Development of Gene Therapies for Cardiovascular and Renal Diseases by Nucleic Acid Medicines

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Abstract: Nucleic acid medicines such as antisense DNA, antisense peptide nucleic acid (PNA), ribozyme, and decoy are expected to be novel therapeutic strategy for sever diseases which are resistant to present therapy. We have developed antisense DNA, antisense PNA and ribozyme targeting platelet-derived growth factor (PDGF) A-chain and transforming growth factor-β1 (TGF-β1) for arterial proliferative diseases such as coronary artery stenosis after angioplasty or stent implantation, hypertensive vascular diseases and atherosclerosis, and progressive renal diseases. Antisense DNA to PDGF A-chain inhibited arterial growth in spontaneously hypertensive rats without lowering blood pressure and inhibited the neointima formation of pig coronary artery after stent implantation. Ribozymes to PDGF A-chain and TGF-β1 specifically inhibited the target transcripts and prevented the neointima formation. Ribozymes to TGF-β1 improved renal damages in hypertensive rats. These nucleic acid medicines targeting PDGF A-chain and TGF-β1 will be feasible gene therapies for the arterial proliferative diseases and progressive renal diseases. Pyrrole-imidazole polyamides are novel gene scilencing compound, which bind to minor grove of double strand DNA by base-specific manner to inhibit gene expression. We developed pyrrole-imidazole polyamide to TGF-β1 and confirmed that the polyamide binds to the TGF-β1 promoter. The polyamide inhibited TGF-β1 promoter activity and decreased expression of TGF-β1 *in vitro* and *in vivo*. The polyamide markedly improved the renal injury in hypertensive rats. The pyrrole-imidazole polyamide will be a novel gene scilencing agent for cardiovascular and renal diseases.

INTRODUCTION

Cardiovascular disease, heart disease and stroke combined, accounts for the highest incidence of mortality among Japanese. Cardiovascular diseases induced by arterial proliferative diseases such as restenosis of coronary artery, atherosclerosis and hypertensive vascular diseases which are basically associated with the arterial damages resulting from the exaggerated growth of vascular smooth muscle cells (VSMC). On the other hand, there are still no effective treatments for progressive renal disease, resulting in 35,000 new hemodialysis patients each year. Gene therapy is now being considered for these diseases.

Gene therapy had been applied for sever diseases such as AIDS or cancer. However, gene therapy is going to be applied for common diseases resistant to the present therapies. Gene therapy can be classified as the suppliment of genes for diseases caused by gene deficient or gene abnormality, and the suppression of gene expression for diseases caused by increases in specific genes or proteins. The later is by the nucleic acid medicines. The nucleic acid medicines are expected to be novel therapeutic strategy since they can be designed freely and applied to any diseases.

Nucleic acid medicines include antisense DNA, antisense peptide nucleic acid (PNA), triple-helix forming oligonucleotides, decoy, ribozyme and small interference RNA (siRNA). We have developed antisense DNA, antisense PNA, ribozyme, and a novel genetherapy agent pyrrole-imidazole polyamide targeting transforming growth factor-β (TGF-β) or platelet-derived growth factor (PDGF) A-chain for arterial proliferative diseases and progressive renal diseases.

MECHANISMS OF ARTERIAL PROLIFERATIVE DISEASES AND PROGRESSIVE RENAL DESEASES

Investigation of responsible factors for the target diseases is important in development of the nucleic acid medicines. To clarify the responsible factors in the arterial proliferative diseases, we investigated mechanisms underlying the exaggerated growth of VSMC from spontaneously hypertensive rats (SHR) which are good model for VSMC in neointima of artery [1]. We have found that SHR-derived VSMC show the synthetic phenotype by which angiotensin II (Ang II) and Ang II-related growth factors such as TGF-β, PDGF A-chain, and basic fibroblast growth factor are endogenously produced from the cells and induce the exaggerated growth [2-7]. In addition, we found abnormality of TGF-β [8-10] and the shorten cell cycle in response to Ang II [11] in SHR-derived VSMC. At least two distinct subtypes of Ang II receptors have been identified on the basis of their differential pharmacological and biochemical properties, and they are designated type 1 receptor and type 2 receptor [12, 13]. VSMC usually express only type 1 receptor [14]. We found type 2 receptor gene transfer down-regulates type 1 receptor in normal VSMC, but not in VSMC from SHR [15, 16]. These abnormalities are considered to be associated with the exaggerated growth of VSMC from SHR.

