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Cardiomyocyte-targeted siRNA delivery by prostaglandin E₂-Fas siRNA polyplexes formulated with reducible poly(amido amine) for preventing cardiomyocyte apoptosis

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Abstract

A cardiomyocyte-targeted Fas siRNA delivery system was developed using prostaglandin E₂ (PGE₂)-modified siRNA polyplexes formed by a reducible poly(amido amine) to inhibit cardiomyocyte apoptosis. PGE₂, which was used as a specific ligand for cardiomyocyte targeting, was conjugated to the terminal-end of the sense siRNA (PGE₂-siRNA). The reducible cationic copolymer, synthesized via Michael-type polyaddition of 1,6-diaminohexane and cystamine bis-acrylamide (poly(DAH/CBA)), tightly condensed the PGE₂-siRNA conjugate to form nanosize polyplexes having a diameter of 100-150 nm. The PGE₂-siRNA/poly(DAH/CBA) polyplexes decomplexed to release PGE₂-siRNA in a cytosolic reducing environment due to the degradation of the reducible poly(DAH/CBA). The cellular uptake of the PGE₂-siRNA/poly(DAH/CBA) polyplex was increased in rat cardiomyocytes (H9C2 cells) due to PGE₂ receptor-mediated endocytosis. When H9C2 cells were transfected with siRNA against Fas, a key regulator of ischemia-induced apoptosis, the PGE₂-Fas siRNA/poly(DAH/CBA) polyplex delivery system led to a significant increase in Fas gene silencing, resulting in inhibition of cardiomyocyte apoptosis. The PGE₂-Fas siRNA/poly(DAH/CBA) polyplex did not induce interferon-alpha in peripheral blood mononuclear cells. These results suggest that the PGE₂-Fas siRNA/poly(DAH/CBA) polyplex formulation may be clinically applicable as a cardiomyocyte-targeted Fas siRNA delivery system to inhibit apoptosis in cardiovascular disease.

Keywords

Cardiomyocyte; siRNA; Fas; Prostaglandin E₂; Reducible cationic polymer

1. Introduction

Cardiovascular disease is one of the leading causes of morbidity and mortality in the world despite technical improvements in prevention, detection, and treatment [1]. In recent years, there has been an increasing interest in the development of gene therapy for cardiovascular

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disease due to significant advances both in the understanding of the molecular mechanism of cardiovascular disease and in gene transfer technology [2]. The molecular mechanisms underlying cardiomyocyte apoptosis, a crucial pathway of myocardial cell injury and death in heart disease, has been one of the areas of greatest interest [3]. Among the various genes which mediate apoptosis, Fas has been identified as an inducer of cardiomyocyte apoptosis caused by ischemic injury [4].

RNA interference (RNAi) is a post-transcriptional gene silencing phenomenon in which 21-23 nucleotide double-stranded RNA (small interfering RNA: siRNA) triggers the degradation of messenger RNA (mRNA) in a sequence-specific fashion [5]. Since RNAi technology can be utilized to inhibit the expression of disease inducing genes, it is a potentially powerful therapeutic strategy for the treatment of gene-related disease [6,7]. The clinical application of RNAi technology to cardiovascular gene therapy, however, has been hindered by the inability to deliver siRNA effectively, due to its inherent instability and poor cellular uptake [8]. Furthermore, cell-selective delivery of therapeutic siRNA is critical to achieve its maximum therapeutic potential in cardiovascular disease. The successful clinical application of siRNA to cardiovascular gene therapy, therefore, depends on the development of a highly efficient and cell-specific gene delivery system.

Specific ligand-modified gene delivery systems are a promising strategy to successfully control gene expression in target cells. The specific ligands bind to cell surface receptors and increase cellular uptake of therapeutic genes via target receptor-mediated endocytosis [9]. There have been many cell-selective siRNA delivery systems constructed using specific ligands for cancer or liver cells [10,11]. Cardiovascular targets for siRNA therapy, however, have no specific vector system, except physical targeting methods. Prostaglandin E₂ (PGE₂) is involved in numerous physiological mechanisms including the contraction and relaxation of smooth muscle, control of blood pressure, and modulation of inflammation [12]. Its diverse physiologic effects are exerted via four distinct PGE₂ receptors (EP1-4) located on the cell surface [13], and PGE₂ receptors (EPs) participate in agonist-induced internalization upon PGE₂ stimulation [14]. Although the expression levels of EPs vary between different species, EP4 has been reported to be highly expressed in the hearts of several species, including humans [15]. Accordingly, we hypothesized that the use of PGE₂ as a specific ligand for cardiovascular-targeted siRNA delivery would increase the efficiency of siRNA transfer to cardiomyocytes.

