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Non-neuronal neuropeptide Y and its receptors during acute rejection of rat pulmonary allografts

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ABSTRACT

This study tested the hypothesis that neuropeptide Y (NPY) and NPY receptors 1 (Y1) and 2 (Y2) participate in lung allograft rejection. Inflammation in grafts may include interaction between blood leukocytes and graft endothelial cells and marked accumulation of intravascular blood leukocytes. Fewer leukocytes accumulate in lung than in kidney allografts. Lung transplantion was performed in the Dark Agouti to Lewis rat strain combination. Intravascular and intraalveolar leukocytes were isolated from the grafts, and we evaluated the mRNA expression of NPY, Y1, and Y2 by real-time RT-PCR as well as the peptide expression of NPY by radioimmunoassay and immunohistochemistry. NPY and Y1 were expressed by pulmonary intravascular and intraalveolar leukocytes. Y1 was up-regulated by pulmonary intravascular and intraalveolar leukocytes during allograft rejection while Y2 could not be detected. Higher NPY expression levels in intravascular leukocytes were observed in lung compared to kidney allografts, which were investigated previously. Our findings suggest that an increased leukocytic expression of NPY in lung compared to kidney allografts results in a reduced accumulation of leukocytes in allograft vessels.

1. Introduction

Graft and thus also patient survival after lung transplantation is still much worse compared to other solid organ transplantations and consists of only 58% after 5 years [1].

In organ transplants, activated macrophages impair graft function and intensify T-cell-mediated rejection [2,3]. Acute vascular rejection includes intravascular leukocyte accumulation and endothelial cell apoptosis. Furthermore, the CD4 and CD8 T cells and macrophages invade the subendothelium and intima of arteries [4].

The lung contains several populations of lineage-different monocytes/macrophages: blood monocytes, alveolar and resident interstitial macrophages. In addition, monocytes extravasate in response to inflammation and differentiate into macrophages. The role of monocytes/macrophages during rejection of lung allografts, however, is not well understood [5,6]. After pulmonary transplantation in the DA to Lew rat strain combination, mononuclear leukocytes (50% monocytes) accumulate in the pulmonary vasculature. Allograft monocytes from pulmonary allografts depict a partially activated immunophenotye, whereas monocytes isolated from the vascular bed of renal allografts transplanted in the same rat strain combination are more numerous and strongly activated [7]. In addition, DA to Lew lung allografts are heavily infiltrated by interstitial and alveolar macrophages [8]. Infiltration of allograft tissue is similar in pulmonary and renal allografts [8,9].

Neuropeptide Y (NPY), a sympathetic co-mediator and modulator of immune functions, may regulate leukocyte accumulation in graft blood vessels and central nervous system [2,10]. In the rat, the NPY gene is located on chromosome 4 in close association with other genes involved in immune regulation such as major histocompatibility complex II and CD36 [11]. Rat blood leukocytes abundantly produce NPY mRNA and peptide, and NPY expression is markedly down-regulated by leukocytes that accumulate in the vascular bed of the graft during acute kidney rejection [12]. The NPY receptors 1 (Y1) and 2 (Y2) are present on most immune cells including monocytes, macrophages, lymphocytes, and neutrophils [13]. The effects of NPY on immune cells that are

Abbreviations: BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; c, control; CT, cycle threshold; d, day; DA, Dark Agouti; ΔC_T, difference in CT values between the gene of interest and porphobilinogen deaminase; h, hour; Lew, Lewis; NPY, neuropeptide Y; RT-PCR, reverse transcriptase polymerase chain reaction; RNA, ribonucleic acid; Y1, neuropeptide Y receptor 1; Y2, neuropeptide Y receptor 2

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transmitted through Y1 include decreasing T-cell activity, activating antigen-presenting cell function, reducing leukocyte adhesiveness, activating and redistributing monocytes, and inducing neointimal proliferation [14–17]. Increased adherence of lipopolysaccharide-stimulated macrophages in response to NPY is mediated by the Y2 receptor [18]. Furthermore, NPY may induce angiogenesis through the Y2 receptor [11].

We hypothesized that neuropeptide Y participates in allograft rejection. The purpose of this study was to evaluate the leukocytic expression of NPY and NPY receptors Y1 and Y2 during acute rejection of rat lung allografts.

2. Material and methods

2.1. Study design

Lung transplantations were performed in the DA to Lew rat strain combination. Graft intravascular leukocytes from transplanted lungs (left lung) and controls (right lung) were isolated by intensive vascular perfusion. In addition, intra-alveolar graft leukocytes were obtained by bronchoalveolar lavage (BAL).

Expression of NPY, Y1, and Y2 mRNA was analyzed by real-time RT-PCR. NPY peptides were measured by radioimmunoassay in BAL fluid (BALF). Immunohistochemical methods were used to identify cells that expressed NPY.

2.2. Animals

Male specified pathogen-free Lew (RT1l) and DA (RT1av1) rats were purchased from Janvier Labs (ST Berthevin, France). Animal experiments were performed as required by the current version of the German Law on the Protection of Animals (Regierungspräsidium Giessen, permit No. V 54 – 19 c 20-15 (1) GI 20/10 Nr. 49/2007) as well as the NIH principles of laboratory animal care. Lew rats served as organ recipients in all transplant experiments. DA rats served as donors to study acute lung allograft rejection. Time-matched isografts and untreated organs served as controls. Native right lungs of pulmonary graft recipients served as additional controls.

