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Peptide Nucleic Acid Antisense Prolongs Skin Allograft Survival by Means of Blockade of CXCR3 Expression Directing T Cells into Graft

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CXCR3, predominantly expressed on memory/activated T cells, is a receptor for both IFN-γ-inducible protein 10/CXC chemokine ligand (CXCL)10 and monokine induced by IFN-γ/CXCL9. It was reported that CXC chemokines IFN-γ-inducible protein 10/CXCL10 and monokine induced by IFN-γ/CXCL9 play a critical role in the allograft rejection. We report that CXCR3 is a dominant factor directing T cells into mouse skin allograft, and that peptide nucleic acid (PNA) CXCR3 antisense significantly prolongs skin allograft survival by means of blockade of CXCR3 expression directing T cells into allografts in mice. We found that CXCR3 is highly up-regulated in spleen T cells and allografts from BALB/c recipients by day 7 of receiving transplantation, whereas CCR5 expression is moderately increased. We designed PNA CCR5 and PNA CXCR3 antisenses, and i.v. treated mice that received skin allograft transplantations. The PNA CXCR3 at a dosage of 10 mg/kg/day significantly prolonged mouse skin allograft survival (17.1 ± 2.4 days) compared with physiological saline treatment (7.5 ± 0.7 days), whereas PNA CCR5 (10 mg/kg/day) marginally prolonged skin allograft survival (10.7 ± 1.1 days). The mechanism of prolongation of skin allograft survival is that PNA CXCR3 directly blocks the CXCR3 expression in T cells, which is responsible for directing T cells into skin allograft to induce acute rejection, without interfering with other functions of the T cells. These results were obtained at mRNA and protein levels by flow cytometry and real-time quantitative RT-PCR technique, and confirmed by chemotaxis, Northern and Western blot assays, and histological evaluation of skin grafts. The present study indicates the therapeutic potential of PNA CXCR3 to prevent acute transplantation rejection. The Journal of Immunology, 2003, 170: 1556–1565.

A acute allograft rejection remains a major problem in solid organ transplantation, because rejection may lead to either acute or chronic loss of graft function. A complex process involving both the innate and acquired immune systems results in allograft rejection. In the early stage of the allogenic response, chemokine receptors, chemokines, and adhesion molecules play essential roles, not only for leukocyte migration into the graft but also in facilitating dendritic and T cell trafficking between lymph nodes and the transplant (1–4).

Several recent studies indicate the importance of the chemokine receptors and their ligands in the allograft rejection process (1–5). For instance, the neutralization of monokine induced by IFN-γ (Mig)/CXCR3 chemokine ligand (CXCL)9 prevents graft T cell infiltration and dramatically prolongs the survival of MHC class II–as well as minor histocompatibility Ag-disparate skin allografts (5, 6). CXCR3–/– mice, which lack the receptor for the three CXC chemokines, Mig/CXCL9, IFN-γ-inducible protein 10 (γ-IP-10)/CXCL10, and IFN-inducible T cell α chemoattractant/CXCL11, display a substantial prolongation of vascularized heart allograft survival (7). Among the three chemokines mentioned above, γ-IP-10/CXCL10 is the first to be produced after transplantation (8). Likewise, mice lacking the receptor of the CC chemokine macrophage-inflammatory protein (MIP)-1/CC chemokine ligand (CCL)3 (CCR1–/– mice) either will not or will only slowly reject cardiac allografts bearing isolated MHC class II or combined MHC class I and II disparities, respectively (9). The activation of donor T cells after small bowel allotransplantation induces production of a Th1 profile of cytokines and γ-IP-10/CXCL10 that enhances infiltration of host T cells and NK cells in small bowel allografts. Blocking this pathway may be of therapeutic value in controlling small bowel allograft rejection (10). With several models of organ transplantation (skin, heart, kidney, and lung), recent data all suggest that recruitment of host leukocytes into the allograft involves chemokine-mediated pathways (11–14).

Antisense oligodeoxynucleotides represent a unique example of gene-specific drugs that can be used to selectively inhibit the expression of target genes. Second- and third-generation oligodeoxynucleotides have been synthesized that possess improved chemical characteristics regarding stability in biological fluids, cellular uptake, and

*Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, People’s Republic of China; †Department of Immunology, Medical College, Wuhan University, Wuhan, People’s Republic of China; ‡Department of Immunology, College of Jining Medical College, Jining, People’s Republic of China; §Department of Immunology, College of Basic Medical Sciences, Anhui Medical University, Hefei, People’s Republic of China

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2 M.I. and W.X. contributed equally to this work.

3 Address correspondence and reprint requests to Dr. Tan Jinquan, Department of Immunology, Tongji Medical College, Medical College, Wuhan University, Dong Hu Road 115, Wuhan 430071, Wuhan, People’s Republic of China. E-mail address: jinquan_tan@hotmail.com; or Dr. Gong Feili, Department of Immunology, Tongji Medical College, Huzhouong University of Science and Technology, Huzhouong Road 13, Wuhan 430030, People’s Republic of China. E-mail address: flgong@tjmu.edu.cn

4 Abbreviations used in this paper: Mig, monokine induced by IFN-γ; CI, chemotactic index; CsA, cyclosporin A; CXCL, CXC chemokine ligand; CCL, CC chemokine ligand; γ-IP-10, IFN-γ-inducible protein 10; MIP, macrophage-inflammatory protein; PNA, peptide nucleic acid.

