Synthetic Pyrrole-Imidazole Polyamide Inhibits Expression of the Human Transforming Growth Factor-β1 Gene


Departments of Internal Medicine (Y.-M.L., N.F., T.U., H.Ma., K.M.), and Advanced Medicine (N.F., S.S., H. Mu.), Nihon University School of Medicine, Tokyo, Japan; Advanced Research Institute of the Science and Humanities, Nihon University, Tokyo, Japan (N.F.); Department of Chemistry, Graduate School of Science, Kyoto University, Kyoto, Japan (H.A., T.B., H.S.); and Gentier Biosystems Incorporation, Tokyo, Japan (K.S.)

Received May 13, 2005; accepted August 22, 2005

ABSTRACT

Pyrrole-imidazole (Py-Im) polyamides can bind to the predetermined base pairs in the minor groove of double-helical DNA with high affinity. These synthetic small molecules can interfere with transcription factor-DNA interaction and inhibit or activate the transcription of corresponding genes. In the present study, we designed and synthesized a Py-Im polyamide to target −545 to −539 base pairs of human transforming growth factor-β1 (hTGF-β1) promoter adjacent to the fat-specific element 2 (FSE2) to inhibit the expression of the gene. Gel mobility shift assay showed that the synthetic Py-Im polyamide binds to its corresponding double-strand oligonucleotides, whereas the mismatch polyamides did not bind. Fluorescein isothiocyanate-labeled Py-Im polyamide was detected in the nuclei of human vascular smooth muscle cells (VSMCs) after 2- to 48-h incubation. Py-Im polyamide significantly decreased the promoter activity of hTGF-β1 determined by in vitro transcription experiments and luciferase assay. In cultured human VSMCs, Py-Im polyamide targeting hTGF-β1 promoter significantly inhibited the hTGF-β1 mRNA and protein. These results indicate that the synthetic Py-Im polyamide designed to bind hTGF-β1 promoter inhibited hTGF-β1 gene and protein expression successfully. This novel agent will be used for the TGF-β-related diseases as a gene therapy.

Pyrrole (Py)-imidazole (Im) polyamides are small synthetic molecules composed of the aromatic rings of the N-methylpyrrole and N-methylimidazole amino acid (Trauger et al., 1996; White et al., 1997; Dervan, 2001). Synthetic polyamides can bind to specific nucleotide sequences in the minor groove of double-helical DNA with high affinity and specificity, suggesting that Py-Im polyamides could be useful tools for molecular biology and, potentially, medicine. Binding site specificity is dependent on the side-by-side pairing of Py and Im: the Py/Im pair targets the GC base pair, Im/Py recognizes the GC base pair, and Py/Py binds both AT and TA base pairs (Trauger et al., 1996; White et al., 1997; Dervan, 2001). Recent studies have shown that the AT degeneracy can be overcome by replacing one pyrrole ring of the Py/Py pair with 3-hydroxypyrrole (Hp); Hp/Py preferentially binds TA pairs (White et al., 1998).

Transcriptional regulation is essential for gene expression. Initiation of transcription requires binding of transcription factors to the cognate DNA response elements in the gene promoter. Py-Im polyamides bind the minor groove and block binding of transcription factors inhibiting gene expression. Gottesfeld et al. (1997) reported inhibition of the transcription of 5S RNA gene by an eight-ring Py-Im polyamide designed to bind the recognition site of zinc-finger protein TFIIIA. To block activity of the human immunodeficiency virus type 1, two polyamides were designed to bind two transcription factor binding sites, and this inhibited virus replication by >99% (Dickinson et al., 1998). Thus, Py-Im polyamides designed to bind transcription factor binding sites can potentially suppress gene expression.

