectrum from the grafted liver (experimental graft) in the recipient and that from the same liver (control liver) immediately after perfusion was analyzed. Yogurt was used to obtain a reference spectrum. The sampling time of each scan was 0.8 s. The spectroscopy was performed under a laparotomy of the recipient. The obtained data were processed by multicomponent analysis with a least-square curve fitting and expressed as an arbitrary unit (AU), since the value from the spectrum difference is a relative concentration against the perfused liver. The factors analyzed were nitrosyl-Hb, oxy-Hb, deoxy-Hb, oxidized cytochrome $a + a_3$, reduced cytochrome $a + a_3$, bile acid, and water.

In vivo production of NO by 3-morpholinosydnonimine administration

3-Morpholinosydnonimine (SIN-1; Biomol Research, Pa., USA), an NO donor, was dissolved in deoxygenated cold Ringer solution (10 mg/ml) immediately before administration and injected via a tail vein at a dose of 2.5–10 mg/kg. To avoid an individual effect on the basal spectrum, a serial measurement of nitrosyl-Hb was performed in a normal LEW rat by a sequence of bolus administrations of SIN-1 at 15-min intervals, and a spectrum was taken every 10 s until 8 min after the SIN-1 challenge. The rat was anesthetized with pentobarbital sodium (50 mg/kg i.p.) during the measurements. The liver was perfused and the basal spectrum taken after the data was acquired.

Determination of nitrite and nitrate in recipient serum

Serum samples stored at −20 °C were analyzed for nitrite and nitrate using the Griess method [4]. The serum was removed from the protein by filtration with Centricon-3 (Grace, USA) and mixed with cadmium powder to deoxidize the nitrate to nitrite. Thereafter, the amount of nitrite was determined on the basis of the Griess reaction, as described previously.

Immunohistochemistry of inducible NO synthetase

A thin cryosection of the liver was fixed with Cytokeep II (Nippon Shoji, Osaka, Japan) and incubated at room temperature for 10 min with 3% goat serum in phosphate-buffered saline (PBS) to prevent nonspecific binding. The section was then incubated for 60 min at room temperature with anti-inducible NO synthetase (-iNOS) rabbit polyclonal antibody (1:300 dilution; Affinity Bioreagents, USA), which was previously absorbed with rat liver homogenate. After washing with PBS, the section was incubated with alkaline phosphatase-conjugated anti-rabbit IgG goat antibody $F(ab \times)_2$ (Capple, N.C., USA). Finally, the staining was completed with an alkaline phosphatase-staining kit (Funakoshi, Japan), followed by Myer's hematoxylin staining. The ratio of the positive region was calculated using image analysis (Macscope, Mitani, Japan).

Detection of iNOS mRNA by reverse transcriptase polymerase chain reaction

A small portion of the liver sample was frozen in liquid nitrogen as soon as it was excised and was stored until use. RNA was extracted with Isogen (Nippon Gene, Japan), and the reverse transcriptase polymerase chain reaction (RT-PCR) was carried out with the RNA PCR kit version 2 (TaKaRa, Japan). After a reverse transcriptase reaction, the amplification was performed with 40 cycles of PCR (94 °C for 30 s, 60 °C for 30 s, 72 °C for 90 s), using a mixture of primers for iNOS (5 × -TAG AAA CAA CAG GAA CCT ACC A, 3 × -ACA GGG GTG ATG CTC CCG GAC A) and beta-actin (5 × -CAT CGT GGG CCG CTC TAG GCA, 3 × -CCG GCC AGC CAA GTC CAG ACG C) [34]. The PCR products were detected by 1.5% agarose gel electrophoresis and visualized with ethidium bromide staining.

Miscellaneous

Serum aspartate aminotransferase (AST), total bilirubin, and creatinine levels were determined with Vision pak (Dinabot, Japan). A histological examination was performed in the formalin-fixed section stained by hematoxylin and eosin. The statistical significance ($P < 0.01$) was judged with ANOVA and Scheffe's test or Gehan's generalized Wilcoxon test.

