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Blocking the CC Chemokine Receptor 5 Pathway by Antisense Peptide Nucleic Acid Prolongs Islet Allograft Survival

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ABSTRACT

Chemokines are important regulators in the development, differentiation, and anatomic location of leukocytes. The CC chemokine receptor 5 (CCR5) is the receptor for the proinflammatory chemokines and plays an important role in islet allograft rejection. Peptide nucleic acid (PNA) is a nucleic acid analog in which the sugar phosphate backbone of natural nucleic acid has been replaced by a synthetic peptide backbone. Studies indicate that PNA inhibits both transcription and translation of targeted genes. Fullly major histocompatibility complex (MHC)-mismatched murine islet transplant models were used to test the in vivo effect of PNA CCR5 by targeting CCR5 in acute allograft rejection. PNA CCR5-treated recipients demonstrated significant prolongation (12.0 ± 1.75 days) of functional allograft survival compared with saline (6.5 ± 0.58 days)- or PNA mismatch-treated recipients (6.5 ± 0.50 days). The PNA CCR5 blocked the expression of CCR5 in spleen CD3+ T cells. Lymphocytes from PNA CCR5-treated mice exhibited a reduced degree of proliferation comparable to that of saline- and PNA mismatch-treated mice. The present study indicated that PNA CCR5 has a substantial therapeutic effect to inhibit acute allograft rejection.

ISLET TRANSPLANTATION offers a physiological approach for restoration of glucose homeostasis, thereby reversing the metabolic and neurovascular complication of diabetes. In the past, there were only a few successes with human islet transplantation; the initial results were disappointing. However, recent reports of greater success have renewed interest in it as a possible therapeutic option for patients with diabetes.[1,2] One of the main obstacles to islet transplantation is the rejection that leads to islet allograft injury. However, current posttransplantation immunosuppression is nonspecific with adverse effects including toxicity to β cells. To overcome them, novel immunosuppressive targets and strategies need to be defined.

Chemokines are important regulators of the development, differentiation, and anatomic location of leukocytes.[3] Through activation of G-protein-coupled cell surface receptors on target cells, chemokines and their receptors play a major role in the process by which leukocytes are recruited from the bloodstream into sites of inflammation. Several have been implicated in allograft rejection.[4,5] The CC chemokine receptor 5 (CCR5), the receptor for the proinflammatory chemokines, plays an important role in allograft rejection.

Synthetic molecules that can bind with high sequence specificity to a chosen target in a gene sequence are of major interest in medicinal and biotechnological contexts. They show promise for the development of gene therapeutic agents. Peptide nucleic acid (PNA) is a nucleic acid analog in which the sugar phosphate backbone of natural nucleic acid has been replaced by a synthetic peptide backbone usually formed from N-(2-amino-ethyl)-glycine units, resulting in an achiral and uncharged mimic. Studies indicate that PNA inhibits both transcription and translation of targeted genes, which holds promise for its use in antigene and antisense therapy.[6] In this study, we investigated the effects of targeting CCR5 using antisense PNA CCR5 on islet allograft survival in murine recipients rendered diabetic using streptozotocin.

MATERIALS AND METHODS

Animals

Adult donor BALB/c(H-2^d) and recipient C57BL/6(H-2^b) mice of 8 to 12 weeks (30–35 g) were used throughout this study. The recipients rendered diabetic by a single intraperitoneal injection of

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250 mg/kg streptozotocin (STZ; in citrate buffer, pH 4.5; Sigma) were considered diabetic when the tail vein blood glucose concentration was more than 300 mg/dL for 2 consecutive days.[7]

Animal Treatment With PNA Antisense

All PNAs were synthesized by The Peptide company. The sequences of PNA antisense were as follows: PNA CCR5 antisense, $_{434}$(5′-Tyr-CCGTTCTGACTTTT-Lys-3′)$_{421}$; PNA mismatch sequence, Tyr-TTTCCAGCTGCTTT-Lys (randomly sanitized). Mice spontaneously received 2.5 mg/kg IV daily of PNA CCR5 antisense or mismatch sequence PNA for 7 days, with the first injection 4 hours the day before transplantation.

Islet Isolation and Transplantation

Mice were anesthetized with ether. After intraductal injection of 3 to 4 mL of cold Hank's balanced salt solution containing 1.5 mL/mL of collagenase V (Sigma, USA), pancreata were surgically procured and digested at 37°C for 15 minutes. Islets were washed with Hank's balanced salt solution and then purified by 4-layer discontinuous gradient centrifugation using Ficoll (Sigma) and hand picked under an inverted microscope.[8]

For each transplantation procedure, recipients were anesthetized with intraperitoneal butaylone. A lateral flank incision was made above the right kidney, and islets inserted under the capsule at the anterior surface through a polypropylene tube. From 300 to 400 islets were used in each transplantation. Tail vein blood glucose was monitored to assess islet graft function. Rejection was defined when nonfasting blood glucose levels were above 200 mg/dL on 2 consecutive days.