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It is considered therefore that the neointimal VSMC in the injured artery after angioplasty show the synthetic phenotype and increasingly produce Ang II and growth factors such as TGF-β and PDGF A-chain which induce the arterioproliferation. In addition, TGF-β involves in pathogenesis of the progressive renal diseases [17]. PDGF is a potent stimulator for mesenchymal cells such as VSMC and renal mesangial cells. We demonstrated contributions of TGF-β [18] and PDGF A-chain [19] in cardiovascular organs and kidney in stroke-prone spontaneously hypertensive rats which show sever hypertension and cardiovascular and renal damages.

Thus we chose TGF-β and PDGF A-chain as target factors for the nucleic acid medicines to treat arterial proliferative diseases and progressive renal diseases.

ANTISENSE

1. Principle of Antisense

Short antisense DNA which is internalized by the cells *via* an endocytotic mechanism, inhibits expression of the target gene primarily by forming hybrids with their mRNA. Consequently, it causes a depletion of the respective protein(s) and, hence, changes the corresponding cellular functions. In addition, antisense DNA may trigger RNase H-mediated degradation of the target RNA and interfere with the processing of pre-mRNA.

2. Modification of Antisense

Since natural oligonucleotides are degraded rapidly in biological systems, the artificial oligonucleotides are designed to be more stable against. Modification of the phosphate linkage has been the first successful strategy for antisense drug developments and Fomivirsene the first antisense drug in therapy, which has resulted in a revolutionary spin off to antisense research leading to a second generation of antisense oligonucleotides [20]. The third generation of antisense oligonucleotides contains structure elements, which enhance the antisense action. Zwitterionic oligonucleotides show remarkable results, first, because the stability against ribozymes is largely increased, and secondly, because the electrostatic repulsion between the anionic sense and the zwitterionic antisense cords is minimized [21]. Promising new target molecules in antisense reseach are oligonucleotide chimares, which enhance the antisense. Thus antisense DNA should be chemically modified for resistance for nuclease.

3. Antisense Treatments for the Arterial Proliferative Diseases

We investigated the effects of an antisense DNA to PDGF A-chain on growth of VSMC from SHR. SHR-derived VSMC show the sysnthetic phenotype and are a model for intimal VSMC. Antisense DNA to PDGF A-chain inhibits growth of VSMC from SHR both *in vitro* and *in vivo* (Fig. **1**) [22, 23]. We examined effects of antisense DNA to PDGF A-chain on the stenosis of coronary artery with stent and found that the antisense inhibited the neointima formation (Fig. **2**).

Biotechnology companies in the United States have developed antisense oligodenoxynucleotides (ODNs) that are resistant to degradation by nucleases by chemical modification to improve stability *in vivo*. We developed an antisense DNA to PDGF A-chain that was completely resistant to nuclease degradation due to an MMI-linkage in collaboration with ISIS Pharmaceuticals (San Diego, CA, USA) [23].

PNA is a DNA mimic in which the entire deoxyribose-phosphate backbone is replaced by a peptide chain. PNA can hybridize with DNA and RNA through Watson and Crick base pairing and helix formation [24]. Antisense PNA has advantages of high stability and resistance to degradation by nucleases, because of the peptide backbone provides superior hybridization properties. However, antisense PNA has a

