Silencing the Formylpeptide Receptor FPR by Short-Interfering RNA

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ABSTRACT

A double-stranded short-interfering RNA (siRNA) was designed to attenuate the expression and function of the formylpeptide receptor FPR, a G protein-coupled receptor mediating migration and activation of phagocytic leukocytes in response to bacterial chemotactic formylpeptides. Retrovirus-based constructs were generated to introduce FPR-siRNA into a rat leukemia cell line transfected to overexpress FPR. Cells infected with FPR-siRNA corresponding to the third extracellular loop of the putative receptor protein, showed significantly reduced expression of FPR mRNA and protein, in association with impaired calcium mobilization and chemotactic responses to peptide agonists. Direct transduction of synthetic FPR-siRNA into human macrophages also inhibited the expression of FPR and abrogated cell chemotaxis and the release of superoxide anions induced by the bacterial formylpeptide. FPR-siRNA additionally abrogated the expression and function of FPR in a human malignant glioma cell line. Our study demonstrates successful application of siRNA to silence a G protein-coupled chemoattractant receptor involved in inflammation and suggests the potential to use this approach in studies of receptor regulation and prevention of undesirable side effects associated with FPR activation.

Phagocytic leukocytes accumulate at sites of inflammation, injury, and bacterial infection presumably in response to locally produced chemotactic factors. In fact, a number of chemoattractants derived from pathogens or host tissues have been identified. “Classical” chemoattractants include bacterial chemotactic formylpeptides represented by formylmethionyl-leucyl-phenylalanine (fMLF), activated complement components, and chemotactic lipids (Murphy, 1994). There are also a number of newly discovered “chemokines” that induce the migration of selected cell population (Rollins, 1997; Le et al., 2000a). Both classical and chemokine family chemoattractants use seven-transmembrane G protein-coupled cell surface receptors (Murphy, 1994; Rollins, 1997; Le et al., 2000a). The N-formylpeptide receptor FPR is a G protein-coupled receptor (Murphy, 1996; Prossnitz and Ye, 1997; Le et al., 2002) that responds to classical chemotactic formylpeptides produced by exogenous pathogens represented by gram negative bacteria (Schiffmann et al., 1975; Marasco et al., 1984) and possibly to mitochondria components of ruptured host cells (Carp, 1982). Because FPR is mainly expressed by phagocytic leukocytes, this receptor has been considered to play a role in mediating the traffic of phagocytic leukocytes to the sites of bacterial infection and tissue injury where formylpeptides are likely generated. This assumption was supported by the observations that mice depleted of the human FPR analog mFPR1 exhibit reduced resistance to infection by Listeria monocytogenes (Gao et al., 1999). However, over the past few years, several novel exogenous and host-derived FPR ligands, which are not formylated and do not show homology in their amino acid sequences, have been identified, suggesting FPR may also participate in biological processes other than antibacterial...
host responses and tissue injury (Murphy, 1996; Prossnitz and Ye, 1997; Le et al., 2002). Activation of FPR by its agonists initiates a cascade of signaling events that culminate in increased cell migration, phagocytosis, release of reactive oxygen intermediates, and new gene transcription (Murphy, 1996; Prossnitz and Ye, 1997; Le et al., 2002). Although these functions of FPR are believed to be beneficial for the clearance of bacteria, excessive production of oxidants may also cause undesirable inflammation and tissue damage. Thus, development of molecules capable of limiting FPR expression and function will not only assist in studies of receptor regulation but also be important for the development of anti-inflammatory agents.

Double-stranded RNA inhibits gene expression in a sequence-specific manner by triggering the degradation of messenger RNA (Billy et al., 2001). This effect, referred to as RNA interference, has been studied most extensively in Caenorhabditis elegans and Drosophila melanogaster (Hammond et al., 2001). After recognition of homologous single-stranded RNA, double-stranded RNA is degraded by the Dicer RNase into short fragments of 21-nt short-interfering RNA (siRNA). siRNA causes post-transcriptional gene silencing and is believed to function in a variety of organisms, including mammalian cells (Elbashir et al., 2001; Paddison et al., 2002b; Yu et al., 2002). With the discovery that siRNA can be effectively produced as hairpin transcripts from RNA polymerase III promoters (Brummelkamp et al., 2002; Paddison et al., 2002a; Yu et al., 2002), intracellular synthesis of siRNA has become feasible, which provides a new tool for studying gene regulation and has the potential as gene-specific therapeutics. In this study, we report the successful use of double-stranded siRNA to attenuate FPR overexpression in rat leukemia cells, primary human macrophages, and human malignant glioma cells.