In our recent studies, we have reported the development of linear and branched types of reducible cationic copolymers (disulfide-containing poly(amido amine)s, SS-PAA), synthesized by Michael-type addition between various amine-containing monomers and cystamine bis-acrylamide, as effective gene carriers [16-19]. The linear/branched SS-PAA formed stable nanosize polyplexes with both plasmid DNA and siRNA and remarkably increased gene-transfer efficiency with significantly lower cytotoxicity in various cell lines compared to the conventional cationic polymer PEI (25-kDa). In the present study, a linear SS-PAA, synthesized by copolymerization between 1,6-diaminohexane and cystamine bis-acrylamide (poly(DAH/CBA)), served as a powerful vector to carry Fas siRNA into rat cardiomyocytes (H9C2 cells). PGE₂ was conjugated to Fas siRNA molecules, and the synthesized PGE₂-Fas siRNA contributed to cardiomyocyte targeting via PGE₂ receptor-mediated intracellular delivery. The structural characteristics of the reducible poly(DAH/CBA), including siRNA polyplex formation and decomplexation, were studied by DLS and gel retardation analysis. The cardiomyocyte-targeted delivery of the PGE₂-Fas siRNA/poly(DAH/CBA) polyplex was examined using a PGE₂-receptor competition assay. Fas gene silencing and the anti-apoptotic effects of the PGE₂-Fas siRNA polyplex were investigated by RT-PCR and FACS analysis. Induction of an interferon- α response by the PGE₂-Fas siRNA/poly(DAH/CBA) polyplex was evaluated in peripheral blood mononuclear cells (PBMCs).

2. Materials and methods

2.1. siRNAs

siRNAs were chemically synthesized and supplied by Dharmacon Co. (Lafayette, CO). The target sequences used for rat Fas siRNA were no.1: ACA CGG ACA GGA AAC ACT A; no. 2: AGG AAA CAC TAT TCT GAT A; no.3: ACC AAA TGC AAG AAA CAA A; no.4: CGG GAA GAC TGG TGC GTG T. Fas siRNA candidates were selected from rat Fas mRNA sequences using siRNA Target Finder (Ambion). Green fluorescent protein (GFP) siRNA (target sequence: GCA CGA CTT CTT CAA GTC C) was used as the control siRNA without sequence homology to the Fas gene. All siRNAs were designed with symmetric 3'dTdT-overhang (Fig. 1a). For the synthesis of the PGE₂-Fas siRNA conjugate, Fas siRNA no.1 was modified with an amino-6 carbon linker at the 3'-overhang region of the sense strand. For the cellular uptake study, the 3'-terminal end of the anti-sense siRNA was further modified with Cy3 dye.

2.2. Preparation of PGE₂-Fas siRNA conjugate

PGE₂-Fas siRNA conjugate was synthesized as shown in Fig. 2. The terminal carboxyl group of PGE₂ was primarily activated in the DCC/NHS reaction. PGE₂ (12.62 µg, 50 nmol, Sigma-Aldrich, St. Louis, MO) dissolved in anhydrous DMSO was reacted with DCC (10.3 µg, 50 nmol) and NHS (5.8 µg, 50 nmol) at room temperature. After the reaction reached completion in 4 h, the reaction solution was filtrated (0.45 µm syringe filter) to remove the insoluble byproduct dicyclohexylurea. The activated PGE₂ was conjugated to the amine-terminated Fas siRNA (75 mg, 5 nmol). After incubation for 2 h at room temperature, the resulting product was dialyzed against PBS buffer (pH 4.0-5.0, MWCO 1000) at 4 °C. The synthesis of PGE₂-Fas siRNA was assessed by reversed phase-high performance liquid chromatography (RP-HPLC) on a C18 column (200 × 4.6 mm, 5 µm, Waters, Water 26955 Alliance liquid-chromatography system) equipped with a UV detector at 260 nm. The conjugation percent of PGE₂-Fas siRNA was more than 98% as determined by HPLC analysis using the external standard for naked siRNA.