Results

Relationship between the generation of NO and increase in nitrosyl-Hb in vivo

An injection of SIN-1 into a normal rat resulted in an increase in nitrosyl-Hb in the liver, the peak levels of which increased dose-dependently (Fig. 1). This indicated that NIRS quantitatively detects the in vivo level of NO.

Nitrosyl-, oxy-, and deoxy-Hb levels and RT-PCR profile of iNOS mRNA after grafting

The nitrosyl-Hb level in the syngeneic graft increased 3 days and 5 days after transplantation and decreased to the basal level on day 8 (Fig. 2). In accordance with this observation, the iNOS mRNA was clearly observed in the grafts both 3 days and 5 days after grafting, but not on day 8.

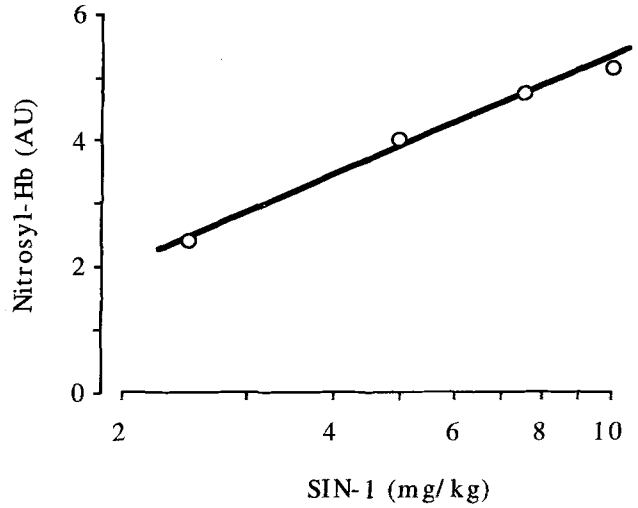

Fig. 1 Correlation between peak levels of nitrosyl hemoglobin (-Hb) and administered doses of 3-morpholinosydnonimine (*SIN-1*), an NO donor. Nitrosyl-Hb (arbitrary units, *AU*) levels were the peak values determined by continuous monitoring with near-infrared spectroscopy (NIRS) after different doses of SIN-1 administration, as described in Materials and methods

The increase in the nitrosyl-Hb level was enhanced after allogeneic transplantation and no decline was observed on day 8. Inflammatory cell infiltration was observed around the portal area in the allogeneic graft without any treatment from day 3 after grafting, and the increase in the nitrosyl-Hb level in this graft correlated well with the onset of graft rejection, determined by the histological findings. In contrast, the levels of oxy-Hb and deoxy-Hb showed no marked difference between the allogeneic and syngeneic groups.

Survival of the recipients treated for ongoing rejection with DSG or FK506

Immunosuppression was performed in the allografted recipients from the onset day of graft rejection (day 3), which was histologically confirmed by multiple focal lymphocyte infiltration around the portal area, and continued until day 14 postoperatively (Table 1). Eight or nine out of 12 recipients survived indefinitely with the treatment of DSG or FK506, whereas no rat survived longer than 15 days without any rejection therapy. In addition, there was no significant difference in recipient survival time between the DSG-treated and FK506-treated groups. Therefore, the antirejection effect of these two drugs was equivalent in terms of allograft survival.