Materials and Methods

Reagents. The chemotactic peptide fMLF was purchased from Sigma-Aldrich (St. Louis, MO). Peptide WKYVMVm (designated W peptide) (Le et al., 1999) was synthesized and purified by the Department of Biochemistry (Colorado State University, Fort Collins, CO). 3H)fMLF was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Anti-phosphorylated (p) ERK1/2 (Thr 202/204) and anti-ERK1/2 antibodies were from Cell Signaling Technologies (Boston, MA). Anti-phosphorylated (p) ERK1/2 (Thr 202/204) and Ye, 1997; Le et al., 2002). Activation of FPR by its agonists initiates a cascade of signaling events that culminate in increased cell migration, phagocytosis, release of reactive oxygen intermediates, and new gene transcription (Murphy, 1996; Prossnitz and Ye, 1997; Le et al., 2002). Although these functions of FPR are believed to be beneficial for the clearance of bacteria, excessive production of oxidants may also cause undesirable inflammation and tissue damage. Thus, development of molecules capable of limiting FPR expression and function will not only assist in studies of receptor regulation but also be important for the development of anti-inflammatory agents.

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Transduction of Synthetic FPR-siRNA in Human Macrophages. FPR-siRNAT28 ([5′-AGAUAUGUUAUGCUUUUU (sense) and 5′CACUGCAACAAUUCUUU (antisense]) was also synthesized, purified, and annealed (Ambion, Austin, TX) for transduction in human macrophages. Human peripheral blood mononuclear cells were isolated from Buffy coats (Transfusion Medicine Department, National Institutes of Health Clinical Center, Bethesda, MD) with iso-osmotic Percoll gradient (Le et al., 1999). Washed monocytes (2 x 10⁶) were resuspended in 900 μl of RPMI 1640 medium (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) and cultured in 24-well plates precoated with poly(2-hydroxyethyl methacrylate) (0.1 ml/cm², 12 mg/ml; Sigma-Aldrich). Transient transfection of FPR-siRNA was performed with 3 μl Oligofectamine (Invitrogen) in RPMI 1640 medium (12 μl) preincubated (5–10 min) at room temperature. During the incubation, 80 μl of RPMI 1640 medium was mixed with 5 μl of FPR-siRNAT28 (20 μM), which targets the positions 926 to 944 (nucleotide) of FPR mRNA corresponding to the third extracellular loop of the putative protein. Oligofectamine and FPR-siRNA mixtures were combined and incubated (20 min) at room temperature for complex formation. This mixture was then added to the cells cultured in the wells of 24-well plates with a final concentration of FPR-siRNA at 100 nM. Human macrophages were washed after overnight FPR-siRNA transduction.
and resuspended in RPMI 1640 with 10% heat-inactivated human AB serum and antibiotics for further experiments.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). DNA-free total RNA was extracted from ETFR cells, human macrophages, or human malignant glioma cells with a RNaseasy Mini kit (Qiagen). Total RNA (0.1 μg) was used for RT-PCR using High Fidelity ProSTAR HF system (Stratagene, La Jolla, CA). Reverse transcription was performed at 42°C for 15 min and terminated by incubation at 95°C for 1 min. Amplification was completed with 40 cycles at 95°C (45 s), 55°C (45 s), and 72°C (1 min), and a final extension at 72°C for 10 min. For human FPR, primers 5’-ATATCAGTGGTGCCCCTTA (sense) and 5’-CTGGCCCATGAAGATAGA (antisense) were used to yield a 151-base pair product. β-Actin or GAPDH was used as control. Polymerase chain reaction products were visualized by ethidium bromide staining in 1.2% agarose gel.

Flow Cytometry. ETFR cells or macrophages were washed with FACS buffer (5 mM EDTA, 0.1% NaN₃, and 1% FCS, in Dulbecco’s phosphate-buffered saline) incubated with a monoclonal antibody against human FPR (BD Biosciences Pharmingen, San Diego, CA) for 30 min on ice, and then stained with a fluorescein isothiocyanate-labeled secondary antibody. The cells were then examined for FPR expression by flow cytometry (BD Biosciences, San Jose, CA).