2.3. Synthesis of poly(DAH/CBA)

The reducible cationic copolymer poly(DAH/CBA) was synthesized as previously described [19]. Briefly, *N*-Boc-1,6-diaminohexane (*N*-Boc-DAH) (0.216 g, 1 mmol) and cystamine bis-acrylamide (CBA) (0.260 g, 1 mmol) were dissolved in 10% aqueous MeOH. The Michael addition polymerization was performed in the dark under a nitrogen atmosphere at 60 °C for 3 days. Excess amount of *N*-Boc-DAH was added to stop the polymerization by consuming any unreacted acrylamide groups. The reaction was performed at 60 °C for 2 h. The resulting solution was precipitated with anhydrous diethyl ether and freeze-dried. The terminal *N*-Boc protecting group was removed under acidic conditions (trifluoroacetic acid/triisobutylsilane/water; 95/2.5/2.5) at room temperature for 1 h. The final product was purified by dialysis (MWCO 1000) against deionized water and lyophilized. Poly(DAH/CBA) was analyzed by using ¹H NMR in D₂O: 3.15 (NCH₂CH₂CH₂CH₂CH₂CH₂NH₂, 2H), 1.48 (NCH₂CH₂CH₂CH₂CH₂CH₂NH₂, 2H), 1.19 (NCH₂CH₂CH₂CH₂CH₂CH₂NH₂, 2H), 1.19 (NCH₂CH₂CH₂CH₂CH₂CH₂NH₂, 2H), 1.48 (NCH₂CH₂CH₂CH₂CH₂CH₂NH₂, 2H), 2.85 (NCH₂CH₂CH₂CH₂CH₂CH₂NH₂, 2H), 2.81 (NCH₂CH₂CO, 4H), 2.52 (NCH₂CH₂CO, 4H), 3.35 (CONHCH₂CH₂S-S, 4H), 2.65 (CH₂S-S-CH₂, 4H). The synthesized poly(DAH/CBA) has an weight average molecular weight (MW) of 3520 (polydispersity index, 1.13), which was determined by size exclusion chromatography (SEC) on an AKTA FPLC system (Amersham Biosciences, Piscataway, NJ, Superose 12 column) with standard poly[*N*-(2-hydroxypropyl) methacrylamide].

2.4. Preparation and characterization of PGE₂-Fas siRNA/poly(DAH/CBA) polyplex

The synthetic PGE₂-Fas siRNA was complexed with poly(DAH/CBA) and formed nanosize polyplexes as shown in Fig. 2. To analyze the particle size of the PGE₂-Fas siRNA/poly(DAH/CBA) polyplex, a dynamic light scattering (DLS) study was carried out at different N/P ratios from 0 to 30. The polyplexes were formed by mixing 1.5 µg of PGE₂-Fas siRNA with poly(DAH/CBA) (0-36 µg) in an aqueous phase (PBS 0.2 ml, pH 7.0) and incubating at room temperature for 30 min. After a 10-fold dilution of each mixture, the hydrodynamic diameter was measured by a DLS instrument (Zeta-Plus, Brookhaven Instrument Co., New York). The polyplex formation was also confirmed by electrophoretic mobility shift assay. The fixed amount of PGE₂-Fas siRNA (0.3 µg) was complexed with varying amounts of poly(DAH/CBA) (0-7.2 µg) at room temperature for 30 min. To examine the cleavage characteristics of poly(DAH/CBA) in a reductive state, the PGE₂-Fas siRNA/poly(DAH/CBA) polyplexes formed at different N/P ratios were incubated with 10 mM glutathione (GSH) at 37 °C for 18 h, which correlates to the intracellular concentration of GSH (typically 5-10 mM). Each sample solution was loaded onto a 2.0% agarose gel and visualized by ethidium bromide staining. Electrophoresis was conducted by applying 120 V for 15 min in 1 × TAE buffer.