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molecular specificity for the target sequence. Among the various alterations of the standard phosphodiester structure, the peptide nucleic acid (PNA) backbone is optimal in terms of specificity and affinity for the target and resistance to degradation. PNA is a structural mimic of natural nucleic acids, composed of a pseudo peptide carrying nucleobases. PNA/DNA hybrids are more stable than dsDNA. PNA is resistant to degradation caused by nucleases and proteases, and it has been shown to interfere in a sequence-specific manner with several DNA- and RNA-based processes (15–20). The therapeutic and diagnostic potentials of PNA have been attracting considerable attention with attempts at treatments, such as those for HIV infection and cancer, and of speeding diagnosis, such as for infectious diseases (15–21).

We have demonstrated that CXCR3 is a dominant factor directing T cells into mouse skin allografts. PNA CXCR3 designed by us directly blocks CXCR3 expression on T cells significantly to prolong mouse skin allograft survival, indicating the therapeutic potential of PNA CXCR3.

Materials and Methods

Animals

C57BL/6 (H-2b) mice as donors and BALB/c (H-2d) mice as receivers were obtained from Institute of Organ Transplantation (Tongji Affiliated University Hospital, Wuhan, People’s Republic of China). Adult males of 8–12 wk of age were used throughout this study.

Reagents and Abs

All recombinant mouse chemokines, eotaxin/CCL11, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, Mig/CXCL9, and γ-10-IP/CXCL10, were purchased from R&D Systems (Abingdon, U.K.). FITC-labeled anti-CD3 was purchased from DAKO (Glostrup, Denmark). PE-labeled rat anti-mouse monoclonal CCR5 (clone C34-3448) and goat anti-mouse polyclonal CCR3 (clone C34-3448) and goat anti-mouse polyclonal CCR5 (clone C34-3448) and goat anti-mouse polyclonal eotaxin/CCL11, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, Mig/CXCL9, and hamster anti-mouse CD69 (clone H1.2F3) mAbs were purchased from BD Pharmingen.

Skin transplant

Full-thickness trunk skin allografts were placed using standard techniques (22, 23). Briefly, skin was harvested from killed donor mice, the s.c. fat was removed, and the donor skin was cut into 0.8-cm² pieces. Recipient mice were anesthetized with pentobarbital and shaved around the chest and abdomen. The skin allograft was placed in a slightly larger graft bed prepared over the chest of the recipient and secured using Vaseline gauze and a bandage. By day 5, the grafts were then visually scored daily for evidence of rejection. The allograft was considered fully rejected when it was a bandage. By day 5, the grafts were then visually scored daily for evidence of rejection. The allograft was considered fully rejected when it was 50% necrotic.

Spleen T cell purification and skin graft infiltrating monocellular cell preparation

Spleen cell suspensions from recipient BALB/c (H-2b) mice were prepared and incubated at 37°C in 5% CO₂ in 10-ml plastic tissue culture flasks. After 45 min, the nonadherent cells were harvested and suspended in RPMI 1640 supplemented with 10% FCS and loaded onto a nylon wool column, and incubated for 45 min. The T cells were then eluted as described earlier (24). The purity of CD3⁺ T cells ranged from 85 to 94% as determined by flow cytometry. The skin graft was finely minced into small pieces (~0.3 mm³) with a scalpel and digested for 2 h in RPMI 1640 medium containing 30 U/ml collagenase (Sigma-Aldrich, Vallensbaek, Denmark) at 37°C, followed by culture with 0.25% trypsin-EDTA solution (Sigma-Aldrich) for 45 min. After passing the graft through a nylon sieve twice, the cell suspension was obtained in medium with 1% FCS before further experimental procedure.

Flow cytometry

As previously described (25), for detection of CCR5 or CXCR3, the spleen T cells were first incubated with a rat anti-mouse CCR5 PE-labeled mAb or goat anti-mouse polyclonal Ab CXCR3 at 5 μg/ml or 5 μg/ml matched isotype goat Ab (DAKO) in PBS containing 2% BSA and 0.1% sodium azide for 20 min, and then washed twice in staining buffer. For CXCR3 detection, secondary swine anti-goat IgG PE-labeled mAb (DAKO) was subsequently added. The cells were then incubated with a goat anti-mouse CD3 (DAKO), rat anti-mouse CD4, CD8, CD14, or CD19 FITC-labeled mAb (BD PharMingen) at 5 μg/ml in PBS containing 2% BSA and 0.1% sodium azide for 20 min, and then washed twice. For detection of CD25, CD45RB, or CD69, the first Ab was chased with matched isotype Ab at 5 μg/ml and added and washed twice, and then an appropriate PerCP-labeled secondary Ab was added. All procedures were conducted at 4°C. The cells were then fixed with 1% paraformaldehyde. The analyses were performed with a flow cytometer (Coulter XL; Coulter, Miami, Florida).