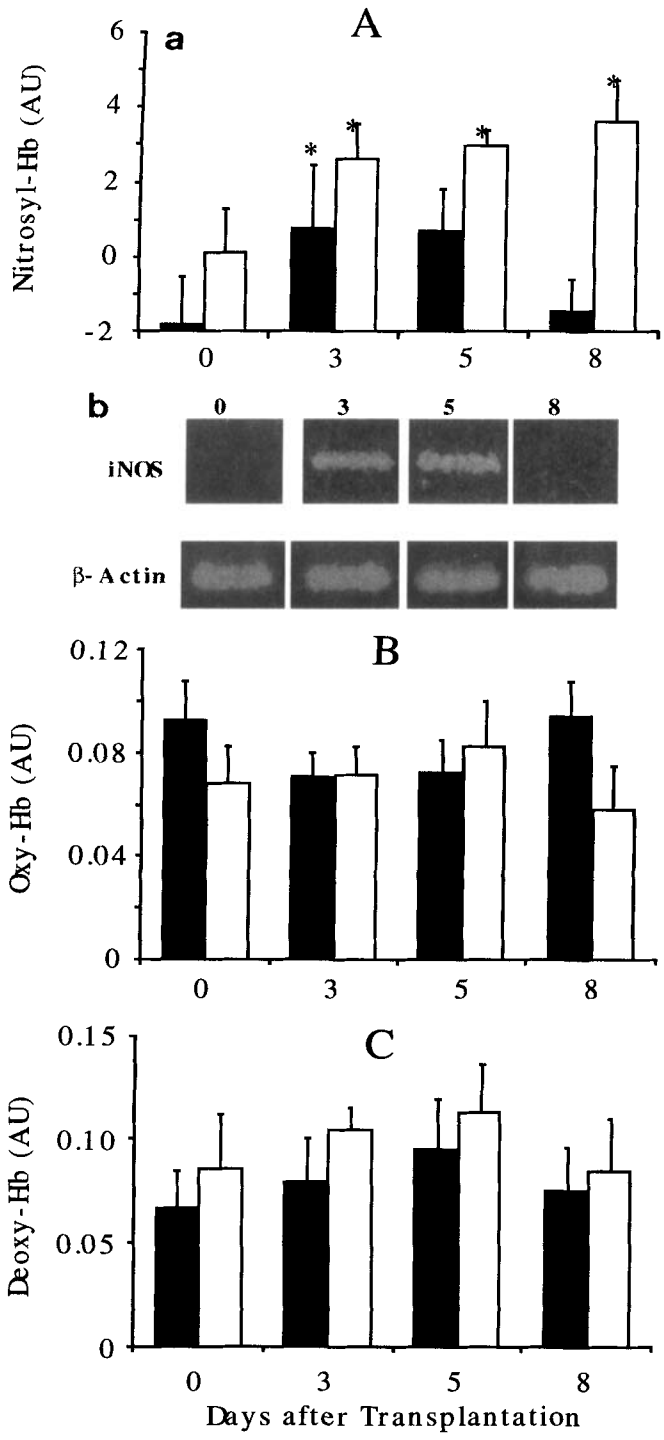

Fig. 2 Time course changes of parameters obtained by NIRS and RT-PCR of inducible NO synthetase (iNOS) mRNA after liver transplantation. *Solid columns* and *solid bars* indicate mean AU ± SD ($n = 5$) with the syngenic combination (LEW-LEW), and *open columns* and *open bars* with the allogeneic combination (DA-LEW). **A-b**, the RT-PCR of iNOS mRNA performed in the syngeneic graft on the day before grafting (day 0) and 3 days, 5 days, and 8 days after grafting. *Statistical significance ($P < 0.01$) against AU at day 0 by Scheffe's test

Table 1 Survival times of LEW rats that received allogeneic DA liver grafts treated with deoxyspergualin (*DSG*) or tacrolimus (*FK506*)

Immunosuppression[a]	Graft survival (days)	Median (day)	P value[b]
Non-treatment	10 × 2, 11 × 4, 12 × 3, 13, 14, 15	11.5	< 0.01
DSG, 5 mg/kg i.p.	39, 70 × 2, 85, > 100 × 8	> 100	< 0.01
FK506, 1 mg/kg i.m.	47, 71, 75, > 100 × 9	> 100	

[a] Recipient rats were administered DSG or FK506 from day 3 to 14 postoperatively