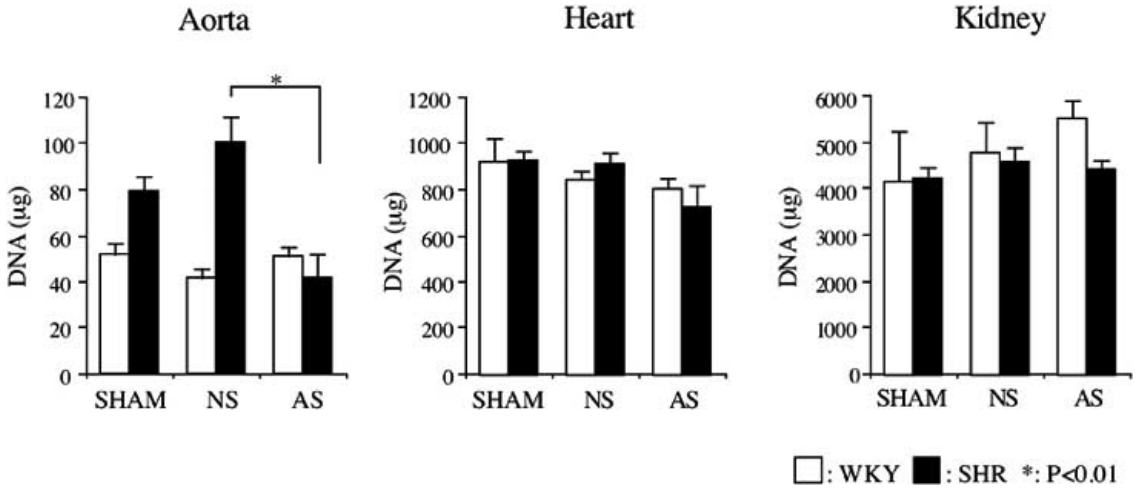

Fig. (1). DNA content of aorta, heart and kidney after infusions of antisense oligodeoxynucleotide (ODN) to PDGF A-chain in WKY rats and SHR. WKY rats (open column) and SHR (closed column) at 15 weeks of age were divided into 3 groups. The sham infusion group (SHAM) was given subcutaneous infusion of water for 4 weeks. The nonsense (NS) and antisense (AS) groups were given subcutaneous infusion of nonsense or antisense ODN (80 ng/g body weight/day) using the pump for 4 weeks. Data are the mean± SEM (n=4). *: $P < 0.01$ between the nonsense and antisense groups. Ref. [22].

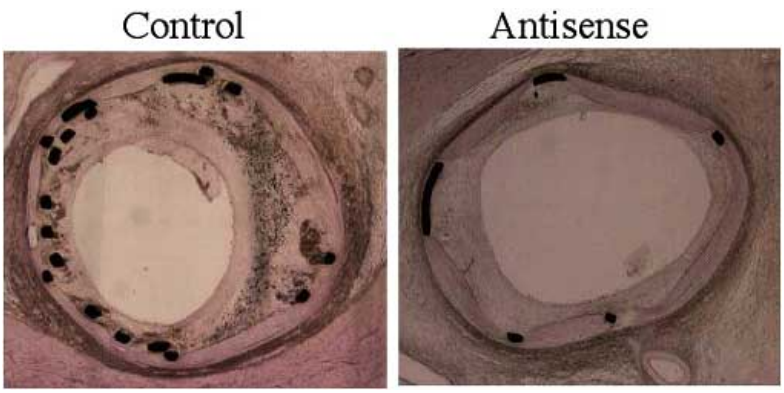

Fig. (2). Effects of antisense oligodeoxynucleotide (ODN) to PDGF A-chain on neointima formation of coronary artery after stent implantation. Control group implanted with stent coated with saline and antisense group implanted with stent coated with 100 µg of antisense ODN. Atisense ODN was diluted to 100 µg in saline, and 100 µl of 20 kDa polyethylenimine reagent was diluted in 50 µl of saline. After standing at room temperature for 45 min, the two regents were combined and incubated at room temperature for 15 min. The combined regents were corted on stent with hydrogel. Four weeks after implantations of the stents hearts were removed. Specimens were cross-sectioned of porcine coronary artery in stent with or without antisense ODN to PDGF A-chain.

disadvantage of poor membrane permeability in comparison to that for antisense DNA [25].

We developed an antisense PNA to PDGF A-chain and found that it inhibited growth VSMC from SHR [26], suggesting that this antisense PNA to PDGF A-chain may be useful for prevention of restenosis.

3. Antisense Treatments for Hypertensive Renal Diseases

Therapeutic target for hypertension is not only for the reduction of blood pressure but also is to improve damages in heart, vessel and kidney. In our previous studies, antisense DNA targeting PDGF A-chain improved nephrosclerosis in stroke-prone SHR without any reduction of blood pressure [19]. These findings suggest that the cardiovascular and renal organ damages in hypertension are partially independent to the high blood pressure and the treatment with antisense DNA targeting PDGF A-chain is effective to treat hypertension.

DECOY

Transcription of various genes can be inhibited by double-stranded ODNs that compete with DNA binding proteins for specific binding sites in promoters and enhancers. Transfection of double-stranded ODN as a decoy is one of the nucleic acid medicines and permits examination of regulation of specific genes [27]. Morishta *et al*. [28] reported that a decoy ODN targeting E2F, which controls cell cycle progression, inhibits restenosis of the coronary artery. In addition, the E2F decoy was also shown to prevent stenosis of venous grafts in the PREVENT I study [29]. On the basis of these results, the E2F decoy has been used in a clinical trial to prevent restenosis of coronary artery after PTCA.