Real-time quantitative RT-PCR assay

Real-time quantitative RT-PCR were performed as described elsewhere (26–28). Briefly, total RNA was purified from purified spleen T cells (1 × 10⁶, purify > 99%, by positive selection of CD3⁺ Dynabeads) or skin grafts. Total RNA was prepared by using Quick Prep Total RNA Extraction kit (Pharmacia Biotech, Upsala, Sweden). The RNA was reverse transcribed by using oligo (dT)₁₂₋₁₈ and Superscript II reverse transcriptase (Life Technologies, Grand Island, NY). Reversion transcription was performed for 60 min at 37°C, and any potential contaminating protein was denatured by incubation for 10 min at 95°C. The real-time quantitative PCR was performed in standard 96-well microplate format (Applied Biosystems, Foster City, CA) with ABI PRISM 7700 Sequence Detector Systems (Applied Biosystems). By using SYBR Green PCR Core Reagents kit (PiN 4304886; Applied Biosystems), fluorescence signals were generated during each PCR cycle by the fluorescence dye to observe the specific reactions of each primer: CCR3 sense, 5'-CTTTTGAGACCACACCTAATGAG-3' and CCR3 antisense, 5'-GACCCCATGCTTACCTGAATG-3'; CXCR3 sense, 5'-AGGCGATTAGCGACAGCAG-3' and CCR5 antisense, 5'-TTCCTCAACAAAGGCTATAGTGA-3'; CCR3 sense, 5'-CAGGCCTGACCTTACGACA-3' and CXCR3 antisense, 5'-GCACGCCCGACGAGAAGA-3'; eotaxin/CCL11 sense, 5'-GGCTGACCTTAAACTCTACAGA-3' and eotaxin/CCL11 antisense, 5'-ACATTGTCTGTTGGTGTCGAT-3'; MIP-1α/CCL3 sense, 5'-GATCTGGCCGTATCCAAAGA-3' and MIP-1α/CCL3 antisense, 5'-CCAAAGACTCTACGACATCGA-3'; MIP-1β/CCL4 sense, 5'-TGCTGGTGTCGGCTTCTC-3' and MIP-1β/CCL4 antisense, 5'-CGAGGAATGGGAGGATCAGA-3'; RANTES/CCL5 sense, 5'-GCAAGTGCTCCAACTTGCA-3' and RANTES/CCL5 antisense, 5'-GATTATTTGTTGACCACTTCTTCTC-3'; γ-IP-10/CXCL10 sense, 5'-GGACGCTTGGGCGCTGCAA-3' and γ-IP-10/CXCL10 antisense, 5'-GCTTCCCTATGGFCCCTCATT-3' and Mig/CXCL9 sense, 5'-TTTCTTGTGTTGCAATCTCATTT-3' and Mig/CXCL9 antisense, 5'-AGCATCGTGCATCTTCCTCATT-3'.

An unknown cDNAs were diluted to contain equal amounts of β-actin cDNA. The standards, "no template" controls, and unknown samples were added in a total volume of 50 μl per reaction. PCR retain conditions were 2 min at 50°C, 10 min at 95°C, and 40 cycles with 15 s at 95°C and 60 s at 60°C. Potential PCR product contamination was digested by uracil-N-glycosylase, because dTTP is substituted by dUTP (26). In the reaction system, uracil-N-glycosylase and AmpliTaq Gold (Applied Biosystems) were applied according to the manufacturer’s instructions (26, 27).

Northern and Western blot assays

For mRNA detection (Northern blot), 5 μg of total RNA from each sample were electrophoresed under denaturing conditions, followed by blotting onto Nytran membranes, and cross-linked by UV irradiation as previously described (29). CCR3 and CXCR3 cDNA probes, labeled by α-[³²P]dCTP, were obtained by PCR amplification of the sequence mentioned above from total RNA from total spleen cells from normal adult mice. The membranes were hybridized overnight with 1 × 10⁶ cpm/ml of ³²P-labeled probe, followed by intensive washing with 0.2× SSC (1× SSC: 0.15 M NaCl and 0.015 M sodium citrate (pH 7.0)) and 0.1% SDS before being autoradiographed. For protein detection (Western blot), the transplanted skin tissues were excised from recipients and snap frozen. After extraction and precipitation, the extracts were resuspended in buffer as previously described (30). Extracts were centrifuged at 10,000 rpm for 5 min at 4°C. Protein concentration was measured by Bio-Rad (Hercules, CA) protein assay. Total protein (60 μg) was loaded onto 16% SDS-PAGE, transferred onto polyvinylidene difluoride membranes after electrophoresis, and incubated with the CCR5 or CXCR3 Ab (0.5 μg/ml). Analyses were conducted using ECL detection (Amersham Pharmacia Biotech, Little Chalfont, U.K.).
Histological evaluation of skin grafts

Skin allografts were harvested from killed recipient mice at time intervals as indicated for histological analysis. For conventional histological evaluation of skin (H&E staining), allografts were fixed with 10% formalin, and paraffin-embedded sections were stained with H&E for 3 min each. For immunohistology, a portion of the skin tissue was placed in OCT compound frozen in liquid nitrogen for immunohistological studies. Sections (8 μm) were dried overnight, fixed in acetone for 10 min, air-dried, and then immersed in PBS for 8 min and then in 0.03% H₂O₂ for 8 min to eliminate endogenous peroxidase activity. The Abs were diluted to 5 μg/ml in 0.05% Tris-HCl buffer with 2% BSA. The slides were then stained for 4 h at room temperature. Control slides were incubated with appropriate isotope Abs as the primary Ab. After three washes in PBS of 5 min each, slides were incubated for 20 min at room temperature with the appropriate biotinylated secondary Ab diluted 1/200 in PBS, followed by three washes in PBS, and then slides were incubated with peroxidase-labeled streptavidin-horseradish peroxidase (DAKO) for 30 min at room temperature. The freshly prepared substrate-chromagen solution with 12 μl 30% H₂O₂ added was applied to each slide and incubated for ~5 min at room temperature. After a final wash in water, slides were counterstained with hematoxylin, rinsed, and immersed in 37 mM NH₄OH for 10 s. All slides were viewed and evaluated in a blinded fashion by a qualified dermatopathologist.