[b] Statistical significance was determined by Gahan's generalized Wilcoxon test

Levels of nitrosyl-Hb, nitrite and nitrate, and iNOS after allografting with rejection therapy

The nitrosyl-Hb level in the liver graft on day 8 after grafting is shown in Fig. 3A. The local level of nitrosyl-Hb in the DSG-treated graft was unexpectedly similar to that in the allograft without immunosuppression. However, the FK506-treated graft showed an extremely low level of nitrosyl-Hb. The ratio of the iNOS-positive area by the immunohistochemical examination corresponded with the result of the nitrosyl-Hb level; the FK506 treatment significantly suppressed the expression of iNOS, whereas DSG did not markedly reduce iNOS expression (Fig. 3C). Both the DSG-treated and FK506-treated groups showed extremely low nitrite and nitrate levels in the peripheral blood on day 8 after grafting, while levels in the nontreated group were significantly higher (Fig. 3B). In addition, there was no significant difference in oxy-Hb and deoxy-Hb levels between the nontreated group and DSG-treated or FK506-treated groups (Fig. 3D, E).

iNOS expression in the allograft by immunohistochemistry

A large number of Kupffer cells and endothelial cells were iNOS-positive in the nontreated graft 8 days after transplantation (Fig. 4A), while the iNOS-positive cells in the DSG-treated graft were localized in the area of focal cell infiltration (Fig. 4B). Furthermore, the graft section from the FK506-treated rat showed no iNOS-positive cells or cell infiltration (Fig. 4C).

Expression of iNOS mRNA by RT-PCR on day 8

The RT-PCR of iNOS mRNA confirmed the expression of the gene product in both the DSG-treated allografts and the nontreated ones, while a slightly decreased expression was seen in the former compared