Transforming growth factor-β1 (TGF-β1) represents a large family of cytokines that are involved in the regulation

Abbreviations: Py, pyrrole; Im, imidazole; TGF-β1, transforming growth factor-β1; VSMC, vascular smooth muscle cell; hTGF-β1, human transforming growth factor-β1; FSE2, fat-specific element 2; bp, base pair(s); DMF, N,N-dimethylformamide; FITC, fluorescein isothiocyanate; DMEM, Dulbecco’s modified Eagle’s medium; PMA, phorbol 12-myristate acetate.
Materials and Methods

General. Reagents and solvents were purchased from standard suppliers and used without further purification. NMR spectra were recorded with a JEOL JNM-A 500 nuclear magnetic resonance spectrometer (JEOL, Tokyo, Japan), and tetramethylsilane was used as the internal standard. Proton NMR spectra were recorded in parts per million (ppm) downfield relative to tetramethylsilane. The following abbreviations apply to spin multiplicity: s (singlet), d (doublet), t (triplet), q (quartet), qu (quintet), m (multiplet), and br (broad). Electrospray ionization mass spectrometry and electrospray ionization time-of-flight mass spectrometry were produced on a API 150 (PerkinElmerSciex Instruments, Boston, MA) and BioTOF II (Bruker Daltonics, Billerica, MA) mass spectrometer.

Designing and Synthesis of Py-Im Polyamide Targeting the TGF-β1 Promoter. The structures of the match, FITC-labeled, and mismatch Py-Im polyamides used in this study are shown in Fig. 1. Py-Im polyamide was designed to bind bp −545 to −539 the hTGF-β1 promoter adjacent to the FSE2 binding site (Fig. 1A). One Im-Py substitution was induced to create the mismatch Py-Im polyamide. Machine-assisted automatic synthesis of Py-Im polyamides was performed with a Pioneer continuous-flow peptide synthesizer (Applied Biosystems, Foster City, CA) on a 0.1-mmol scale (200 mg of Fmoc-b-Ala-CLEAR Acid Resin, 0.50 mEq/g; Peptide Institute, Osaka, Japan). Automatic solid phase synthesis consisted of an N,N-dimethylformamide (DMF) wash, removal of the Fmoc group with 20% piperidine/DMF, a methanol wash, protection with acetic anhydride/N-diisopropylethylamine (4 Eq each) for 60 min, a methanol wash, protection with acetic anhydride/pyridine if necessary, and a final DMF wash. We generally obtained moderate yields (10–39%) of Py-Im polyamides. After removal of Fmoc group of Fmoc-b-alanine-CLEAR Acid Resin, the resin was washed successively with methanol. The coupling step was performed with Fmoc-amino acid followed by a wash with methanol. These steps were repeated several times until all sequences were introduced. After the coupling steps were completed, the N-terminal amino group was protected or coupled with FITC and washed with DMF, and the reaction vessel was drained. The synthetic polyamides were purified by high-performance liquid chromatography using a Chemobond 5-ODS-H column (Chemco Scientific, Osaka, Japan). 1% AcOH/CH,CN, 0 to 50% linear gradient, to 40 min, 254 nm. H NMR [dimethyl sulfoxide (DMSO)-d6] d: 1.50 (4H, m), 1.78 (2H, t, J = 7.0 Hz), 2.01 (3H, s), 2.21 (4H, t, J = 7.0 Hz), 2.27 (4H, m), 2.59 (4H, t, J = 7.0Hz), 3.77 (3H, s), 3.80 (3H, s), 3.80 (6H, s), 3.81 (3H, s), 3.82 (3H, s), 3.83 (3H, s), 3.84 (3H, s), 3.84 (3H, s), 3.84 (3H, s), 3.93 (3H, s), 3.95 (3H, s), 6.75 (1H, s), 6.82 (1H, d, J = 2.0 Hz), 6.85 (3H, m), 6.90 (1H, d, J = 2.0 Hz), 7.01 (1H, d, J = 2.0 Hz), 7.11 (1H, d, J = 2.0 Hz), 7.12 (1H, d, J = 2.0 Hz), 7.16 (2H, d, J = 2.0 Hz), 7.17 (3H, d, J = 2.0 Hz), 7.19 (1H, d, J = 2.0 Hz), 7.21 (1H, d, J = 2.0 Hz), 7.25 (2H, d, J = 2.0 Hz), 7.41 (1H, s), 7.45 (1H, s), 8.03 (3H, m), 8.22 (1H, m), 9.83 (1H, s), 9.86 (2H, m), 9.86 (1H, s), 9.87 (1H, s), 9.88 (2H, s), 9.91 (1H, s), 9.93 (1H, s), 10.21 (1H, s), 10.28 (1H, s); electrospray ionization-mass spectrometry mass-to-charge ratio (m/e) calcd for C₈₉H₇₃N₂₄O₁₇ (539) to 545, found 545.

