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In vivo near-infrared monitoring of nitric oxide production and tissue oxygen sufficiency in rat liver allografts during the acute rejection reaction

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Abstract We established a new technique of in vivo near-infrared (NIR) spectroscopy that can estimate both nitric oxide (NO) production and tissue oxygen sufficiency in living organs during the alloimmune response. The present study was aimed at evaluating the potential of this technique for monitoring the rejection response utilizing the rat model of orthotopic liver transplantation without arterialization. The relative changes of nitrosyl-hemoglobin, oxy-hemoglobin and oxidized-cytochromeoxidase in the graft livers were quantified by use of this method. Nitrosyl-hemoglobin in the allogenic grafts was elevated at the onset of the rejection response and was suppressed when the rejection reaction was treated by the administration of 15-deoxyspergualin. Oxy-hemoglobin and oxidized-cytochromeoxidase were decreased in accordance with parenchymal disorder determined histologically. These results demonstrated that the new technique of in vivo NIR spectroscopy can assess simultaneously both the immune respons and graft function after liver transplantation.

Key words Near-infrared spectroscopy · Nitric oxide · Liver transplantation · Allograft rejection Monitoring

Introduction

It has been reported that a large amount of nitric oxide (NO) is synthesized during immune reactions in a sponge-matrix allograft model as well as in vascularized organ allografts [4, 8, 9]. This suggests that acute rejection can be quantitatively estimated by the level of NO in the graft. However, measurement of NO in biological specimens is difficult because of the instability of NO. We established a new technique of in vivo near-infrared (NIR) spectroscopy that can measure nitrosyl-hemoglobin (nitrosyl-Hb) formed from immunologically produced NO and erythrocyte hemoglobin. In vivo NIR spectroscopy has been used to monitor continuously changes in the steady state of oxy-hemoglobin (oxy-Hb) and deoxyhemoglobin (deoxy-Hb) and in the redox state of cytochromeoxidase (Cyt a, a_3) in living tissues [6, 13, 16]. In the present study, this technique was applied to rat liver allografts for assessing simultaneously NO production and tissue oxygen sufficiency as a means to monitor the rejection response following liver transplantation.

Materials and methods

Animals

Inbred strains of male Lewis (RT-1^l) and ACI (RT-1^a) rats weighing 200–300 g maintained on a standard diet were used in this experiment.

Liver transplantation

Orthotopic liver transplantation was performed using the cuff technique previously described by Kamada and Calne [7]. Reconstruction of the hepatic artery was not performed. In vivo NIR spectroscopy was performed on the grafts on postoperative days (POD) 2, 4, 6, and 8, and thereafter the recipients were sacrificed for blood sampling and histological inspection of the graft.

Experimental groups

The experimental animals were divided into three groups: group 1, the syngenic combination (Lewis-to-Lewis) as control; group 2, the allogenic combination (ACI-to-Lewis) trated with no immunosuppression as the acute rejection model; group 3, the allogenic combination treated with 15-deoxyspergualin (DSG) administered intraperitoneally at a daily dose of 5 mg/kg starting from POD 3 as the rejection-treated model.

In vivo NIR spectroscopy

NIR multi-wavelength spectroscopy is a noninvasive technique developed for in vivo assessment of tissue oxygen sufficiency. It has been used to monitor continuously regional blood volume changes, heme saturation, and the redox state of Cyt a, a_3 in intact tissues [6, 13, 14, 16]. This method can also be used for the detection of nitrosyl-Hb by the following manner. For NIR measurement, the recipient's abdominal cavity was opened to expose the graft on given days under light ether anesthesia. NIR light from a halogen lamp at 150 W was directed through a flexible bundle of quartz optical fibers into the liver, and the reflected light was conveyed through another bundle to the spectrometer (Fig. 1). The tips of these two bundles were fixed at a position approximately 3 mm above the graft. The spectrometer (MCPD-1000, Otsuka Electrical CO., Japan) was equipped with 400 channel photodiode arrays and the reflected light was measured in the NIR range at 4-s intervals. The difference in the spectrum from the grafted liver and a perfused liver measured immediately before removal was subjected to multi-component analysis. In the range of 700–1000 nm, the difference in the spectrum was analyzed by the curve-fitting method based on the least squared method using the standard spectra of purified oxy-Hb, deoxy-Hb, nitrosyl-Hb, oxidized-Cyt a, a_3, reduced-Cyt a, a_3, water, and rat bile. The relative changes in each component were obtained in arbitrary units through this multi-component analysis calculated by singular value decomposition.