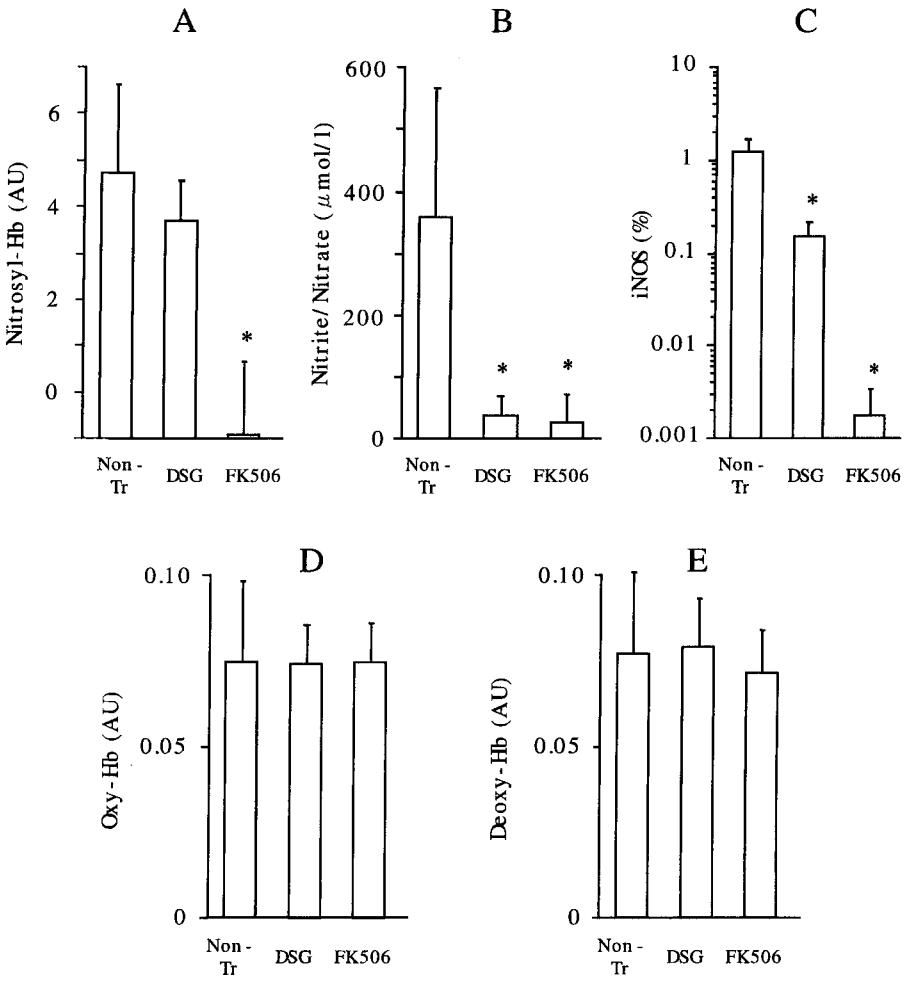

Fig. 3 NO-related parameters on day 8 after liver transplantation followed by treatment with deoxyspergualin (*DSG*) or tacrolimus (*FK506*). DSG or FK506 were administered 5 mg/kg per day (i.p.) or 1 mg/kg per day (i.m.) from day 3. *Non-Tr* denotes control (nontreated) group. Each column indicates mean ± SD ($n = 9$). *Statistical significance ($P < 0.01$) against nontreated control group by Scheffe's test

with the latter (Fig. 5). The graft treated with FK506 did not show the RT-PCR product at all, while the band of internal standard that originated from beta-actin mRNA was as clearly detectable as in the other two groups.

Liver and kidney functions in the untreated recipients with ongoing rejection

Serum AST, total bilirubin, and creatinine (S-Cr) levels in the nontreated group were markedly increased 8 days after transplantation (Fig. 6). Although administration of DSG or FK506 was started from postoperative day 3 to rescue the allograft from ongoing rejection, the treatments were fully effective in preventing the elevation of these parameters.

Discussion

There are conflicting opinions regarding whether the biological action of NO is advantageous or disadvantageous to living tissues [19]; when produced in a large amount, NO may cause tissue damage [20], while a small amount may have a protective effect on bystander tissues [21]. The Griess method, a conventional procedure to determine the blood NO level, is inaccurate in estimating endogenous NO. For instance, a vascular smooth muscle is thought to be relaxed with the extremely low level of NO produced by constitutive NO synthetase (cNOS) [20]. Therefore, a highly sensitive assay system is required to investigate the biological action of NO in more detail. The method used in this study is capable of real-time detection of subtle changes in the NO level without any invasion into living organs. The NO level is estimated as the spectrum of nitrosyl-Hb by NIRS, since NO, when produced, rapidly binds with hemoglobin to form a stable end product (nitrosyl-Hb) in physiological conditions.