Chemotaxis Assays. Chemotaxis assays were performed using 48-well chemotaxis chambers (Neuro Probe, Gaithersburg, MD) (Le et al., 1999; Hu et al., 2001). Chemotaxants at different concentrations were placed in the wells of the lower compartment of the chamber. ETFR cells, human macrophages, or U87 human malignant glioma cells were placed in the wells of the upper compartment. ETFR cells, human macrophages, or U87 cells, were washed with RPMI 1640 medium with 10% FCS, 2 mM glutamine, and resuspended in RPMI 1640 medium, 10% FCS, and 2 mM glutamine for 60 min at room temperature, washed, and resuspended in saline buffer at a density of 0.5 × 10⁶ cells/ml. The cell suspension (2 ml) was placed in a cuvette in a fluorescence spectrometer (PerkinElmer Life and Analytical Sciences, Beaconsfield, England) and activated by adding 20 μl of stimulants. The fluorescence intensity was calculated based on the ratio at 340- and 380-nm wavelengths with a FL WinLab program (PerkinElmer Life and Analytical Sciences).

Binding Assays. A single concentration of [³H]fMLF was added simultaneously with different concentrations of unlabeled fMLF to ETFR cell suspension (2 × 10⁶ cells/200 μl) in RPMI 1640 medium, 1% bovine serum albumin, and 0.05% NaN₃) in duplicate samples. The samples were incubated under constant rotation for 30 min at 4°C. After incubation, the samples were filtered through Whatman GF/C discs (Whatman, Kent, UK) on a 12-well manifold followed by extensive washing with ice-cold phosphate-buffered saline. The discs were air-dried at 65°C, submerged in liquid scintillation cocktail, and counted for β emission. The binding isotherms were generated with a computer-aided program LIGAND (P. Munson, Division of Computer Research and Technology, National Institutes of Health, Bethesda, MD) (Munson and Rodbard, 1984).

Western Immunoblotting. ETFR cells in the presence or absence of FPR-siRNA T28 were grown on 60-mm dishes and cultured overnight in FCS-free medium. After treatment with sMLF, the cells were lysed with 1× SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 50 mM dithiothreitol], sonicated for 10 to 15 s, and heated at 100°C for 5 min. The cell lysate was centrifuged at 14,000 rpm and 4°C for 10 min, and protein concentration of the supernatant was measured by Coomassie Protein Assay Reagent (Pierce Chemical, Rockford, IL). Western blotting of phosphorylated ERK1/2 was performed according to the instruction of the supplier of the anti-phospho-ERK1/2 antibody (Cell Signaling Technology Inc.). Briefly, proteins were electrophoresed on a 10% SDS-polyacrylamide
gel electrophoresis precast gel (Invitrogen), and transferred onto Immobilon P membranes (Millipore Corporation, Billerica, MA). The membranes were blocked with blocking buffer (1× phosphate-buffered saline and 0.05% Tween 20 with 3% nonfat dry milk), and then were incubated with primary antibodies overnight at 4°C. After incubation with a horseradish peroxidase-conjugated secondary antibody, the protein bands were detected with a Super Signal chemiluminescent substrate stable peroxide solution (Pierce Chemical) and BIOMAX-MR film (Eastman Kodak, Rochester, NY). For detection of total ERK1/2, the membranes were stripped with Restore Western blot stripping buffer (Pierce Chemical) followed by incubation with an anti-pan-ERK1/2 antibody.

**Superoxide Anion Assay.** Human macrophages were suspended in Hanks’ balanced salt solution at 10⁶/ml. The cells (80 μl) were transferred into a chemiluminescence tube and mixed with 100 μl of Diogenes complete enhancer solution (National Diagnostics, Atlanta, GA). Stimulants at different concentration (20 μl) were added to the cells, and chemiluminescence was measured with a luminometer (Analytical Luminescence, San Diego, CA). The results are expressed as integrated luminescence in relative light units.

**Statistical Analysis.** All experiments were performed at least three times, and representative results are presented. Paired Student’s t test was used to compare the significance of differences in cell migration. p values equal to or less than 0.05 were considered statistically significant.