Animal treatment with PNA antisense

All PNA s were purchased from Applied Biosystems. The sequences of PNA antisense were as follows: PNA CCR5 antisense, 5′-NH₂-CCGTTCGTACCTTTCOOH-3′; PNA CXCR3 antisense, 5′-NH₂-ACTTGAAGGCTTTTGCG-3′; and PNA mismatch sequence, NH₂-TTCCAGCTGTCTTTCOOH (randomly sanitized).

Mice spontaneously received 10 mg/kg (unless other dosages were indicated) daily of PNA CCR5 antisense, PNA CXCR3 antisense, mismatch PNA, or DNA CXCR3 antisense (with identical sequence) via i.v. injections. The treatments were started 5 days before transplantations took place. Another group of animals received either normal physiological saline or cyclosporin A (CsA; 1 mg/kg) daily injections i.v. as negative or positive controls. All treatments lasted until the results were obtained or animals were sacrificed.

Chemotaxis assay

The chemotaxis assay was performed in a 48-well microchamber (Neuro Probe, Bethesda, MD) (31). Briefly, chemokines were diluted in RPMI 1640 with 1% FCS and placed in the lower wells (25 μl). Twenty-five milliliters of the cell suspension were added to the upper well of the chamber, which was separated from the lower well by a 5-μm pore size, polycarbonate, polycylnylrylidene-free membrane (Nucleopore, Pleasanton, CA). The chamber was incubated for 60 min at 37°C in an atmosphere containing 5% CO₂. The membrane was then carefully removed, fixed in 70% methanol, and stained for 5 min in 1% Coomassie brilliant blue. The cells that migrated and adhered to the lower surface of the membrane were counted using a light microscope. Approximately 10% of the cells will migrate spontaneously (known as migrating cells on negative control) (32), corresponding to between 300 and 400 cells. The results were expressed as chemotactic index (CI), that is, the ratio between the numbers of migrating cells in the sample and in the medium control (31, 33), with SD.

T cell proliferative responses

Spleen T cell proliferative responses were assessed by culturing responder and Con A mitogen (1.25–10 μg/ml; Sigma-Aldrich) in RPMI 1640 medium containing 5% FBS, 1% penicillin/streptomycin, and 5 × 10⁻⁴ M 2-ME in a 96-well plate (9). Cultures were incubated at 37°C in 5% CO₂ for 3 days and were pulsed with [³H]thymidine for 6 h before harvesting; mean cpm and SD were calculated using 12 wells/group.

Statistical analysis

For pairwise comparisons, the groups were analyzed using a t test, and for multiple comparisons, a one-way ANOVA with Dunnett’s method, setting the saline group as the control, was used. In both cases, p < 0.05 was considered significant.

Results

CXCR3 is a dominant factor directing T cells into mouse skin allografts

We analyzed the chemokine receptor expression in spleen T cells from mice at different time intervals after transplantation. There rarely were CCR5⁺ cell fractions in freshly isolated spleen CD3⁺ T cells (<8%) (Fig. 1A). After 3 days of allograft transplantation, there was a significant increase in the CCR5⁺ cell fraction in spleen T cells (57%), and there were 60% by day 5 and 66% by day 7. CXCR3 was also rarely expressed in freshly isolated CD3⁺ spleen T cells (<10%) (Fig. 1B). There were 41% of CXCR3⁺ cell fractions in spleen T cells after 3 days of allograft transplantation, and there were 79% by day 5 and 98% by day 7. In phenotypical characterization of activated/memory subsets of CXCR3-bearing spleen T cells, we detected by day 7 after transplantation that CD25 and CD69 expressions significantly increased compared with those of the cells from native mice (from 5 to 58% and from 6 to 62%, respectively), whereas CD45RB did not significantly change (from 6 to 7%).

CCR5 mRNA was detected at a low level in freshly isolated CD3⁺ spleen T cells (~0.6 × 10³ copies/50 ng cDNA). After allograft transplantation, CCR5 mRNA expression slightly increased in the cells. By days 3, 5, and 7, there were ~0.8 × 10³, 1.0 × 10³, and 1.8 × 10³ copies/50 ng cDNA, respectively (Fig. 2A). CXCR3 mRNA expression after allograft transplantation was significantly increased. CXCR3 mRNA was detected at a low level in freshly isolated CD3⁺ spleen T cells (~0.5 × 10³ copies/50 ng cDNA). By days 3, 5, and 7, there were ~2.2 × 10³, 4.5 × 10³, and 3.5 × 10³ copies/50 ng cDNA in the cells, respectively (Fig. 2B). The changes of CCR5 and CXCR3 mRNA expressions had also been confirmed by Northern blot. In the upper panels, freshly isolated CD3⁺ spleen T cells expressed CCR5 and CXCR3 mRNA at low levels, whereas CXCR3 mRNA was significantly increased by days 3, 5, and 7 after transplantation (Fig. 2D), and CCR5 mRNA was slightly increased (Fig. 2C). The lower panels show

![FIGURE 1.](http://www.jimmunol.org/)}
that comparable total RNA amounts from different days were added. CCR3 mRNA was expressed at a low level in freshly isolated CD3^+ spleen T cells. It was not changed by days 3, 5, and 7 after allograft transplantation in the cells (data not shown). We also observed a similar pattern of CCR5 and CXCR3 expression in allografts at the different time intervals after allograft transplantation by real-time quantitative RT-PCR assay and Northern blots (data not shown).