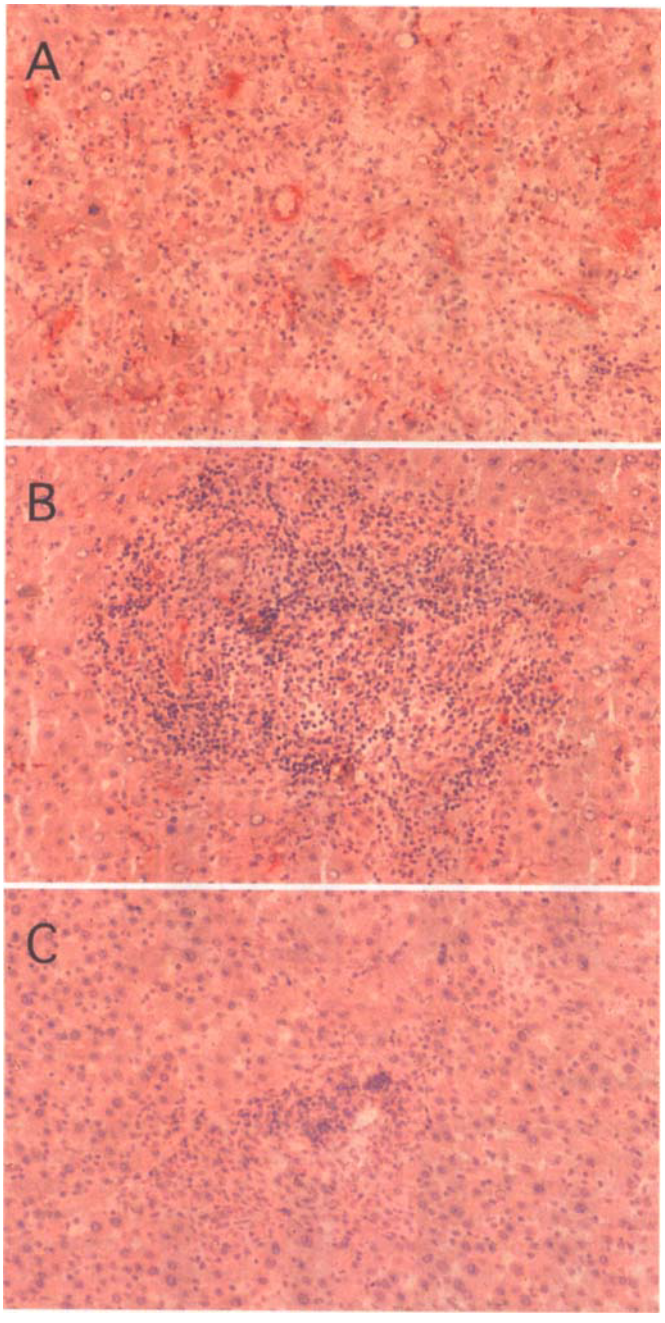

Fig. 4 Immunohistochemistry of transplanted DA liver on day 8 from nontreated (**A**), DSG-treated (**B**), and FK506-treated (**C**) recipient. The sections were stained for iNOS with alkaline phosphatase staining kit (*red*), followed by hematoxylin-eosin

The application of NIRS to biological materials originated from a report by Jöbsis in 1977 that demonstrated the oxygen saturation of hemoglobin and cytochrome $a + a_3$ in cat brains and dog hearts [8]. A number of subsequent investigations have been conducted for in vivo assessment of tissue oxygen sufficiency using brains, hearts, livers, intestines, and muscles [5, 6, 9, 10, 26, 30, 35]. However, most of these studies demonstrated critical problems in their accuracy and reproducibility based on their using only a few wavelengths of a photon. In contrast, by measuring a wide-ranging spectrum of NIR wavelengths with continuous wave spectroscopy and analyzing the obtained data with multicomponent analysis, a large variety of components, including hemoglobin, myoglobin, and cytochromes, can be evaluated in living tissues.

To define the accuracy of the NIR technique for measuring nitrosyl-Hb, we administered SIN-1, a compound of sydnonimines, to normal rats, and the level of nitrosyl-Hb was continuously detected in their liver. Sydnonimines are reported to release NO in the presence of both hydroxyl oxide and oxygen without the need for any cofactor [3, 7, 28]. Our previous work reported that the peak level of nitrosyl-Hb was detected 60 s after SIN-1 administration and thereafter reduced to a stable level in approximately 120 s [11]. The present study clearly shows that the production of nitrosyl-Hb depended on the administration doses of SIN-1. Taken together, these results demonstrate that the nitrosyl-Hb level detected by NIRS reflects the amount of NO produced in the living organ. However, the degradation rate of nitrosyl-Hb should be considered in this context; nitrosyl-Hb is mainly catabolized by respiratory exchange in the lungs [27]. It is also possible that blood circulation in the graft undergoing rejection could be disturbed due to tissue edema, increased vascular tone, or vascular thrombosis, resulting in blood congestion. These may delay the removal of nitrosyl-Hb from the graft. In this study, the levels of oxy-Hb and deoxy-Hb did not differ among the various experimental groups, suggesting that the respiratory conditions and local circulation were well maintained during the observation period.