**Results**

To identify siRNA constructs (Fig. 1) that might effectively and specifically attenuate the transcription of FPR, we tested several FPR-siRNA constructs in ETFR cells that overexpress human FPR and mount robust responses when they are stimulated with the bacterial chemotactic peptide fMLF. ETFR cells stably transduced with a retrovirus-based FPR-siRNA construct T28, targeting FPR mRNA at the nt positions of 926 to 944, showed a significantly reduced expression of FPR as detected by RT-PCR (Fig. 2A). Semiquantitative measurement of the mFPR synthesis revealed an average 80% reduction of FPR mRNA transcription in ETFR cells expressing FPR-siRNAT28 compared with mock-transduced cells. This was associated with a decreased expression of FPR protein on the cell surface as determined by flow cytometry analyses (Fig. 2B). To further examine the capacity of T28 to affect cell surface expression of FPR, we measured the binding of [³H]fMLF to ETFR cells. The wild-type and mock transduced ETFR cells possess considerable levels of binding sites for [³H]fMLF. With the transduction of FPR-siRNAT28, ETFR cells exhibited more than 60% reduction in the number of binding sites for [³H]fMLF on the cell surface, with minimal change in the affinity (Fig. 2C). These results indicate that the FPR-siRNA construct T28 effectively inhibited the expression of FPR at both mRNA and protein levels in cells overexpressing this receptor. Two additional FPR-siRNA constructs T10 and T16 (Fig. 1), which target FPR mRNA at the positions of nt 392 to 410 and 605 to 623, respectively, did not show significant effects in reducing the mRNA (Fig. 2A) or cell surface expression of FPR in ETFR cells (data not shown), suggesting the importance of selectivity for individual potential FPR-siRNA.

We next examined the effect of FPR-siRNA on two major FPR functions in ETFR cells, namely, Ca²⁺ mobilization and chemotaxis in response to the agonists. ETFR cells transfected with FPR-siRNAT28 showed a significantly reduced Ca²⁺ mobilization induced by the bacterial chemotactic peptide fMLF (Fig. 3A), as well as a synthetic peptide W peptide (W pep) (Fig. 3B), a potent FPR agonist derived from random peptide library (Le et al., 1999). The inhibition of Ca²⁺ mobilization in ETFR cells by stable transduction of T28 was more pronounced when the FPR agonists were used in low nanomolar concentration range. It is of interest that although FPR-siRNAT10 and T16 did not exhibit significant inhibition on FPR mRNA expression in ETFR cells, both constructs showed certain levels of attenuation of agonist-induced Ca²⁺ mobilization in ETFR cells, albeit with lower efficacy compared with T28. As evidence to further confirm
the effect of FPR-siRNA on FPR function, ETFR cells expressing FPR-siRNA T28 showed a markedly reduced chemotactic response to the FPR agonists fMLF and W peptide (Fig. 4, A and B). In contrast to the effect on FPR expression and function, FPR-siRNA T28 construct did not show any inhibition of the expression and function of an FPR variant receptor FPRL1 (data not shown), which is a low-affinity receptor for fMLF and exhibits a distinct agonist profile compared with FPR (Murphy, 1996; Prossnitz and Ye, 1997; Le et al., 2002). FPR-siRNA T10 or T16, which showed minor inhibition of agonist-induced Ca\(^{2+}\) mobilization in ETFR cells, did not affect the chemotaxis of these cells to fMLF or W peptide. The capacity of FPR-siRNA to affect the agonist-induced phosphorylation of ERK1/2 MAP kinases coupled to the signaling cascade of FPR was further investigated. As shown in Fig. 4C, fMLF induced a rapid and transient phosphorylation of ERK1/2 in ETFR cells, which was reduced considerably by the presence of siRNA T28. Thus, T28 is a selective and highly efficacious siRNA for human FPR that attenuates both FPR expression and its key functions.

To determine the effect of FPR-siRNA in a physiologically more relevant cell type, we used human macrophages by directly transducing siRNA T28. After 72 h, macrophages transduced with T28 showed a reduced cell surface expression of FPR compared with mock-transduced cells. A further reduction in cell surface expression of FPR was observed when macrophages were transduced with FPR-siRNA for 6 days (Fig. 5A). This was associated with a markedly reduced expression of FPR mRNA as determined by RT-PCR (Fig. 5B). Functionally, macrophages transduced with T28 for 6 days completely lost their chemotactic response to fMLF compared with mock-transduced cells (Fig. 6A). In addition, the capacity of these macrophages to rapidly release superoxide anion in response to fMLF was abrogated (Fig. 6B). In agreement with results obtained with ETFR cells, this shows that synthetic siRNA is also capable of effectively attenuating the expression and function of FPR in human primary macrophages, which are active participants of antibacterial host responses and chronic inflammation.