RIBOZYME

1. Principle of Ribozyme

In the central dogma of gene expression RNA was solely considered the molecule to direct peptide synthesis. However, RNA can also act as an enzyme and is capable of catalyzing RNA splicing and cleavage as well as several other chemical reactions. Most genes have exons that are interrupted by stretches of noncoding DNA called introns. Transcripts of such genes undergo cleavage-ligation reactions to produce the mature functional mRNA.

Ribozymes are RNA molecules that hybridize to and cleave enzymaticaly target RNAs [30]. Hammerhead ribozyme, which is the smallest ribozyme, is approximately 30 nucleotides long and catalytes site-specific cleavage of phosphodiester bonds [31]. The hammerhead ribozyme motif comprises three base-paired helices connected by two single-stranded regions. In general, the single-stranded regions, which contain the catalytic domain, are largely invariant, whereas the stems are less conserved. Hammerhead ribozyme consists of antisense arms (stems I and III) and a catalytic consensus sequence with a flanking stem II and loop section (Fig. **3A**). The catalytic consensus sequence cleaves the target RNA. Once the target has been cleaved, the ribozyme dissociates from the cleaved transcript and repeats the process with another RNA molecule.

2. Ribozyme Treatments for Arterial Proliferative Diseases

We designed and synthesized a 38-base chimeric DNA-RNA hammerhead ribozyme with two phosphorothioate linkages at the 3' terminal that cleaves rat PDGF A-chain mRNA at the GUC sequence (Fig. **3B**). We then examined its effects on the exaggerated growth of VSMC from SHR and found that the ribozyme effectively and specifically inhibited the exaggerated growth of VSMC from SHR. This effect was mediated by cleavage of the rat PDGF A-chain mRNA resulting in reduced production of rat PDGF-AA protein production [32, 33]. To evaluate the effect of this chimeric DNA-RNA hammerhead ribozyme to PDGF A-chain on restenosis after angioplasty, we examined the effects of this ribozyme on neointima formation in rat carotid artery after balloon injury *in vivo*. The ribozyme reduced neointima formation by 55% (Fig. **4**), and inhibited expression

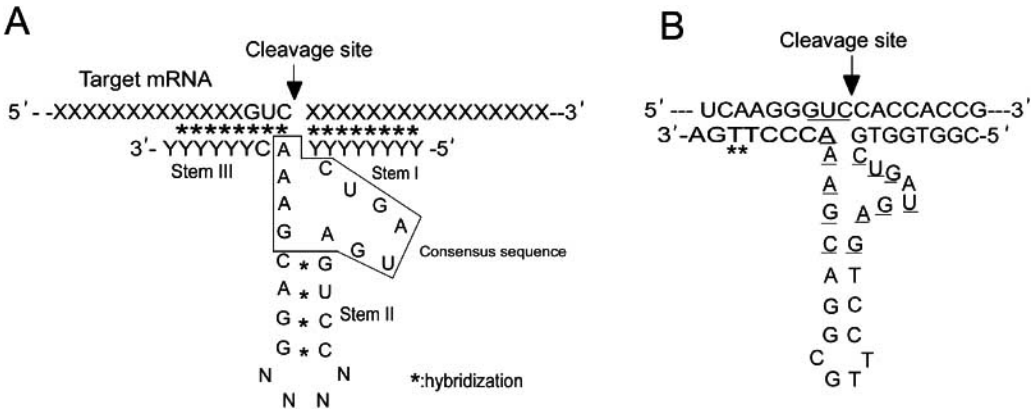

Fig. (3). (A) Basic structure of a hammerhead ribozyme. Box includes consensus sequence which has cleavage activity. (B) Sequences of the DNA-RNA chimeric hammerhead ribozyme to rat PDGF A-chain mRNA. The ribozyme (lower) cleaves the GUC codon of PDGF A-chain mRNA (upper) as indicated by arrow. Ribonucleotides of ribozyme are underlined. Phosphoro-thioate linkages are marked by star.

of PDGF A-chain mRNA and production of PDGF-AA protein in injured vessels, suggesting that ribozyme blocks neointima formation through degradation of PDGF A-chain mRNA [34]. One of advantage of ribozyme as gene therapy is its specificity to target molecule. We examined effects of the chimeric ribozyme to PDGF A-chain on expression of transcripts in the injured rat carotid artery by microarray analysis, and found that the ribozyme to PDGF A-chain completely inhibited only expression of PDGF A-chain, suggesting the specificity of ribozyme to target molecule (Fig. **5**).