The nitrosyl-Hb and iNOS mRNA levels in the syngeneic grafts significantly increased 3 days and 5 days after transplantation, suggesting that the surgical damage based on ischemia-reperfusion produced NO in these grafts. In our preliminary study, the nitrosyl-Hb was observed at a high level on days 3 and 5 in the allografts treated for ongoing rejection with DSG or FK506, as seen in the control allografts. The increase in nitrosyl-Hb in the allografts with or without rejection therapy during the early stage (days 3 and 5) after grafting is probably due to both ischemia-reperfusion injury and the onset of acute rejection, although its high level on day 8 in the control allografts was caused only by the immune response; its level markedly decreased in the syngeneic grafts on day 8. To avoid the surgical influence, we applied NIRS on day 8 after grafting for determining the effect of DSG and FK506 on local NO production in the allografts being rejected.

When treated with FK506, the allografts did not induce iNOS on day 8, which was detected by immunohis-

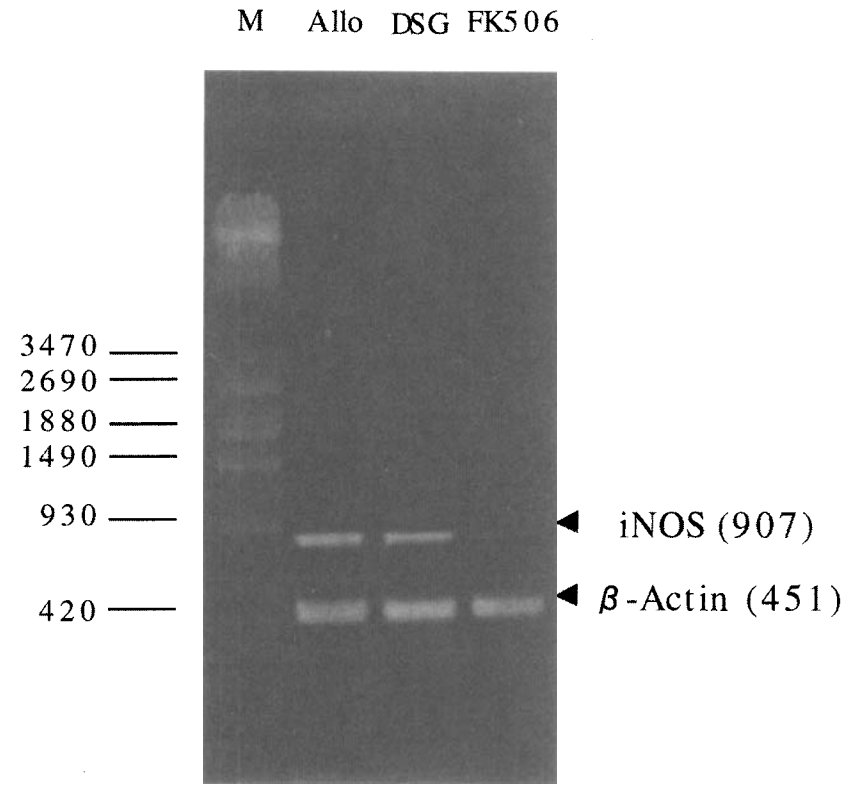

Fig. 5 RT-PCR profile of iNOS mRNA in DA liver grafts from nontreated, DSG-treated, and FK506-treated recipient. Liver samples were taken on day 8 after grafting. Both DSG-treated allografts and nontreated ones markedly expressed the RT-PCR product, although a slightly decreased expression was seen in the former compared with the latter. The allograft treated with FK506 did not show any iNOS mRNA. The *numbers in parentheses* are the size of PCR products for iNOS or beta-actin, respectively