2.3. Organ transplantion

Rats that underwent lung transplantion by using a cuff technique [8] weighed 220 to 280 g and were shortly anesthetized by inhalation of isoflurane (Forene, Abbott, Wiesbaden, Germany) followed by intraperitoneal injection of ketamine hydrochloride (90 mg/kg body weight) (Ketavet, Pfizer, Karlsruhe, Germany), and medetomidine hydrochloride (0.1 mg/kg body weight) (Domitor, Pfizer, Karlsruhe, Germany). All rats received heparin (1000 IU/kg intravenous) (Ratiopharm, Ulm, Germany) and 1 dose of ampicillin (150 mg intraperitoneal) (Ratiopharm), but no immunosuppression was given. After surgery, anesthesia was antagonized by atipamezol hydrochloride (0.5 mg/kg intraperitoneal) (Antisedan, Pfizer).

2.4. Bronchoalveolar lavage and perfusion of the vasculature

On day 4 after lung transplantion, the trachea was cannulated and the right main bronchus was clamped to perform BAL of the transplanted lung (5 mL; 6 repetitions) with calcium- and magnesium-free phosphate-buffered saline (4 °C) containing potassium chloride (2.68 mM), potassium dihydrogen phosphate (1.47 mM), sodium chloride (136.89 mM), and disodium hydrogen phosphate (8.1 mM) (Dulbecco's phosphate-buffered saline [pH 7.3], PAA Laboratories, Pasching, Austria). Thereafter, the left bronchus was clamped and the right native lung underwent BAL.

To harvest intravascular leukocytes on day 4 after transplantion, graft recipients were anesthetized and heparinized, and graft blood vessels were perfused with ice-cold phosphate-buffered saline containing ethylenediaminetetraacetic acid (2.7 mM) and bovine serum albumin (0.1%) as previously described [19]. For isolation of peripheral blood mononuclear leukocytes, isopycnic Percoll gradient centrifugation was performed [7,20]. Cells were counted in Türck solution (Merck, Darmstadt, Germany). All samples were stored at − 80 °C until use.

2.5. RNA isolation and real-time RT-PCR

Total RNA was isolated from cells using a commercial kit (Qiagen RNeasy Miniprep kit, Qiagen, Hilden, Germany) according to the instructions from the manufacturer. For reverse transcription, Moloney Murine Leukemia Virus reverse transcriptase (Promega, Mannheim, Germany) and random hexamer primers (Promega) were used. Changes of gene expression for NPY, Y1, and Y2 were analyzed in duplicates by real-time RT-PCR (StepOne Real-Time-PCR System, Applied Biosystems, Carlsbad, California CA, USA) using Platinum SYBR qPCR SuperMix-UDG (Invitrogen, Karlsruhe, Germany). Controls omitting the template were included in all repetitions. Products were separated on agarose gels together with DNA ladder 100 bp (Promega). Products were sequenced (MWG Biotech, Ebersberg, Germany) after purification from the agarose gels (MinElute PCR Purification Kit, Quiagen).

Porphobilinogen deaminase mRNA expression was used for normalization. Relative gene expression values were calculated by the eq. $2^{-\Delta CT}$, where ΔCT is the difference in cycle treshold (CT) values between the gene of interest and porphobilinogen deaminase. Data for each experimental group were reported in relation to the expression in the control, which was set to 1 arbitrary unit. Primers (0.6 μM) (Table 1) were selected for intron spanning cDNA sequences (MWG Biotech). The real-time RT-PCR included initial denaturation for 5 min at 95 °C, 45 cycles of 20 s at 60 °C, and 1 cycles of 10 s at 72 °C.

2.6. Immunhistochemistry

Organs were fixed with 4% buffered paraformaldehyde, embedded in paraffin, and cut into sections (6 μm). Slides were incubated with protease type XIV (0.5 mg/mL) (Sigma-Aldrich) in *tris*(hydroxymethyl) aminomethane hydrochloride (50 mM, pH 7.6) with sodium chloride

Table 1
Primers used for real time polymerase chain reaction[a].

Gene	Primer	Sequence	Amplicon size (bp)
rNPY (NM_012614.2)	Sense antisense	5′-GCTCTATCCCTGCTCGTGTGTT-3′ 5′-GTAGTGTCGCAGAGCGGAGTAG-3	123
rY1 (NM_001113357.1)	Sense antisense	5′-GTGAGACCAAACGAATCAACG-3′ 5′-CAGAGCAGGAACAGCAGATTG-3′	129
rY2 (NM_023968.1)	Sense antisense	5′-CCCGGATCTGGAGTAAGCTAAA-3′ 5′-GTGGAGCACATCGCAATAATGT-3′	235
rPBGD (NM_013168)	Sense antisense	5′-GGCGCAGCTACAGAGAAAGT-3′ 5′-AGCCAGGATAATGGCACTGA-3′	126

[a] Abbreviations: bp, base pair; NPY, neuropeptide Y; PBGD, porphobilinogen deaminase; r, rat; Y1, NPY receptor 1; Y2, NPY receptor 2.