Histological Examination

For histological evaluation, the transplanted grafts were harvested on the indicated day. Tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 μm, and then stained with hematoxylin and eosin. At day 7, 5 islet grafts from each recipient type were excised. Immunohistochemical staining was performed by the SP method with a heat-induced antigen retrieval step. Rabbit anti-mouse insulin (Boster Biotechnology Limited Corporation, China) was applied at dilutions of 1:100. Detection was first performed by rehydrating through xylene and graded alcohol. Before application of the primary antibodies, the sections were incubated in 3% H_2O_2 for 10 minutes to block activity of endogenous peroxidase. After incubating with the primary antibodies overnight at 4°C, the sections were incubated with biotin-labeled secondary antibody for 20 minutes at room temperature, and then incubated with streptomyces-avidin-peroxidase for 10 minutes at room temperature. Finally, the sections were dyed in DAB and then redyed in hematoxylin.

In Vitro Lymphocyte Responses

In vitro T-cell proliferative responses were assessed by mixed lymphocyte response (MLR). We cultured 1×10^6/mL responder (C57/BL6) and 1×10^6/mL gamma-irradiated (500 rad/min) inactivated stimulator splenocytes (BALB/c) in 96-well flat-bottomed plates. Cultures incubated for 72 hours were pulsed with [^{3}H]thymidine (^{3}H-TdR) for 16 hours before harvesting. The cells were counted on a β-scintillation counter. All wells were analyzed in quadruplicate; the mean counts per minute (cpm) and SD were calculated.

RNA Isolation and cDNA Synthesis

After transplantation, 5 islet grafts per group were harvested at day 7 or at rejection. Total RNA was extracted from islet grafts with TRIzol Reagent (TaKaRa Biotechnology Co) as described by the manufacturer. For reverse transcriptase-polymerase chain reaction (RT-PCR), 1 μg of total RNA was reverse transcribed with 1 μL of oligo(dt) primer (0.5 μg/μL). Subsequently, 10 μL of RT premix was added. It consisted of 4 μL of Superscript 5× reaction buffer, 3.5 μL of diethyl pyrocarbonate-treated water, 0.4 μL of 25 mmol/L of dNTP, 1 μL of 100 mmol/L of dithiothreitol, 0.5 μL (20 units) of RNasin, and 0.5 μL (100 units) of Superscript Moloney murine leukemia virus RT.

Quantitative Real-Time PCR Analysis

The following primers were used[9]: RANTES, 5′-CCTCACCATCATCCTCACTGCA-3′, 5′-TCTTCTCTGGGTTGGCACACAC-3′ (215 bp); MIP-α5′-GAAGAGTCCCTCGATGTGGCTA-3′, 5′-CCCTTTTCTGTTCTGCTGACAAG-3′ (472 bp); CCR5, 5′-CAAGACAATCCTGATCGTGCAA-3′, 5′-TCCTACTCCCAAGCTGCATAGAA-3′ (125 bp); β-actin, 5′-GGGAAATCGTGCGTGACATTA-3′, 5′-GATCTCTTCTGCATCCTGTC-3′ (350 bp). Quantitative real-time PCR analysis was performed using the ABI Prism 7700 sequence detector system (PE Applied Biosystems, CA, USA). Primer sets were purchased from PE Applied Biosystems. PCR was carried out with the TaqMan Universal PCR Master Mix (PE Applied Biosystems) using 1 μL of cDNA in a 20 μL final reaction volume. The PCR reactions were cycles 45 times after initial denaturation (95°C, 15 minutes) with the following parameters: denaturation at 94°C for 15 seconds; annealing at 56°C for 30 seconds; extension at 72°C for 1 minute. The expression levels of each mRNA were divided by the mRNA levels of the housekeeper gene β-actin.

Western Blot Assays

For protein detection, the islet grafts were excised from recipients and snap frozen. The tissues were resuspended in schizolysis buffer and homogenate. Then it was added to membrane protein extract containing 1% Triton overnight at 4°C. Extracts were centrifuged at 10,000 rpm for 5 minutes at 4°C. Protein concentration was measured by Bio-Rad protein assay. The protein (50 μg) was loaded onto 12% SDS-PAGE, transferred onto colloxylin membranes after electrophoresis. The protein incubated with the primary antibody of rabbit monoclonal anti-mouse CCR5 Ab (0.5 μg/mL) (Boster Biotechnology Limited Corporation, China) overnight at 4°C. The membranes were washed twice for 5 minutes each in TBS-Tween. Secondary antibody conjugates (AKP conjugate goat anti-rabbit) were diluted 1:5000 and incubated for 2 hours at room temperature. Analyses were conducted using electrochemiluminescence (ECL) detection. Densitometry index analysis of band was made by gel imagery system.

Flow Cytometry

The spleen cells were washed once with PBS and resuspended in 100 μL PBS containing 2% bovine serum albumin (BSA) with a cell concentration of 1×10^6/mL. For bicolor analysis, CCR5 FITC-labeled MAb and CD3 PE-labeled MAb (Biolegend) were used together to stain cells. After 30 minutes at 4°C, cells were washed twice with PBS and then analyzed on FACScan to determine the level of surface expression.