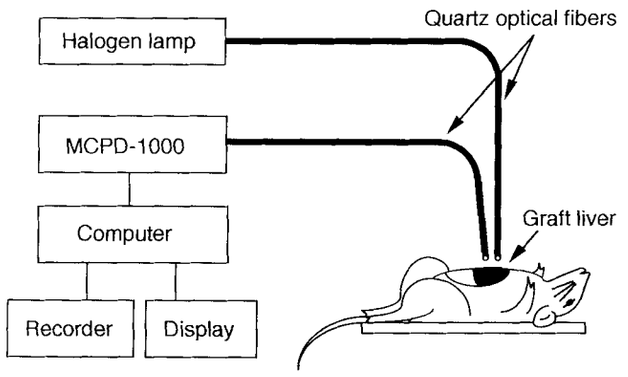

Fig. 1 Block diagram of in vivo near-infrared spectroscopy apparatus

Serum NO_3^- determination

The levels of serum nitrate in the syngenic and allogenic recipients (groups 1 and 2) were determined by use of high-performance liquid chromatography equipped with an ion exchange column (TSK gel IC-Anion PW, TOSO CO., Japan) after deproteinization (using ULTRACENT-10 and TOYOPAC IC-SP, TOSO CO., Japan) of the serum. All serum samples obtained for nitrate levels were measured for creatinine, which never exceeded 0.8 mg/dl.

Histological examination

Specimens of the grafted livers were fixed in 10% formalin and stained with hematoxylin and eosin for light microscopy.

Statistical analysis

Statistical analysis was performed using paired *t*-tests, and a P value of less than 0.05 was considered significant. Values are expressed as mean ± SEM.

Results

Histological findings

The histological studies of the graft confirmed the presence of acute rejection in the allogenic groups (groups 2 and 3) and the absence of the characteristic findings of rejection in the syngenic group (group 1). In group 2, no evidence of rejection was observed on POD2. Mononuclear cell infiltration in portal tracts with or without venous endothelialitis and bile duct damage was observed on POD4. Extensive cell infiltration in portal tracts and parenchymal was on observed POD6 and a further remarkable mononuclear cell infiltration in portal tracts

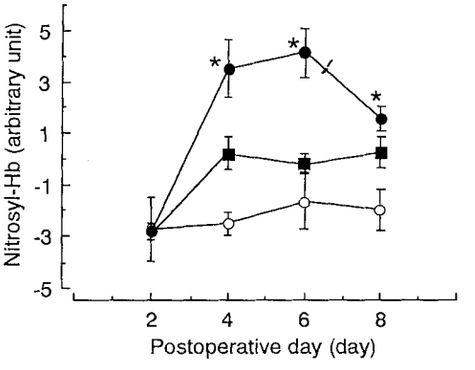

Fig. 2 Time course of nitrosyl-hemoglobin level of grafted liver. Group 1, the syngenic grafts ($n = 5$) (○); group 2, the allogenic grafts ($n = 5$) (●); group 3, the allogenic grafts treated with 15-deoxyspergualin ($n = 5$) (■). Results are expressed as mean ± SEM. * $P < 0.01$ vs. group 1