We further investigated the mRNA expression of different CC and CXC chemokines in the grafts at different time intervals after allograft transplantation. We measured the following panel of chemokines: eotaxin/CCL11 (a ligand for CCR3), RANTES/CCL5 (a ligand for CCR3 and CCR5), MIP-1α/CCL3 (a ligand for CCR3), MIP-1β/CCL4 (a ligand for CCR5), γ-IP-10/CXCL10 (a ligand for CCR3), and Mig/CXCL9 (a ligand for CXCR3). All chemokine mRNAs were detected at low levels in mouse skin (~0.5 × 10^3 copies/50 ng cDNA) (Fig. 3). After allograft transplantation, the eotaxin/CCL11, RANTES/CCL5, and MIP-1β/CCL4 mRNA expressions were significantly changed in the skin grafts at days 3, 5, and 7 (~0.8 × 10^3 copies/50 ng cDNA) (Fig. 3, A, B, and D). MIP-1α/CCL3 mRNA expression was slightly increased in the grafts at days 3, 5, and 7 (~1.7 × 10^3 copies/50 ng cDNA) (Fig. 3C). γ-IP-10/CXCL10 and Mig/CXCL9 mRNA expressions were highly up-regulated in the grafts at days 3, 5, and 7 (~2.5 × 10^3 copies/50 ng cDNA) (Fig. 3, E and F).

There were only a few lymphocytes nesting s.c. in skin allografts in H&E staining (Fig. 4A). These cells were expressing almost no CCR5 (Fig. 4B) or CXCR3 (Fig. 4C). By day 3 (data not shown), day 5 (data not shown), and day 7 after transplantation, H&E-stained sections of allografts showed widespread s.c. necrosis and leukocyte infiltration (Fig. 4D). Most of the infiltrating leukocytes were T cells, identified morphologically. Immunohistologically, CCR5 was moderately expressed (Fig. 4E), whereas CXCR3 was highly expressed in infiltrating cells (Fig. 4F).

To quantitatively characterize the subpopulation of infiltrating cells that were expressing CXCR3, we analyzed the cells from the allograft at different time intervals after transplantation. There rarely were CXCR3-positive CD4^+ T cells (0.5%), CD8^+ T cells (1.2%), CD19^+ B cells (0.4%), and CD14^+ monocytes (0.3%) in native skin biopsies (Fig. 5A). At days 3 and 5 after allograft transplantation, CXCR3^+ cell fractions significantly increased in the infiltrating CD4^+ and CD8^+ T cells, but not in the CD19^+ B cells and CD14^+ monocytes (data not shown). CXCR3 was highly expressed on CD4^+ (38%) and CD8^+ (31%) T cells from the grafts at 7 days after transplantation, whereas the expression was not significantly changed on CD19^+ B cells (1.2%) and CD14^+ monocytes (2.5%) (Fig. 5B). CD25 and CD69 expression on CXCR3-expressing graft-infiltrating CD3^+ T cells from the grafts also significantly increased, compared with the cells from native skin biopsy, whereas CD45RB did not significantly change (data not shown).

We examined the abilities of eotaxin/CCL11, RANTES/CCL5, MIP-1α/CCL3, MIP-1β/CCL4, γ-IP-10/CXCL10, and Mig/CXCL9...
PNA CXCR3 antisense regulates progression of acute skin allograft rejection in mice

The findings mentioned above led us to investigate the effects of CXCR3 PNA antisense on progression of acute skin allograft rejection in mice. After a number of pioneer experiments in vitro and in vivo (data not shown), we designed and selected CCR5 and CXCR3 PNA sequences to treat mouse skin allograft transplantation in vivo. The high dosage (10 mg/kg/day) of PNA CXCR3 significantly prolonged mouse skin allograft survival compared with the treatment with normal physiological saline, whereas the low dosage (0.5 mg/kg/day) failed to prolong mouse skin allograft survival (Table 1). The high dosage (10 mg/kg/day) of PNA CCR5 marginally but significantly prolonged mouse skin allograft survival, whereas low dosage (0.5 mg/kg/day) had no such effect. Meanwhile, PNA mismatch and DNA CXCR3 did not have any effects on prolonging mouse skin allograft survival. PNA CXCR3 and PNA CCR5 together (each 10 mg/kg/day) seem to be more effective. Without any treatment, skin allografts in recipients were rejected between days 6 and 8 (Fig. 7). In contrast, significant prolongation of allograft survival up to day 18 was observed in recipients treated with the PNA CXCR3 at a dosage of 10 mg/kg/day.

