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

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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 (15 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.

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)7/CXC 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 allotransplantation 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

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

3. 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 synthetic 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 cancers, 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 reconstituent mouse chemokines, eotaxin/CCL11, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, Mlg/CXCL9, and γ-IP-10/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 C54-3448) and goat anti-mouse polyclonal CXCR3 (clone SC-6226) were purchased from BD PharMingen (San Diego, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Rat anti-mouse CD25 (clone 3C7), CD45RB (clone 16A), and hamster anti-mouse CD69 (clone H.12F3) 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 grafted skin was scored daily for evidence of rejection. The allograft was considered fully rejected when it was >50% necrotic and confirmed histologically.

Spleen T cell purification and skin graft infiltrating mononuclear cell preparation

Spleen cell suspensions from recipient BALB/c (H-2d) 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 using RPMI 1640 supplemented with 10% FCS and loaded onto a nylon wood column, and incubated for 45 min. The T cells were then eluted as described earlier (16). The purity of CD3⁺ T cells was >99% by immunofluorescence analysis on the cell suspension (15).

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 incubated matched isotype Ab at 5 µg/ml was 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, purity >99%), by positive selection of CD3⁺ T lymphocyte 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). Reverse 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 special 96-well microplate (Applied Biosystems, Foster City, CA) with ABI PRISM 7700 Sequence Detector Systems (Applied Biosystems). By using SYBR Green PCR Core Reagents kit (P/N 4304886; Applied Biosystems), fluorescence signals were generated during each PCR cycle via the 5’ to 3’ endonuclease activity of AmpliTaq Gold (26) to provide real-time quantitative PCR information. The target genes were generated by connecting the following sequences of the specific primers: CCR3 sense, 5'-GGCTTGGACGACACCACTTGAAG-3' and CCR3 antisense, 5'-GACCCCGACCTTCTGATGCA-3'; CCR5 sense, 5'-AGGCCATGCGGCAACAG-3' and CCR5 antisense, 5'-TTCCTCAACAAAAAGGCATAGTA-3'; CXCR3 sense, 5'-CAGCCTGGAAC TTCAGAGAAGCACTC-3' and CXCR3 antisense, 5'–GCAGCCCGCCAGCA AGAAGA-3'; eotaxin/CCL11 sense, 5'-GGCCGTACCTCAAACTCATCGAA AA-3' and eotaxin/CCL11 antisense, 5'-ACATTTCTGGGTTCGGATGT-3'; MIP-1α/CCL3 sense, 5'-GATCGTGGCTGACTCAGAAAG-3' and MIP-1α/CCL3 antisense, 5'–CCAGAGACTCTCCAGCGATCGT-3'; MIP-1β/CCL4 sense, 5'–TGCTGGTGCGGTCTCCAGA-3' and MIP-1β/CCL4 antisense, 5'-CAGGGAATGGGAGGCTGAGA-3'; RANTES/CCL5 sense, 5'-GCAAGTCTCACAATTTGG-3' and RANTES/CCL5 antisense, 5'-GATGTATTTGCTGACACTTTCTC-3'; γ-IP-10/CXCL10 sense, 5'-GGACGGTTCGCGTCAAA-3' and γ-IP-10/CXCL10 antisense, 5'-GCTTCTCCATGAGGCTCCAT-3'; and Mig/CXCL9 sense, 5'-TTTCTCCTTTGGGCCATCATT-3' and Mig/CXCL9 antisense, 5'-AGCATCTGGCATCCTCATT-3'. All 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 was electrophoresed under denaturing conditions, followed by blotting onto Nytran membranes, and cross-linked by UV radiation as previously described (29). CCR3 and CXCR3 cDNA probes, labeled with 32P-dCTP, were obtained by PCR amplification of the sequence mentioned above from total RNA from normal 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 transfected 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.).