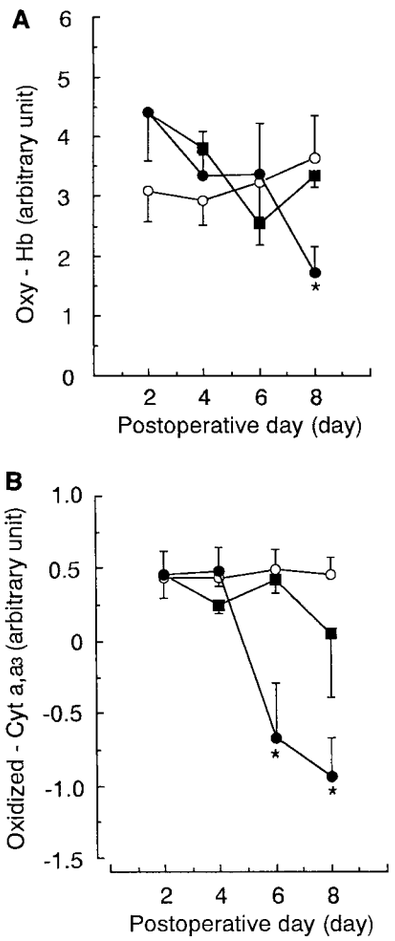

Fig. 3 **A** Time course of oxy-hemoglobin level of grafted liver. **B** Time course of oxidized-cytochromeoxidase level of grafted liver. Group 1, the syngenic grafts ($n = 5$) (○); group 2, the allogenic grafts ($n = 5$) (●); group 3 the allogenic grafts treated with 15-deoxy-spergualin ($n = 5$) (■). Results are expressed as mean ± SEM. * $P < 0.05$ vs. group 1

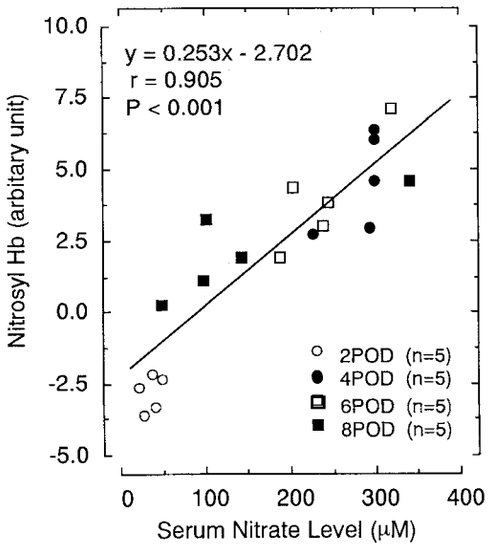

Fig. 4 Correlation between nitrosyl-hemoglobin level measured by near-infrared spectroscopy and serum NO_3^- level in the allografted recipients. A significant correlation was observed between them

and parenchyma with a confluent dropout of hepatocytes was observed on POD8. In group 3, the sinusoids contained varying numbers of mononuclear cells, but always fewer than in group 2. There were some scattered individual necrotic hepatocytes, but foci of necrosis were not seen on POD8. Thus, although mild or moderate rejection remained during the observation period, the significant therapeutic effect of DSG was observed in group 3.

In vivo NIR spectroscopy

Summarized NIR optical data from the three experimental groups are presented in Figs. 2 and 3. Nitrosyl-Hb in group 2 was significantly higher on POD4, 6, and 8 compared to that in group 1, which remained at a constant level throughout the observation period. In group 3, nitrosyl-Hb was suppressed (Fig. 2). Oxy-Hb and oxidized-Cyt a, a_3 levels remained constant during the observation period in group 1. In group 2, oxy-Hb showed a significantly lower level on POD8, and oxidized-Cty a, a_3 decreased on POD 6. In group 3, both oxy-Hb and oxidized-Cyt a, a_3 showed improvement (Fig. 3).