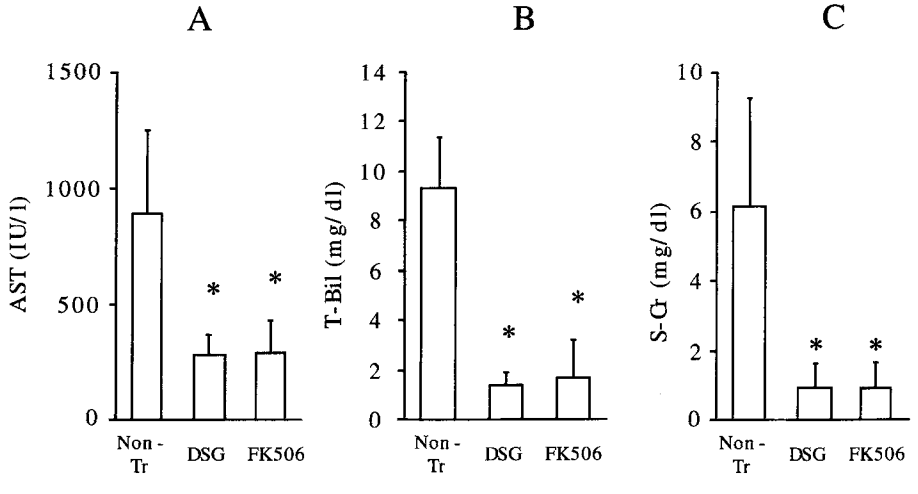

Fig. 6 Liver and kidney functions in the recipients treated for ongoing graft rejection with or without DSG or FK506. Serum samples were taken on day 8 after grafting. *Statistical significance ($P < 0.01$) against data from the nontreated control group by Scheffe's test

tochemistry and RT-PCR. This result corresponded with the levels of nitrosyl-Hb detected by NIRS and of nitrate and nitrite detected by the Griess method. Although there was no significant difference in recipient survival time between the FK506-treated group and the DSG-treated group, the DSG treatment did not reduce the nitrosyl-Hb level and slightly decreased the iNOS production in the graft, while the nitrate and nitrite level remained low in the peripheral blood. The iNOS, which is translated from iNOS mRNA transcribed based on genetic information, produces NO; it is therefore obvious that the expression of iNOS mRNA, production of iNOS protein, and NO synthesis occur at different stages of gene expression. Therefore, it is reasonable that the expression levels of these gene products are slightly different to each other. A small decrease in iNOS mRNA and its protein expression did not affect the production of nitrosyl-Hb in the DSG-treated allograft on day 8, suggesting that a slightly decreased expression of iNOS does not lead to the reduction of NO synthesis.

In addition, the measurement of circulating nitrite and nitrate is a conventional procedure to determine the blood NO level, and its blood level is affected by renal function [18]. A marked increase in peripheral nitrite and nitrate on day 8 in the control allografted recipients may be related to renal dysfunction (Fig. 6), or probably multiple organ failure, which was induced by fatal hepatic failure at the end stage of acute rejection. The recipients with well-functioning liver grafts under the rejection therapy using DSG or FK506 did not have impaired kidney function, resulting in a low blood concentration of nitrite and nitrate. Therefore, the level of nitrite and nitrate in peripheral blood did not reflect the local production of NO in the grafted liver. Taken together, it is suggested that DSG does not affect the production of NO, which may contribute to the down-regulation of the immune response, as was proposed by the in vitro study [16].

FK506 and DSG have completely different mechanisms of action: the former inhibits both the blast formation of lymphocytes during the recognition phase of the immune response and the effector cells during acute rejection, while the latter inhibits only the activated cytotoxic lymphocytes during graft rejection [29]. Therefore, when ongoing rejection was treated with DSG alone, the infiltrated mononuclear cells, including the helper T cells, did not completely disappear. The surviving lymphocytes may produce several kinds of cytokines, which initiate iNOS production from bystander cells such as hepatocytes and endothelial cells.

In conclusion, the present results demonstrate that DSG and FK506 have a different effect on NO production in allografted liver with ongoing rejection. They also indicate that circulating nitrite and nitrate levels do not reflect the local level of NO produced in the graft undergoing rejection.

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