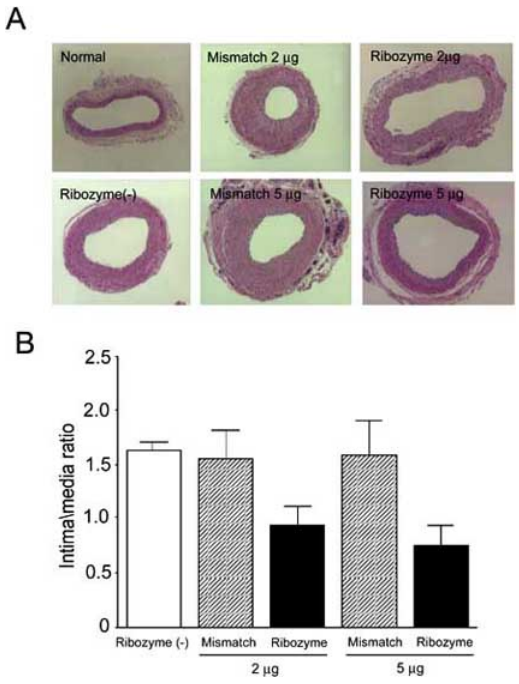

Fig. (4). Effect of chimeric DNA-RNA ribozyme against PDGF A-chain on neointima formation in rat carotid artery 2 weeks after balloon injury. Two or 5 µg of ribozyme or mismatch ribozyme was incubated within the artery lumen for 10 min. (**A**) Specimens were cross-sectioned at 3 µm and stained with hematoxylin and eosin. (**B**) Intimal and medial cross-sectional areas of four cross sections of artery obtained from each rat were measured. The intima/media cross-sectional area ratios were determined. Data are the mean± SEM (n=4). * $P < 0.05$ *vs* balloon injury without ribozyme. Ref. [34].

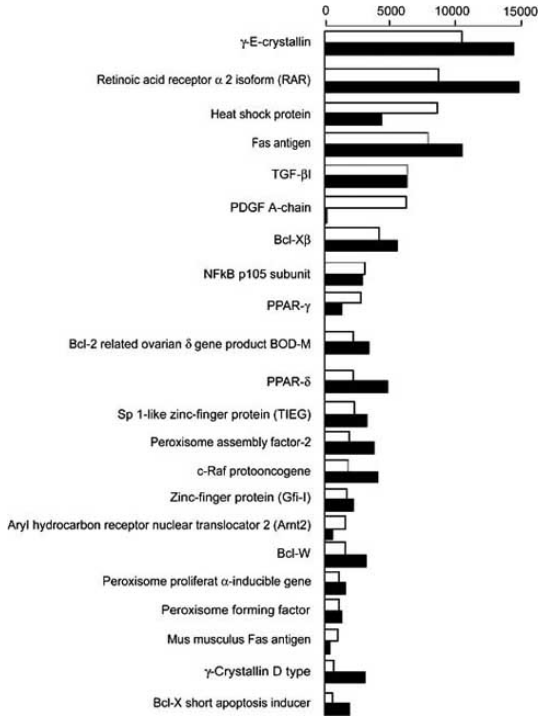

Fig. (5). Increments in transcript of cytokines, growth factors, and transcription factors analyzed by microarray (R-U34 GeneChip Array, Affymetrix) in carotid artery 6 hours after balloon injury treated with (closed column) or without (open column) 5 µg of chimeric DNA-RNA ribozyme to PDGF A-chain. Final transcript values are means of duplicate values in injured vessel minus the average values of non-injured vessel. Ref. [34].

In addition, we constructed a replication-deficient recombinant adenovirus that expresses a ribozyme specific for PDGF A-chain mRNA as endogenous delivery for ribozyme in tissue. Transfection of this vector significantly reduced proliferation of VSMC from SHR in a dose-dependent manner [35]. We also examined the effect of adenovirus-encoded ribozyme to PDGF A-chain on neointima formation in rat carotid artery after balloon injury. Decreased expression of PDGF A-chain mRNA and protein in rat carotid artery was observed at 14 days after balloon injury, suggesting sustained suppression of expression of PDGF A-chain due to expression of the ribozyme encoded by the adenovirus vector system. Adenovirus-encoded ribozyme to PDGF A-chain reduced the neointima formation by 70%, which is greater than the efficacy of the chimeric DNA-RNA ribozyme to PDGF A-chain [36]. Therefore, the hammerhead ribozyme to PDGF A-chain encoded by an adenovirus may be a potential gene therapy for prevention of restenosis.