Cell Culture and Distribution of Py-Im Polyamide. Human VSMCs (Cambrex Bio Science Rockland, Inc., Rockland, ME) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA) and 50 μg/ml streptomycin (Invitrogen). To examine the distribution of growth, differentiation, and morphogenesis in a wide range of cell types (Lyons and Moses, 1990; Sporn and Roberts, 1992). TGF-β1 has been reported to be involved in several cardiovascular diseases such as stroke, ischemic heart disease, and glomerulosclerosis, owing to its effects on the growth of vascular smooth muscle cells (VSMCs) and extracellular matrix formation (Grant et al., 1999; Joki et al., 2000; Kobayashi et al., 2001). TGF-β1 plays a pivotal role in chronic inflammatory changes of the interstitium and accumulation of extracellular matrix during renal fibrogenesis (Border and Noble, 1997; Blobe et al., 2000). We designed a Py-Im polyamide targeting the hTGF-β1 promoter adjacent to the fat-specific element 2 (FSE2) to inhibit expression of the human TGF-β1 (hTGF-β1) gene; we then examined the effect of this polyamide on hTGF-β1 gene expression.
Py-Im polyamide in cells, VSMCs were plated on two-well chamber slides (LabTek; Nalge Nunc International, Tokyo, Japan) at a density of 3000/cm². Cells were incubated with 1 nM FITC-conjugated Py-Im polyamide in DMEM for 2 h and fixed for 20 min (glutaraldehyde). Nuclear staining was achieved with Hoechst 33324 and visualized with an Olympus microscope (Olympus, Tokyo, Japan) using appropriate filters.

**hTGF-β1 Promoter Activity.** A 2.2-kb fragment of the hTGF-β1 promoter was inserted into a pGL3 basic (Promega, Madison, WI) vector (pGL3-TGF-β1). One microgram of pGL3-TGF-β1 was transfected into human VSMCs in serum-free medium with the LipofectAMINE reagent for 6 h. After transfection, cells were incubated with 1 µM Py-Im targeting human TGF-β1 or mismatch polyamide in the presence or absence of 1 µM phorbol 12-myristate acetate (PMA) in DMEM containing 0.5% calf serum for 24 h. Luciferase activity was measured in cell extracts with the Dual-Luciferase reporter gene assay system (Promega).

**Gel Mobility Shift Assay.** A double-stranded DNA fragment corresponding to bp −548 to −537 of human TGF-β1 promoter was labeled with [γ-32P]ATP by T4 polynucleotide kinase (Promega) according to the standard method (Sambrook et al., 1989). The labeled double-stranded DNA was then incubated with 10 nM mismatch polyamide or 1, 2, 4, and 10 nM Py-Im polyamides at 37°C for 15 min. DNA-Py-Im complexes were separated by electrophoresis on 20% polyacrylamide gels and visualized by autoradiography.

**Reverse Transcription-Polymerase Chain Reaction and Western Blot Analysis.** Total RNA was extracted from cultured cells as described previously (Mocharla et al., 1990) and was reverse-transcribed with oligo dT (Takara Biochemicals, Osaka, Japan) and avian myeloblastoma virus reverse transcriptase (Takara Biochemicals) at 37°C for 40 min. Then, 1.5 µl of reverse-transcribed material was amplified with Taq DNA polymerase (Takara Biochemicals), as described previously using each specific primers (Ando et al., 2004). Western blot analysis was performed as described previously using each specific antiserum (Ando et al., 2004).