Serum nitrate levels

In group 1, serum nitrate levels detected on POD 2, 4, 6, and 8 were 22.4 ± 2.7, 23.0 ± 2.9, 22.3 ± 10.4, and 38.7 ± 8.6 μ*M*, respectively. In group 2, serum nitrate levels on POD2, 4, 6, and 8 were 35.7 ± 5.5, 275.2 ± 17.5, 239.2 ± 25.4, and 146.8 ± 56.8 μ*M*, respectively. There was a significant correlation between the nitrosyl-Hb level and the serum nitrate level in group 2 (Fig. 4).

Discussion

The rejection response in a vascularized allograft organ is characterized by infiltration of mononuclear cells, including activated lymphocytes and macrophages. The macrophages activated by exposure to cytokines and/or to endotoxin are known to produce NO [10, 15]. Activation

induces the expression of NO synthase, the enzyme that catalyzes formation of NO from L-arginine and molecular oxygen. It has been reported that a large amount of NO is synthesized during the alloimmune response in vivo; however, the biological significance of the generation of NO remains unknown [4, 8, 9]. It would be useful to establish a method that can directly estimate NO production and elucidate NO metabolism in vivo during the alloimmune response. In the present study, a new technique of in vivo NIR spectroscopy was applied to the standard rat model of orthotopic liver transplantation for the investigation of NO production at the site of allograft livers during the rejection reaction. NO has a strong affinity for transit metals such as iron atoms in heme proteins and thereby induces the formation of nitrosyl-Hb at the site of allografts undergoing rejection. This new technique of in vivo NIR spectroscopy can detect nitrosyl-Hb formed from immunologically produced NO and erythrocyte hemoglobin in intact grafts.

To ascertain that the nitrosyl-Hb levels measured by NIR spectroscopy reflected in vivo NO synthesis, the serum NO_3^- levels were quantified. It has been demonstrated that the elevated serum and urinary NO_3^- levels are, in fact, due to in vivo immunologically produced NO [2, 3]. In the present study, a significant correlation was observed between the liver nitrosyl-Hb level measured by NIR spectroscopy and the serum NO_3^- level in allogenic grafted recipients with normal kidney function. This demonstrated that NIR spectroscopy was highly efficient for monitoring NO production in living allografts.

The elevation of the nitrosyl-Hb level was observed in the early phase of acute rejection (on POD4) in the allogenic group, whereas it was not detected in the syngenic group. As DSG specifically inhibits lymphocyte clone expansion at the onset of rejection [1, 11, 12], the DSG treatment of liver allografts with ongoing rejection showed a tendency to suppress the elevation of the nitrosyl-Hb level in accordance with the reversal of the rejection as determined histologically. This indicated that the nitrosyl-Hb level could be of value for earlier diagnosis of rejection and evaluation of the effect of rejection therapy.

Our previous study has showed that in vivo NIR spectroscopy can be used to evaluate the viability of a grafted liver by monitoring the level of hemoglobin oxygenation and the redox state of Cyt a, a_3 in the graft [14]. In the present study, oxy-Hb and oxidized-Cyt a, a_3 levels in the allografts were decreased at the advanced stage of rejection (on POD6 or 8) in accordance with parenchymal disorder as determined histologically, and these levels showed improvement when the rejection reaction was treated by the administration of DSG. Thus, in vivo NIR spectroscopy also proved to be of value in the evaluation of graft dysfunction due to the rejection response.

In the present study, general anesthesia and a laparotomy were performed in experimental animals for blood sampling and histological inspection, and NIR spectrophotometry was conducted directly on the grafted liver. However, considering the good transparency of biological tissues to NIR light, noninvasive NIR monitoring in clinical liver transplantation should become possible in the near future. This is supported by previous investigations that have demonstrated that NIR spectroscopy can be used for the noninvasive measurement of cerebral blood volume in human infants [13, 16].

In conclusion, this new technique of in vivo NIR spectroscopy could assess simultaneously both the immune response and graft function following liver transplantation through the monitoring of NO production and tissue oxygen sufficiency in intact grafts. It should also be useful for the earlier diagnosis of rejection and evaluation of the effect of rejection therapy.

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