FPR-siRNA additionally seemed to be a very useful approach to the study of FPR function expressed in malignant tumor cells, which were of the nonhematopoietic origin. We previously reported that a malignant human glioma cell line U87 expressed FPR and responded to fMLF by increased chemotaxis and Ca\(^{2+}\) mobilization (Le et al., 2000b). Stable transfection of U87 cells with FPR-siRNA T28 almost abolished the expression and function of FPR in these tumor cells (Fig. 7). Thus, siRNA provides a novel strategy to “silence” FPR not only in cells of the innate host defense but also in malignant tumor cells.

**Discussion**

RNA interference has become a powerful tool to inhibit gene expression in the studies of posttranscriptional level regulation and its potential application in gene therapy of...
diseases has been appreciated (Billy et al., 2001; Hammond et al., 2001; Elbashir et al., 2001; Brummelkamp et al., 2002; Paddison et al., 2002a,b; Yu et al., 2002). Among numerous genes that have been targeted with siRNA silencing are at least two chemokine receptors CXCR4 and CCR5, which are G protein-coupled receptors and serve as essential coreceptors for HIV-1 entry of host cells (Martinez et al., 2002; Anderson et al., 2003; Butticaz et al., 2003; Lee et al., 2003). siRNA silencing CXCR4 and CCR5 render host cell resistance to HIV-1 infection. Since CXCR4 has been implicated in mediating metastasis of several human malignant tumors (Muller et al., 2001), “knockdown” of CXCR4 by an inducible siRNA resulted in markedly reduced migratory response of a human breast cancer cell line to the CXCR4 chemokine ligand in vitro (Chen et al., 2003). Our study extended the use of siRNA technology to the human formylpeptide receptor FPR and has shown the effectiveness of siRNA to reduce the expression of FPR mRNA and important functions of this receptor in proinflammatory responses. It should be noted that the efficacy of siRNA can vary in different cell types and may depend on the level of gene transcription. In our study, siRNA seems to more potently attenuate the expression and function of FPR in primary macrophages and a malignant glioma cell line than in ETFR cells, which express higher levels of FPR. In our experiments, FPR-siRNA targeting FPR mRNA coding for the third and second transmembrane regions of FPR protein, respectively, were lesser effective. Since the third extracellular loop of FPR protein is an important domain involved in ligand interaction (Quehenberger et al., 1993), our results suggest potentially more efficient knockdown of FPR by siRNA targeting mRNA segments that encode crucial ligand recognition domains. Further research is warranted to more thoroughly screen the siRNAs targeting diverse mRNA segments of FPR, which may yield constructs with greater potency. It is plausible that a combinations of several siRNA targeting different mRNA segments may prove even more efficient in attenuating a receptor gene that is expressed at high levels in a given cell type.

Silencing FPR by siRNA may have important pathophysiological applications. In addition to its expression in myeloid cells, FPR was detected in several cell types of nonhematopoietic origin. Activation of FPR on human hepatocytes and fibroblasts by the bacterial peptide fMLF induced release of acute phase proteins (McCoy et al. 1995; VanCompernolle et al., 2003). More intriguingly, several malignant human astrocytoma cell lines as well as a type II alveolar epithelial tumor cell line express FPR, which mediates chemotaxis and activation of such tumor cells by chemotactic agonists (Le et al., 2000b; Rescher et al., 2002). The biological significance of the expression of FPR by nonhematopoietic and malignant tumor cells remains to be determined. Thus, the use of siRNA may facilitate the understanding of the role of FPR in biological responses other than inflammation and bacterial infection, which mainly involve phagocytic leukocytes. siRNA may have advantages...
over antibodies or receptor antagonists as being noncytotoxic and less likely to induce host antibody responses. In addition, the relative simplicity in design and screening as well as targeted delivery render siRNA a potentially very effective means of studying the in vivo role of FPR, in particular, for the biological consequences of its expression by nonhematopoietic cells and malignant tumor cells. Furthermore, the use of specific siRNA can be extended to studies of additional chemoattractant receptors involved in inflammation, immune responses, leukocyte trafficking, and malignancy.

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References


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