2.5. Cell culture and transfection

H9C2 cells, a clonal line derived from rat heart (ATCC, Manassas, VA), was maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator with 5% CO₂. All cell culture products, including FBS, Dulbecco's phosphate buffered saline (PBS), and Dulbecco's modification of Eagle's medium (DMEM), were supplied from Invitrogen (Carlsbad, CA). Cells were seeded in 50-mm diameter plastic Petri dishes at a density of 3×10^5 cells per dish and incubated for 24 h. The cell culture medium was replaced with serum-free medium prior to transfection. Lipofectamine (Invitrogen, Carlsbad, CA) was used only in the selection of the Fas siRNA target sequence. To select the Fas siRNA target sequence, four different Fas siRNAs (50 nM) were condensed with Lipofectamine according to the manufacturer's instructions and transfected to cells in normoxic condition for 4h at 37 °C. The transfection medium was then discarded and supplemented with fresh 10% serum containing medium. Cells were further incubated for 24 h prior to RT-PCR analysis.

The anti-apoptotic activity of Fas siRNA formulations based on poly(DAH/CBA) was measured under hypoxic conditions. First, cells were transfected with unmodified and PGE₂-modified Fas siRNA no. 1 (50 nM) complexed by poly(DAH/CBA) at a N/P ratio 20. After 24 h of incubation, cells were exposed to the medium containing 1 mM hypoxia-mimic cobalt chloride (CoCl) for 18 h to apply hypoxic stress. Then cells were stimulated with 1 µg anti-Fas antibody/ml (MBL International Corporation, Watertown, MA) for 6 h to induce Fas-mediated apoptosis *in vitro*. The gene silencing activity of various Fas siRNA formulations was evaluated by RT-PCR detection of Fas mRNA, and the apoptotic cell death in H9C2 cells was measured by a propidium iodide (PI) staining flow cytometric assay.

2.6. RT-PCR analysis

Total RNA was isolated from the transfected H9C2 cells using the RNeasy® Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's recommendation. One microgram of the isolated RNA was reverse transcribed using SuperScript™ III (Invitrogen, Carlsbad, CA) and random hexamers, according to the manufacturer's protocol. PCR was performed (Invitrogen, Carlsbad, CA) on 2 µl of cDNA using Platinum Taq Polymerase (Invitrogen). All reactions were conducted under the following thermal cycling conditions: *Taq* activation; 95 °C for 5 min, PCR amplification; 36 cycles at 95 °C for 30 s, at 57 °C for 30 s, and at 72 °C for 30 s, final extension; 1 cycle 72 °C for 5 min. For the study of rat Fas siRNA selection under normoxic conditions, the number of amplification cycles was increased from 36 to 40. The

primers for rat Fas and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were: Fas forward, 5'-CTG CAG ATA TGC TGC TGT GGA TCA-3'; Fas reverse, 5'-TTT GGT GTT GCT GGT GCG T-3'; GAPDH forward, 5'-CAT GCC GCC TGG AGA AAC CTG CCA-3'; GAPDH reverse, 5'-TGG GCT GGG TGG TCC AGG GGT TTC-3'. The PCR primers to detect four subtypes of PGE₂ receptors were: EP1 forward, 5'-CGC AGG GTT CAC GCA CAC GA-3'; EP1 reverse, 5'-GCG TAG CTC CCG GCA CAG TG-3'; EP2 forward, 5'-TTC AAT GAC TCC AGG CGA GT-3'; EP2 reverse, 5'-AGG ACA GTA CTG GAC GTA CT-3'; EP3 forward, 5'-GGT ATG CCA GCC ACA TGA AGA C-3'; EP3 reverse, 5'-CAA GAT CTG GTT CAG CGA AGC C-3'; EP4 forward, 5'-TTC CGC TCG TGG TGC GAG TGT TC-3'; EP4 reverse, 5'-GAG GTG GTG TCT GCT TGG GTC AG-3'. The sizes of the PCR products for Fas, GAPDH, EP1, EP2, EP3, and EP4 were 494, 296, 222, 540, 530 and 424 bp, respectively. The PCR products were separated in a 1.0% agarose gel by electrophoresis and visualized with ethidium bromide staining.

