Antiproliferative Effects of NKH477, a Forskolin Derivative, on Cytokine Profile in Rat Lung Allografts

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Objective NKH477 was recently identified as a water-soluble forskolin derivative and was reported to prolong survival of murine cardiac allografts. However, the mechanism of the efficacy is not clear in vivo. The aim of this study was to investigate the immunosuppressive effects of NKH477 on acute lung allograft rejection in the rat model and its mechanism of action in vivo.

Methods Left lungs were transplanted orthotopically from Brown-Norway donors to Lewis recipients. Recipient rats were untreated or treated daily with different doses of NKH477. Grafts were excised on Day 3 or Day 5 to determine histopathological rejection and expressions of interleukin (IL)-2, IL-4, IL-10, and interferon (IFN)-γ by enzyme-linked immunosorbent assay. The cytokine expression at Day 3 or Day 5 was also evaluated in recipient spleens by immunohistochemistry. Furthermore, mesenteric lymph node cells from recipients at Day 5 were cultured alone or stimulated with donor antigens for 72 hours to determine cell proliferation by means of thymidine incorporation.

Results NKH477 significantly extended allograft survival time in a dose-dependent manner and reduced histopathological rejection. Treatment with NKH477 inhibited IFN-γ and IL-10 expression, whereas expression of these cytokines were markedly upregulated in the untreated allografts. Expression of IL-2 and IL-10 also increased in the spleen of untreated allorecipients. NKH477 suppressed expression of both cytokines in the spleen. In addition, lymphocyte proliferation was inhibited in NKH477-treated recipients as compared with untreated recipients.

Conclusion These results suggest that NKH477 exerts an antiproliferative effect on lymphocytes in vivo with an altered cytokine profile in rat recipients of lung allografts.

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Cyclic adenosine monophoshate (cAMP) is an intracellular second messenger that has various modulatory effects on the immune system. Forskolin increases intracellular levels of cAMP by activating adenylate cyclase. Forskolin and other cAMP-elevating agents decrease the production of interleukin (IL)-2 and IL-2 receptor α-chain expression through transcriptional regulation of the genes.[1,2] Therefore, the increase in intracellular cAMP is related to suppression of T cell proliferation and leads to the impairment of cytotoxic T lymphocyte (CTL) generation, which is considered to play an important role in the course of acute rejection.[1,3,4] Thus, forskolin has been widely used in in vitro studies to investigate the influence of intracellular cAMP elevation on lymphocyte function.

However, despite its immunosuppressive properties, the hydrophobic nature of forskolin and putative nonspecific pharmacological activities have hampered an attempt to develop in vivo or clinical applications for immunosuppression. NKH477, (+)-(3*R*, 4*aR*, 5*S*, 6*S*, 6*S*, 6*aS*, 10*S*, 10*aR*, 10*bS*)-5-acetoxy-6-(3-dimethylaminopropionyloxy)-dodecahydro-10,10*b*-dihydroxy-3,4*a*,7,7,10*a*-pentamethyl-3-vinyl-1*H*-naphtho-[2,1-*b*]pyan-1-one monohydrochloride, is a novel water-soluble forskolin derivative that was introduced as a positive inotropic agent in heart failure.[5,6] Like forskolin, NKH477 directly activates the catalytic unit of adenylate cyclase and increases intracellular cAMP.[5,7–9] It has been shown to improve cardiac failure mainly through

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its beneficial effects on diastolic cardiac function.[6] Subsequently, prolonged survival of murine cardiac allografts was demonstrated.[10] Although immunomodulation by NKH477 was shown in vitro, the in vivo mechanism of its efficacy was not addressed previously in the report. Furthermore, because the inotropic effects of NKH477 directly acting on cardiomyocytes might contribute to the improvement of allograft survival, the animal model that uses cardiac allografts itself is not ideal to investigate the immunosuppressive activity of NKH477.