**Statistical Analysis.** Values are reported as mean ± standard error of the mean (S.E.M.). Statistical analysis was done with Student’s t test for unpaired data or with two-way analysis of variance or Duncan’s multiple range test. p < 0.05 was considered statistically significant.

**Results**

**Binding of Py-Im Polyamide to Double-Stranded Oligonucleotides.** Gel shift assay allowed the determination of the binding affinity and specificity of polyamides for double-stranded DNA. The designed Py-Im polyamide bound the appropriate 12-bp double-stranded oligonucleotide in a dose-dependent manner, whereas mismatch polyamide did not show binding (Fig. 2).

**Distribution of Py-Im Polyamide in Cultured VSMCs.** When human VSMCs were cultured with one micromolar of FITC-labeled Py-Im polyamide for 2 h, strong fluorescent signals were detected in nuclei of these cells (Fig. 3). The intensity of the fluorescent signal was maintained in the nuclei for more than 48 h.

**Effect of Py-Im Polyamide on hTGF-β1 Promoter Activity.** One micromolar PMA significantly increased luciferase activity in human VSMCs transfected with pGL3-TGF-β1. One micromolar of synthetic Py-Im polyamide targeting the hTGF-β1 promoter significantly decreased luciferase activity in cultured human VSMCs, whereas the mismatch polyamide had no effect on luciferase activity (Fig. 4).

**Effect of Py-Im Polyamide on Expressions of hTGF-β1 mRNA and Proteins in Cultured Human VSMCs.** Incubation of the human VSMCs with 1 µM PMA significantly increased (p < 0.05) expression of hTGF-β1 mRNA. One micromolar of Py-Im polyamide to hTGF-β1 promoter significantly decreased level of hTGF-β1 mRNA, whereas the mismatch polyamide had no effect on hTGF-β1 mRNA expression (Fig. 5).

One micromolar PMA also significantly increased production of hTGF-β1 protein. One micromolar Py-Im polyamide specific for the hTGF-β1 promoter significantly decreased expression of human TGF-β1 protein, whereas the mismatch polyamide had no effect on hTGF-β1 protein levels (Fig. 6).

**Discussion**

Based on previous efforts by Kopka’s and Lown’s groups over a period of four decades on minor groove sequence information readout by small molecules (Kopka et al., 1985a,b; Lown et al., 1986), Trauger’s and Dervan’s groups have de-
veloped minor groove-binding Py-Im hairpin polyamides that precisely recognize each of the four Watson-Crick base pairs according to the binding rule of Py-Im polyamides (Trauger et al., 1996; Dervan, 2001). To develop a novel gene therapy for connective tissue proliferative diseases, such as renal sclerosis, pulmonary fibrosis, liver cirrhosis, or atherosclerosis, we designed and synthesized a Py-Im polyamide targeting the hTGF-β1 promoter and examined its effects on hTGF-β1 expression in viable cells.

Fig. 4. Effects of Py-Im targeting hTGF-β1 on hTGF-β1 promoter activity. One microgram of pGL3-TGF-β1 was transfected into human VSMCs with LipofectAMINE reagent. Twenty-four hours after transfection, cells were incubated with 1 nM Py-Im targeting human TGF-β1 or mismatch polyamide in the presence or absence of 1 μM PMA. Luciferase activity was measured in these cell extracts with the Dual-Luciferase reporter gene assay. *, p < 0.05 compared with control.

Fig. 5. Effects of Py-Im polyamide targeting hTGF-β1 on expression of hTGF-β1 mRNA in human VSMCs. Human VSMCs were incubated with 1 μM Py-Im targeting human TGF-β1 or mismatch polyamide in the presence or absence of 1 μM PMA. A, reverse transcription-polymerase chain reaction analysis of expression of hTGF-β1 mRNA. B, the ratios of hTGF-β1 mRNA to 18S rRNA were evaluated by densitometry. Data are shown as the mean ± S.E.M. (n = 4). *, p < 0.05 versus mismatch.