2.7. Flow cytometry

The cellular uptake of different siRNA formulations was examined by flow cytometry using Cy3 dye-modified Fas siRNA. H9C2 cells seeded in 50-mm dishes were incubated with Cy3-labeled Fas siRNA formulations (50 nM), including naked Fas siRNA, Fas siRNA/poly(DAH/CBA), and PGE₂-Fas siRNA/poly(DAH/CBA) polyplexes, in the transfection medium with or without 0.1 mM free PGE₂. After 4 h incubation, cells were trypsinized, washed two times with cold PBS, and fixed with 75% EtOH solution for 30 min at 4 °C. The extents of intracellular uptake of Cy3-labeled Fas siRNA were monitored by flow cytometry (FACS Caliber, Becton-Dickinson, Mountain View, CA) using FL2 channel (Ex. 488 nm/Em. 575 nm) for Cy3 dye. Data were processed using Windows Multiple Document interface software (WinMDI).

To measure apoptotic cells containing hypodiploid DNA content (sub-G₀/G₁), cells were analyzed by propidium iodide (PI) staining and flow cytometry [20]. The transfected cells were trypsinized and washed with cold PBS. Cells were stored in 0.1 mM PI solution (Sigma-Aldrich) for 1 h at 4 °C in the dark. The fluorescence profiles of the PI-stained cells were collected in FL2 channel with flow cytometry (Becton-Dickinson) and further processed with WinMDI.

2.8. Interferon-alpha assay

Peripheral blood mononuclear cells (PBMCs) isolated from whole blood from healthy human donors were supplied by SeraCare Life Sciences Inc. (Gaithersburg, MD). For interferon-alpha (IFN- α) stimulation assay, 2×10^5 freshly thawed cells were seeded in 96-well plates and cultured in RPMI 1640 medium with 10% FBS. Various siRNA formulations, including naked Fas siRNA, PGE₂-Fas siRNA, Fas siRNA/poly(DAH/CBA), PGE₂-Fas siRNA/poly(DAH/CBA), and poly (DAH/CBA) alone, were added to cells at a final siRNA concentration of 3 μ g/ml. Synthetic double-stranded RNA polyinosinic:polycytidylic acid (poly(I:C)) is an artificial mimic of viral RNA which triggers an immune response in PBMCs [21]. Poly(I:C) (Sigma-Aldrich) was used as a control to stimulate production of IFN- α . The culture supernatants were collected after 24 h and assayed for INF- α by sandwich ELISA (BD Biosciences, San Diego, CA) according to the manufacturer's instructions.

3. Results and discussion

3.1. Selection of the Fas siRNA sequence

Cardiomyocyte apoptosis following myocardial ischemia/infarction plays a critical role in the progression of cardiovascular disease [22]. Apoptosis, a programmed cell death characterized by membrane blebbing, cell shrinkage, and DNA fragmentation, can be induced by two distinct

signaling pathways: (1) the mitochondrial pathway and (2) the death receptor-mediated pathway. The death receptor Fas has been shown to be a key regulator of apoptotic cell death in many cell types [4]. It has been reported that Fas is constitutively expressed in myocardium and dramatically upregulated in cardiomyocytes after exposure to ischemia/infarction [23, 24]. In particular, Fas-induced apoptosis can enlarge infarct size during exacerbations of heart failure [25]. Recent reports indicate that Fas gene silencing with RNAi attenuates hypoxia-induced apoptosis *in vitro* and *in vivo* [26,27]. Based on the potency of Fas specific RNAi, in this study we investigated the anti-apoptotic effect of a novel cardiomyocyte-targeted siRNA delivery system in H9C2 cells with a Fas siRNA sequence. Since the selection of siRNA sequences for a target gene is a crucial step in RNAi-mediated gene silencing [28], we examined four Fas siRNA candidates to select a high-potential siRNA target site in the rat Fas mRNA sequence (Fig. 1a). Of the four siRNAs synthesized, Fas siRNA no. 1 (bases 315-333) potently suppressed the expression of Fas in H9C2 cells (Fig. 1b). The RT-PCR result revealed that Fas siRNA no. 1 inhibited Fas mRNA production by 70% compared to the untreated control group. On the basis of this result, we selected Fas siRNA no. 1 as the most highly functional siRNA and used it for the further studies to develop the cardiomyocyte-targeted Fas siRNA delivery system.