In this study, we therefore used a rat orthotopic lung transplantation model to directly assess immunosuppressive activity of NKH477, and asked whether it inhibited acute rejection and prolonged the lung allograft survival. To elucidate in vivo mechanisms of action, the influence of NKH477 on immunomodulatory cytokines was evaluated in the secondary lymphoid organ as well as in the grafted lungs. In addition, alloantigen-induced lymphocyte proliferation was compared between untreated and NKH477-treated animals. For the first time, we addressed in vivo mechanisms of action of NKH477 and showed that NKH477 exerts an antiproliferative effect in vivo with an altered cytokine profile to inhibit the acute rejection of rat orthotopic lung allografts.

MATERIAL AND METHODS

Animal

Specific-pathogen-free inbred male Lewis rats (LEW) ($RT1^l$) were obtained from Charles River Japan (Yokohama, Japan). Brown-Norway (BN) ($RT1^n$) rats were obtained from Seac Yoshitomi (Fukuoka, Japan). LEW rats weighing 250 to 280 g served as recipients; BN or LEW rats weighing 220 to 250 g served as donors. All animals received humane care in compliance with the *Principles of Laboratory Animal Care* and the *Guide for the Care and Use of Laboratory Animals* prepared and formulated by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication no. 86-23, revised 1985).

Orthotopic Lung Transplantation

Donor left lungs were transplanted orthotopically into the left hemithorax of recipients using a modified technique of Marck et al and the pulmonary artery and vein were anastomosed using the cuff technique of Reis et al.[11,12] The mean graft ischemic time was 45 ± 10 minutes.

Treatment

NKH477 was provided as a pure powder by Nippon Kayaku (Tokyo, Japan). It was dissolved in saline and adjusted to pH 4 with 0.01 N HCl. The solution was prepared daily and stored at 4°C until time of use. NKH477 was administered orally to recipients once a day from Day 0 to Day 10 after transplantation or until the day of rejection.

Experimental Groups

The recipients were divided into the following 5 groups: recipients bearing isografts (LEW to LEW) (Group 1), untreated allografts (BN to LEW) (Group 2), allografts with NKH477 treatment at a daily dose of 1, 2, or 3 mg/kg body weight (Group 3, 4, or 5, respectively). Within Groups 1, 2, and 5, the recipients were further subdivided into 3 groups and used for graft survival data, histological data, and analysis of cytokine profile.

Graft Function and Rejection

The grafted lung was monitored daily by chest roentogenography. From each roentogenogram, a ventilation score of the graft, ranging from 6 for a normal-looking lung to 0 for an opaque lung, was determined as described by Prop et al. [13] The grafts were considered rejected if the ventilation score dropped to 1 or 0.

Histopathology

Recipients in Groups 1, 2, and 5 were killed on Day 3 or Day 5 ($n = 5$ for each group at each time point). At the time of death, animals were sedated and heparinized. The transplanted lung were removed and flushed through the pulmonary artery with 10% neutralized buffered formalin. Tissues were embedded in paraffin. In order to determine grades of histological rejection, sections of lung grafts were stained with hematoxylin and eosin and evaluated by 2 pathologists blinded to the individual treatment groups. Histological rejection grades were based on the acute rejection classification approved by the International Society for Heart and Lung Transplantation.[14]

Enzyme-Linked Immunosorbent Assay (ELISA)

Transplanted lungs of recipients in Groups 1, 2, and 5 were removed on Day 3 or Day 5 ($n = 5$ for each group at each time point). Grafted lungs were dispersed and homogenized with Polytron (Brinkmann Instruments, Westbury, NY) in 2 ml of normal saline and centrifuged at 1500 rpm for 30 minutes at 4°C. The supernatant was stored at −80°C until time of use. The supernatant was used to quantify the expression of interferon (IFN)-γ, IL-2, IL-4, and IL-10 with the Cytoscreen immunoassay kits (BioSource International, Camarillo, CA) according to the protocol supplied by the manufacturer.

Immunohistochemistry

Recipient spleens, removed at the time of death, were sliced approximately 5 mm thick, embedded in OCT compound (Miles, Elkhart, IN), and snap-frozen