To explore the mechanism of the observation mentioned above, we analyzed the surface chemokine receptor expression on spleen T cells from mice that received different treatments by day 7 after skin allograft transplantation. The PNA CCR5 significantly blocked the expression of CCR5 in spleen CD3 + T cells (<5%), whereas there was a considerable number of CCR5 + fractions in spleen T cells from animals treated with normal physiological saline (71%) and from animals treated with mismatched PNA (68%) (Fig. 8A). CXCR3 was also rarely expressed in CD3 + spleen T cells from animals treated with PNA CXCR3 (<5%), whereas there were 96 and 89% of CXCR3 + cell fractions from animals treated with normal physiological saline and with mismatched PNA, respectively (Fig. 8B). PNA CXCR3 significantly and indirectly reduced CD25 (to 6%) and CD69 (to 5%) but not CD45RB (to 6%) expression in activated/memory subsets of CXCR3-bearing spleen T cells compared with the cells from untreated mice, whereas PNA CCR5, PNA mismatch, and normal physiological saline did not (data not shown).

CCR5 mRNA was detected at a considerable amount in CD3 + spleen T cells from animals treated with normal physiological saline by day 7 (~1.6 × 10 3 copies/50 ng cDNA). There were only ~0.2 × 10 3 copies/50 ng cDNA for CXCR5 mRNA in CD3 + spleen T cells from animals treated with PNA CCR5, but ~1.2 × 10 3 copies/50 ng cDNA for CXCR5 mRNA in CD3 + spleen T cells from animals treated with PNA CXCR3, and 1.6 × 10 3 copies/50 ng cDNA in the cells from animals treated with mismatched PNA (Fig. 9A). PNA CXCR3 significantly inhibited CXCR3 mRNA expression. There were a few copies of CXCR3 mRNA in CD3 + spleen T cells from animals treated with PNA CXCR3 by day 7 (~0.1 × 10 3 copies/50 ng cDNA). There were ~3.8 × 10 3 copies/50 ng cDNA for CXCR3 mRNA in CD3 + spleen T cells from animals treated with normal physiological saline, ~2.1 × 10 3 copies/50 ng cDNA for CXCR3 mRNA in the cells from animals treated with PNA CCR5, and ~6.0 × 10 3 copies/50 ng cDNA in the cells from animals treated mismatched PNA (Fig. 9B). The changes of CCR5 and CXCR3 mRNA expressions in CD3 + spleen T cells had also been confirmed by Northern blot (Fig. 9, C and D). In the upper panels, CCR5 and CXCR3 mRNA expressed at low level, indicating that PNA CCR5 and PNA CXCR3 significantly and specifically inhibit CCR5 and CXCR3 mRNA expression in the respective treatments. The lower panels show that comparable total RNA amounts from different days were added.

There were only a few leukocytes infiltrating s.c. and skin structures were almost normal anatomically at day 7 after allograft transplantation from PNA CXCR3-treated animals (Fig. 10Aa). Immunohistologically, these cells were expressing a few CCR5 (Fig. 4). Histological analyses of native skin from C57BL/6 (H-2 b ) donor mice (A–C) vs skin allografts (D–F) from BALB/c (H-2 d ) recipients. Allografts were harvested at day 7 after transplantation and fixed with 10% buffered formalin. Sections were prepared and stained by H&E staining (A and D) as described in Materials and Methods. Frozen sections were prepared and stained with anti-CCR5 mAb (B and E; b and e are isotype Ab controls) or anti-CXCR3 pAb (C and F; c and f are isotype Ab controls) as described in Materials and Methods. The positively staining cells in representative areas of the slides are shown in brown color. Magnification: A and D, ×100; B, C, E, and F, and b, c, e, and f, ×200.

FIGURE 4. Histological analyses of native skin from C57BL/6 (H-2 b ) donor mice (A–C) vs skin allografts (D–F) from BALB/c (H-2 d ) recipients. Allografts were harvested at day 7 after transplantation and fixed with 10% buffered formalin. Sections were prepared and stained by H&E staining (A and D) as described in Materials and Methods. Frozen sections were prepared and stained with anti-CCR5 mAb (B and E; b and e are isotype Ab controls) or anti-CXCR3 pAb (C and F; c and f are isotype Ab controls) as described in Materials and Methods. The positively staining cells in representative areas of the slides are shown in brown color. Magnification: A and D, ×100; B, C, E, and F, and b, c, e, and f, ×200.
FIGURE 5. Double color flow cytometric analysis of the distribution and regulation of CXCR3 on skin graft-infiltrating cells. The infiltrating mononuclear cells were prepared as described in Materials and Methods from either native skin of BALB/c (H-2\textsuperscript{b}) mice or allografts from recipients (B) after transplantation for 7 days. The graphs are showing CXCR3\textsuperscript{+} and CD4\textsuperscript{+} (a), CD8\textsuperscript{+} (b), CD19\textsuperscript{+} (c), or CD14\textsuperscript{+} (d) cells after gating within infiltrating mononuclear cells. The graphs (e) are isotype controls. The procedure for cell staining was described in Materials and Methods. The percentages of CXCR3\textsuperscript{+} cells are indicated in the graphs. Data are from a single experiment, which is representative of five similar experiments performed.