Fig. 6. Effects of Py-Im polyamide targeting human TGF-β1 on production of human TGF-β1 protein by human VSMCs. Human VSMCs were incubated with 1 μM Py-Im targeting human TGF-β1 or mismatch polyamide in the presence of 1 μM PMA. A, Western blot analysis of hTGF-β1 protein levels. B, the ratio of hTGF-β1 to α-tubulin was evaluated by densitometry. Data are shown as the mean ± S.E.M. (n = 4). *, p < 0.05 versus mismatch.

FITC-conjugated Py-Im polyamide permeated cell membranes and accumulated and remained in the nuclei of cultured human VSMCs. In comparison with our previous observations of the cellular distributions of antisense oligonucleotides and ribozymes (Fukuda et al., 1997; Kotani et al., 2003), the Py-Im polyamide exhibited greater permeability and stability in cultured human VSMCs. These properties of Py-Im polyamides indicate that these molecules are suitable gene therapy agents.

Studies of Py-Im polyamides have recently focused on the structural characterization of the transcription factor-DNA complexes in promoter sequences. The hTGF-β1 gene promoter lacks both a TATA box and an initiator region, but it does contains several positive and negative regulatory elements (Kim et al., 1989). We designed a Py-Im polyamide specific for bp −545 to −539 of the hTGF-β1 promoter sequence, and this Py-Im polyamide inhibited hTGF-β1 promoter activity and expression of hTGF-β1 mRNA and protein in cultured human VSMCs. The target region of this polyamide is located just adjacent to the FSE2 in the hTGF-β1 promoter. In preadipocytes, the FSE2 represses expression of the adipocyte P2 gene (Kim et al., 1989; Romeo et al., 1997). Because the expression of most mammalian genes is likely to depend on the combined action of numerous proteins bound to promoter and enhancer sequences, it is likely that the Py-Im polyamide specific for the hTGF-β1 promoter nonspecifically
blocks target factor binding in the region of the FSE2 element and thus inhibits hTGF-β1 promoter activity.

Factors such as chromatin packing, polyadenylation, splicing, mRNA stability, and translation initiation could influence gene expression (Kozak, 1992; McKeown, 1992; Decker and Parker, 1994; Berger and Felsenfeld, 2001). Synthetic Py-Im polyamides can access their target sites in the nucleosome and may influence chromatin structure (Gottesfeld et al., 2001). It has been reported that pyrimidine opens the heterochromatic brown satellite to allow binding of GAGA factor, resulting in a phenotypic change in Drosophila melanogaster. Because polyamides can be readily synthesized and designed to target any sequence of interest, such polyamides may be useful in studies of genome function and eventually in gene therapies.

It is believed that gene silencing occurs via inhibition of gene expression by the Py-Im polyamides binding to regulatory sequences. Therefore, the inhibition of transcription by Py-Im polyamides at coding regions is difficult because the polyamides are removed from duplex DNA during transcription. Recently, Shinohara et al. (2004) had demonstrated the sequence-specific gene silencing in mammalian cells by alkylating Py-Im polyamides. Extensive inhibition of gene expression by alkylating Py-Im polyamides targeting a coding region is a powerful tool on the gene suppression therapy for cancers or viral infection. Inhibition of gene expression by nonalkylating Py-Im polyamides targeting a regulatory sequence is more physiological compared with that by alkylating polyamides, because this method abolishes the enhancing effect of the targeting transcription factor and preserves the basic expression of the target gene. Suppression of gene expression by nonalkylating polyamides should be a more efficient method for the therapy of chronic nonmalignant disease.

As gene-suppressive agents, nucleic acid medicines such as antisense DNA and ribozymes are easily degraded by nucleases in vivo. However, Py-Im polyamides are chemical compounds that are resistant to nucleases. Therefore, it is possible that Py-Im polyamides could be used as novel, orally administrated gene therapy agents. In conclusion, the synthetic Py-Im polyamide designed to bind the hTGF-β1 promoter inhibited both hTGF-β1 gene and protein expression, suggesting that the Py-Im polyamide may be a novel gene therapy for treatment of TGF-β-related diseases.

References