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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 kept 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 isotype 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 the slides were incubated with peroxidase-conjugated streptavidin-HRP (DAKO) for 30 min at room temperature. The freshly prepared substrate-chromogen 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 PNAs were purchased from Applied Biosystems. The sequences of PNAs antisenses were as follows: PNA CCR5 antisense, 5′-NH2-CCGTCTGCAGTTT-COOH-3′; PNA CXCR3 antisense, 5′-NH2-ACTGCCTGGTTTTCG-COOH-3′; and PNA mismatch sequence, NH2-TTCTCCAGCTGGTTT-COOH (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 (freshly isolated spleen CD3+ cells) at 1 × 10^6 cells/ml was added to the upper well of the chamber, which was separated from the lower well by a 5-μm pore size, polycarbonate, polyvinylpyrrolidone-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 proliferation 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^-3 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 × 10^3 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 × 10^3, 1.0 × 10^3, and 1.8 × 10^3 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 × 10^3 copies/50 ng cDNA). By days 3, 5, and 7, there were ~2.2 × 10^3, 4.5 × 10^3, and 3.5 × 10^3 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...
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 CXCR3), and Mig/CXCL9 (a ligand for CXCR3). All chemokine mRNAs were detected at low levels in mouse skin (~0.5 × 10³ 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 × 10³ 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 × 10³ 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 × 10³ 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 7 (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. Immunohistochemically, 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 to recruit T cells into the skin grafts. The graphs shown are representative of four similar experiments.
to induce spleen T cell chemotaxis after allograft transplantation, because CCR5 and CXCR3 overexpression was found in these cells after transplantation. MIP-1α/CCL3, RANTES/CCL5, γ-IP-10/CXCL10, and Mig/CXCL9 induce a very weak chemotactic migration in freshly isolated native CD3+ spleen T cells (CI of ~1.8 ± 0.3) (Fig. 6). The chemotaxis toward MIP-1α/CCL3 (Fig. 6A) and RANTES/CCL5 (Fig. 6B) was slightly increased by day 5 after allograft transplantations (CI of ~2.5 ± 0.5) and returned to native level by day 7 (CI of ~1.9 ± 0.3). The chemotaxis toward γ-IP-10/CXCL10 (Fig. 6C) and Mig/CXCL9 (Fig. 6D) was highly increased. The increase started by day 3 after allograft transplantation (CI of ~2.7 ± 0.6) and persisted by day 5 (CI of ~4.1 ± 0.4) and day 7 (CI of ~4.2 ± 0.5) after transplantations. The eotaxin/CCL11 induces no significant chemotaxis of spleen T cells (data not shown). The pattern of chemotaxis toward MIP-1β/CCL4 (data not shown) was similar to that toward RANTES/CCL5 and MIP-1α/CCL3.

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 I). 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 CCR5 mRNA in CD3+ spleen T cells from animals treated with PNA CCR5, but ~1.2 × 10^3 copies/50 ng cDNA for CCR5 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 4.0 × 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 structure was 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. 10Ab).
CCR5-, PNA mismatch- and normal physiological saline-treated (data not shown). This phenomenon was not seen in the PNA five similar experiments performed.

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-2d) mice (A) or allografts from recipients (B) after transplantation for 7 days. The graphs are showing CXCR3+ and CD4+ (a), CD8+ (b), CD19+ (c), or CD14+ (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+ cells are indicated in the graphs. Data are from a single experiment, which is representative of five similar experiments performed.

FIGURE 6. The migration of CD3+ spleen T cells toward MIP-1α/CCL3 (A), RANTES/CCL5 (B), γ-IP-10/CXCL10 (C), or Mig/CXCL9 (D). The spleen CD3+ T cells were from either native BALB/c (H-2d) mice [□] or BALB/c (H-2d) recipients that received allograft transplantation for 3 (□), 5 (▲), or 7 (●) days. All results were determined as described in Materials and Methods, expressed as CI ≥ 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

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

* Effect of PNA treatment on mouse skin allograft rejection.  
** All treatments started 3 days prior to the allograft transplantation (n = 15 for each group of BALB/c (H-2d) recipients).  
* Survival time was determined as described Materials and Methods.  
* Normal physiological saline was applied in equal volume to active PNA treatment.  
* A dosage of 0.5 mg/kg/day was considered low and one of 10 mg/kg/day was considered high.  
* p < 0.05  
**p < 0.005.
Mig/CXCL9, respectively (Fig. 11), implying that PNA CXCR3 affects 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 transplantsations.

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.

**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 CXCR3 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/CXCL. Thus, a rather complex picture is now beginning to take shape of how T cells selectively enter...
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...
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 cells than oligonucleotides. 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 CXC3R inhibits 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 CCR5 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.

Acknowledgments

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References