10ab) and seldom CXCR3 (Fig. 10Ac). The results from Northern blots (Fig. 10, B and C) and Western blots (Fig. 10, D and E) also indicated that PNA CCR5 and PNA CXCR3 significantly and specifically inhibit CCR5 and CXCR3 mRNA and protein expression in the skin grafts. We observed the same pattern of CCR5 and CXCR3 mRNA expression in allografts from animals receiving different treatments using real-time quantitative RT-PCR assays as that seen in the CD3\textsuperscript{+} spleen T cells (data not shown). Quantitative characterization of CXCR3 expression in the subpopulation of infiltrating mononuclear cells from allografts at day 7 after transplantation in PNA CXCR3-treated animals showed that CXCR3 expression was totally abolished on CD4\textsuperscript{+} T cells (0.4%), CD8\textsuperscript{+} T cells (0.3%; Fig. 10F), CD19\textsuperscript{+} B cells, and CD14\textsuperscript{+} monocytes (data not shown). This phenomenon was not seen in the PNA CCR5-, PNA mismatch- and normal physiological saline-treated animals (data not shown). PNA CXCR3 significantly and indirectly reduced CD25 and CD69 but not CD45RB expressions in CXCR3-expressing graft-infiltrating CD3\textsuperscript{+} T cells, whereas PNA CCR5, PNA mismatch, and normal physiological saline did not (data not shown).

Compared with the results shown in Fig. 3, the different treatments had not changed the chemokine expression patterns (eotaxin/CCL11, RANTES/CCL5, MIP-1\textalpha/CCL3, MIP-1\beta/CCL4, \gamma-IP-10/CXCL10, and Mig/CXCL9) in the allografts from the animals that received different treatments by day 7 after transplantation, despite PNA CXCR3 having an obvious effect of prolonging skin allograft survival (data not shown). This leads us to interpret that PNA CXCR3 regulate the progression of skin allograft rejection by means of a blockade of CXCR3 expression directing T cells into grafts, but without change in chemokine ligand expression.

PNA CCR5 and PNA CXCR3 significantly and specifically inhibit the chemotactic migration of spleen T cells toward MIP-1\alpha/CCL3 and RANTES/CCL5, and toward \gamma-IP-10/CXCL10 and

FIGURE 6. The migration of CD3\textsuperscript{+} spleen T cells toward MIP-1\alpha/CCL3 (A), RANTES/CCL5 (B), \gamma-IP-10/CXCL10 (C), or Mig/CXCL9 (D). The spleen CD3\textsuperscript{+} T cells were from either native BALB/c (H-2\textsuperscript{b}) mice or BALB/c (H-2\textsuperscript{b}) recipients that received allograft transplantation for 3 (C), 5 (A), or 7 (D) days. All results were determined as described in Materials and Methods, expressed as CI \pm SD, and based on triplicate determination of chemotaxis on each concentration of chemokines indicated. The illustrated data are from a single representative experiment of six experiments performed. * Significant differences between native vs allograft-transplanted animals. All p < 0.05.

Table I. The survival time of mouse skin allograft in response to different treatments\textsuperscript{*}

<table>
<thead>
<tr>
<th>Treatment\textsuperscript{b}</th>
<th>Dosage (mg/kg/day)</th>
<th>Survival time\textsuperscript{c} (days SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal physiological saline</td>
<td>—\textsuperscript{d}</td>
<td>7.5 \pm 0.7</td>
</tr>
<tr>
<td>PNA CCR5</td>
<td>0.5\textsuperscript{e}</td>
<td>7.6 \pm 1.5</td>
</tr>
<tr>
<td>PNA CCR5</td>
<td>10\textsuperscript{f}</td>
<td>10.7 \pm 1.1*</td>
</tr>
<tr>
<td>PNA CXCR3</td>
<td>0.5</td>
<td>8.8 \pm 2.2</td>
</tr>
<tr>
<td>PNA CXCR3</td>
<td>10</td>
<td>17.1 \pm 2.4**</td>
</tr>
<tr>
<td>PNA CCR5 plus PNA CXCR3</td>
<td>10 each</td>
<td>19.9 \pm 2.8**</td>
</tr>
<tr>
<td>PNA mismatch</td>
<td>10</td>
<td>7.2 \pm 0.8</td>
</tr>
<tr>
<td>DNA CXCR3</td>
<td>10</td>
<td>7.4 \pm 0.5</td>
</tr>
<tr>
<td>CsA</td>
<td>1</td>
<td>20.4 \pm 1.5**</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Dosage of 0.5 mg/kg/day was considered low and one of 10 mg/kg/day was considered high.
\textsuperscript{b} All treatments started 5 days prior to the allograft transplantation taking place (n = 15 for each group of BALB/c (H-2\textsuperscript{b}) recipients).
\textsuperscript{c} Survival time was determined as described Materials and Methods.
\textsuperscript{d} Normal physiological saline was applied in equal volume to active PNA treatment.
\textsuperscript{e} Effect of PNA treatment on mouse skin allograft rejection.
\textsuperscript{f} p < 0.05.
\textsuperscript{g} p < 0.005.
**Discussion**