3. Ribozyme Treatments for Hypertensive Renal Diseases

A number of publications have identified TGF-β1 as a critical factor in kidney diseases such as glomerulosclerosis [37] and mesangioproliferative glomerulonephritis [38]. We designed chimeric DNA-RNA hammerhead ribozyme targeting TGF-β1 and confirmed suppression of TGF-β1 expression in VSMC from SHR [39]. In addition, we examined effects of the chimeric ribozyme to TGF-β1 on hypertensive renal damage in SHR-SP and Dahl salt-sensitive rats, which show increased expression of TGF-β1 in kidney cortex. One intraperitorial injection of chimeric DNA-RNA hammerhead ribozyme to TGF-β1 considerably improved wall thickness of renal capillary artery and glomerulosclerosis in SHR-SP (Fig. **6**). The ribozyme to TGF-β1 significantly inhibited expression of TGF-β1 and extracellular matrix molecules such as type I collagen and fibronectin. These findings suggest that the ribozyme to TGF-β1 also will be feasible for progressive renal diseases.

PYRROLE IMIDAZOLE POLYAMIDES (PY-IM)

Pyrrole-Imidazole (Py-Im) polyamides are small synthetic molecules composed of aromatic rings N-methylpyrrole (Py) and N-methylimidazole (Im) amino acid [40-42]. Aromatic Py and Im can be coupled and adopt a U-shape conformation in the presence of γ-aminobutyric acid. Such synthetic polyamides can bind to specific base pairs in the minor groove of the DNA double helix with high affinity and specificity. Base pair specificity is dependent on the pairing of Py and Im. The Py/Im pair targets C-G base pairs. Im/Py recognizes G-C base pairs, and a Py/Py binds both A-T and T-A base pairs [40-42]. It was recently reported that the A-T degeneracy can be overcome by replacing one pyrrole ring of the Py/Py pair with 3-hydroxypyrrole (Hp); Hp/Py preferentially binds T-A pairs [43].

We synthesized a Py-Im polyamide to target the promoter of human TGF-β1 adjacent to the fat-specific element 2. Gel mobility shift assays showed that the synthetic Py-Im polyamides bound to the corresponding double-stranded oligonucleotides, whereas the mismatch polyamides did not. FITC-conjugated Py-Im polyamide has been detected in the nuclei of VSMC. Py-Im polyamide significantly decreased the activity of the TGF-β1 promoter (Fig. **7**) [44]. In cultured human VSMC, this polyamide inhibited expression of the TGF-β1 mRNA and protein. Taken together these findings indicate that a synthetic Py-Im polyamide designed to bind TGF-β1 sequence could be used as a gene therapy to inhibit TGF-β1 activity.

Py-Im polyamides are resistant to nucleases and delivered into cells and tissues without vectors and delivery