Allograft rejection is mediated to a significant degree by the influx of monocytes and T cells (2). The induction of chemokines by "stressed" tissues during allograft rejection is accompanied by the infiltration of leukocytes bearing the respective chemokine receptors (2). The chemokine receptors expressed by different T cell populations have been linked to Th1/Th2-like effector responses of receptor-expressing cells (3). Allograft rejection is thought to be primarily the result of a Th1 type immune response. The Th1 effector cells often express CXCR3 and CCR5, while Th2 cells can express CCR3, CCR4, and CCR8 (2). In particular, recent studies from different independent groups have documented that CXCR3 and the CCR5 ligands γ-IP-10/CXCL10 and Mig/CXCL9 were most significantly associated with acute allograft rejection in heart (7, 8, 34, 35), lung (36), kidney (13) and liver (37). CCR5 was also found to be highly expressed on infiltrating mononuclear cells during allograft rejection (12, 13, 38). In the present study, we have demonstrated that CXCR3 is a dominant factor directing T cells into skin allografts during the process of rejection. To our knowledge, this is the first report on CXCR3 overexpression on mouse spleen T cells induced by skin allograft transplantation, and provides direct evidence of the biological activity of T cells to participate in acute skin graft rejection via CXCR3 and its ligands γ-IP-10/CXCL10 and Mig/CXCL9. Thus, a rather complex picture is now beginning to take shape of how T cells selectively enter skin allografts and affect the process of skin allograft rejection by means of interfering with the T cell chemotaxis toward γ-IP-10/CXCL10 and Mig/CXCL9, which is the result of blockade of CXCR3 expression in the cells. PNA mismatch does not affect chemotactic migration of CD3+ spleen T cells after allograft transplantations.

The proliferative responses of spleen T cells from animals that received different treatments after allograft transplantation were assessed. The spleen T cells from the animals that received PNA CCR5 and PNA CXCR3 treatments showed identical mitogen-induced cell proliferation to that of animals receiving saline treatment, whereas mitogen-induced cell proliferations in animals that received CsA treatment were significantly inhibited (Fig. 12), implying that PNA CXCR3 only inhibit CXCR3 expression in T cells to regulate the process of skin allograft rejection but do not interfere with other T cell functions such as proliferation. In this case, CsA does affect the proliferation of T cells.

**Materials and Methods.** The procedures for cell purification and total RNA isolation were described in Materials and Methods. The hybridization signals for CCR5 or CXCR3 mRNA from different cells are shown in upper panels. The 28S rRNAs in lower panels confirm comparable amounts of loaded total RNA.
different transplanted organs, nest on sites, are further activated, and transmigrate to final destinations to cause physiological and pathophysiological events under continuous interaction with chemokines and cytokines.

Most studies in the transplantation field have so far concentrated on chemokines rather than their receptors and on rather few chemokines. Whether they are in fact playing a biologically significant role and whether their inhibition by any of several strategies would be of actual therapeutic value are largely unknown (1). Tools are now becoming available to analyze these roles in a meaningful manner, including commercially available multiprobe RNA protecting assays, anti-chemokine and anti-chemokine-receptor mAbs, and gene-knockout animals. It is a reasonable expectation that the fundamental significance of chemokine receptors and their ligands for therapeutic targeting will become available from experimental systems (5–9). However, many data from in vitro experiments demonstrating the presence of multiple ligands for a given chemokine receptor, and often multiple receptors for a given chemokine, have led to concerns of biologic redundancy (1). The biologic redundancy of chemokine receptors led us to consider the selection of target chemokine receptor regarding the treatment of allograft rejection. In the present study, we have found that CXCR3 is highly up-regulated in spleen T cells and allografts from BALB/c recipients by day 7 after receiving transplantation, whereas CCR5 expression is moderately increased at mRNA and protein levels. This finding could be a meaningful event along the lines of accumulating knowledge of the roles of chemokines and chemokine receptors in allograft rejection and providing important insight into the physiological and pathophysiological processes under continuous interaction between host and allografts.

Recently developed PNAs, which are synthetic homologs of nucleic acids in which the phosphate-sugar polynucleotide backbone...
All results were determined as described in Materials and Methods, expressed as mean ± SD, and based on triplicate determination of chemotaxis on each concentration of chemokines indicated. The illustrated data are from a single representative experiment of six performed. * Significant differences between native vs allograft-transplanted animals. All p < 0.05.

is replaced by a flexible polyamide, allow the formation of a PNA-DNA hydrogen-bonded double helix, which is more stable than the one formed by DNA-DNA interaction (39). PNAs are resistant to nucleases and proteases (40) and consequently are more stable in one formed by DNA-DNA interaction (39). PNAs are resistant to one formed by DNA-DNA interaction (39). Though potentially capable of blocking gene expression in a selective and specific manner (41), PNAs have never been shown to be effective antigenic agents in intact live cells in culture because of their limited ability to reach cell nuclei (42). Our results from flow cytometry, Northern blots, and Western blots are demonstrating that PNA CXCR3 significantly and specifically inhibit CXCR3 mRNA and protein expression in the spleen T cells and skin grafts. The prolongation effect on skin allograft survival of PNA CXCR3 is obviously better than that of PNA CXCR5 in the animal experiment, indicating that the mechanism of prolongation of skin allograft survival is that PNA CXCR3 directly blocks the CXCR3 expression in T cells, which is responsible for directing T cells into skin allograft to induce acute rejection. Taking a number of previous observations into account (43), these indications of the potential of PNA CXCR3 to prevent acute transplantation rejection in the present study could lead to exciting new therapeutic approaches.

In summary, we have demonstrated that CXCR3 is a dominant factor directing T cells into mouse skin allografts. CXCR3 PNA antisense significantly prolongs mouse skin allograft survival, indicating the therapeutic potential of PNA CXCR3 to prevent acute transplantation rejection.

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References