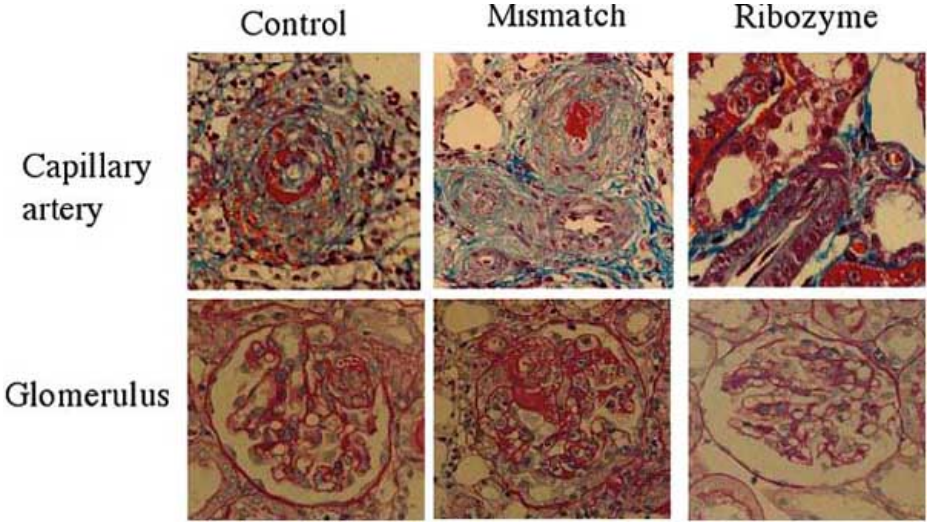

Fig. (6). Histological findings in renal cortex of salt-loaded SHR-SP and Dahl salt-sensitive rats treated with chimeric DNA-RNA ribozyme to TGF-β1. Twelve-week-old male SHR-SP or 7-week-old male Dahl salt-sensitive rats were loaded with 1% salt water for 6 or 8 weeks, respectively. After 4 weeks of salt loading, the mismatch or ribozyme group received an intraperitoneal injection of mismatch ribozyme or chimeric DNA-RNA ribozyme to TGF-β1 (200 μg/body weight). Renal cortex was fixed and stained with Masson trichrome for SHR-SP or hematoxylin-eosin for Dahl-S rats. Bar = 20 μm.

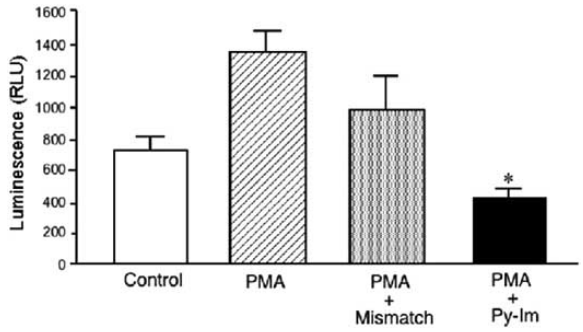

Fig. (7). Effects of Py-Im polyamide targeting TGF-β1 on TGF-β1 promoter activity. One microgram of pGL3-TGF-β1 was transfected into human VSMC with lipofectamine reagent. Twenty-four hours after transfection, cells were incubated with 1 nM Py-Im polyamide targeting TGF-β1 or mismatch polyamide in the 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. Ref. [44].

reagents. FITC-labeled Py-Im polyamide administered intervenously in rats delivered into several organs including aorta and kidney, and excretion into urine detected by HPLC. Specificity of the Py-Im polyamides to target molecules can be obtained by designing to span the boundary of the transcription binding site and the promoter sequence. These findings suggest that Py-Im polyamide will be feasible novel gene therapy agents for cardiovascular and progressive renal diseases.

PERSPECTIVES

Antisense and ribozyme technology have been useful but are not always reliable or robust. siRNA has emerged as a novel technology that offers great hope and promise to knock down a target gene [45, 46]. Triple-helix forming oligonucleotides are short, synthetic single-stranded DNAs that recognize polypurine:polypyrimidine regions in double stranded DNA in a sequence-specific manner and form triplex and act to an anti-gene [47] In addition, several modifications for antisense DNA and ribozymes have been developed to get resistance to nucleases. Locked nucleic acid (LNA) is a class of nucleic acid analogs possessing unprecedented binding affinity toward complementary DNA and RNA [48]. These strong nucleic acid medicines are expected to be useful nucleic acid medicines to suppress the target molecules completely.

Py-Im polyamides will be developed through genomic engineering in a field now known as chemical genomics. Chemical genomics may become the specific field that connects molecular medicine and diagnosis. The recently completed sequence of the human genome and genomes of several pathogenic organisms provides a large number of potential targets for nucleic acid medicines or Py-Im polyamides that may be of benefit in clinical medicine. Because nucleic acid medicines and Py-Im polyamides can be stabilized to prevent biological degradation, they are emerging as new and broadly useful classes of therapeutic agents. We anticipate that gene therapies with nucleic acid medicines may be common for many diseases in the future.

ABBREVIATIONS

PNA = Peptide nucleic acid

PDGF = Platelet-derived growth factor

TGF = Transforming growth factor

siRNA = Small interference RNA

VSMC = Vascular smooth muscle cells

SHR = Spontaneously hypertensive rats

Ang II = Angiotensin II

ODNs = Oligodenoxynucleotides

Py-Im = Pyrrole-Imidazole

Py = N-methylpyrrole

Im = N-methylimidazole

LNA = Locked nucleic acid

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