3.2. Synthesis of PGE₂-Fas siRNA conjugate

The use of specific receptor-ligand interactions provides a potential method for targeted siRNA delivery, because it can improve the efficiency, specificity, and safety of the corresponding delivery systems [9-11]. PGE₂ could be a promising ligand for cardiomyocyte-targeted Fas siRNA delivery due to the specificity of the PGE₂ receptor-ligand interaction. We introduced the PGE₂ moiety into the Fas siRNA no. 1 sequence at the 3'-end of the sense strand (Fig. 2). The terminal carboxyl group of PGE₂ was activated by DCC/NHS and conjugated to the primary amine-modified Fas siRNA molecule. The synthesis of PGE₂-Fas siRNA was monitored by RP-HPLC analysis performed using UV detection at 260 nm (Fig. 2, right panel). PGE₂, which was eluted after 2.98 min using UV absorption at 214 nm (data not shown), was not detected by UV absorbance at 260 nm. Naked Fas siRNA was eluted at 2.27 min, but PGE₂-Fas siRNA, having more hydrophobic moiety (PGE₂), showed a later eluting peak at 2.85 min. The HPLC results revealed that the PGE₂-Fas siRNA conjugate was successfully synthesized, and the conjugating yield of PGE₂-Fas siRNA was more than 98% as determined by HPLC analysis.

3.3. Characterization of PGE₂-Fas siRNA/poly(DAH/CBA) polyplex

The reducible cationic polymers (disulfide-containing poly(amido amine)s, SS-PAA)s form small and stable DNA (or siRNA) complexes promoting cellular uptake and release of DNA (or siRNA) by reduction of disulfide bonds in the cytoplasm [16-19,29]. To prepare nanosize PGE₂-Fas siRNA polyplexes, the PGE₂-Fas siRNA conjugate was complexed with the reducible cationic copolymer poly(DAH/CBA) (Fig. 2), which we have previously synthesized and characterized [19]. The unique structural characteristics of the reducible poly(DAH/CBA) were investigated using DLS and gel electrophoresis (Fig. 3). The hydrodynamic diameters of PGE₂-Fas siRNA polyplexes were measured at various N/P ratios from 1 to 30 (Fig. 3a). At an N/P ratio over 10, poly(DAH/CBA) effectively condensed PGE₂-Fas siRNA into small polyplexes with a diameter of less than 150 nm. In particular, the PGE₂-Fas siRNA polyplex at the N/P ratio of 20 was 134.7 ± 19.9 nm in diameter, which was optimized and used for the transfection experiments. The formation of the PGE₂-Fas siRNA polyplex was further confirmed by electrophoretic mobility shift assay (Fig. 3b, left panel). Poly(DAH/CBA) could completely condense the PGE₂-Fas siRNA conjugate at and above the N/P ratio of 10, which agreed well with the results obtained by DLS. To investigate the reductive degradation of poly(DAH/CBA) with dissociation of PGE₂-Fas siRNA, the PGE₂-Fas siRNA/poly(DAH/CBA) polyplexes were pre-incubated in the medium containing 10 mM glutathione (GSH) and then

electro-phorated on an agarose gel (Fig. 3b, right panel). With reductive stimulation, poly(DAH/CBA) was unable to condense PGE₂-Fas siRNA due to the cleavage of the disulfide linkage, resulting in release of PGE₂-Fas siRNA from the polyplexes. These results suggest that poly(DAH/CBA) could be completely cleaved after successful intracellular delivery of the polyplexes, followed by release of the fully functional siRNA into the cytoplasm (Fig. 2). Gene delivery studies using reducible cationic polymers have revealed that the triggered release characteristics of the polymers lead to increasing pDNA gene expression inside the nucleus and siRNA regulation in the cytoplasm [16,19].

3.4. Cardiomyocyte-targeted delivery of PGE₂-Fas siRNA/poly(DAH/CBA) polyplex

We hypothesized that the use of the PGE₂ receptor-ligand interaction would increase the efficiency of siRNA delivery into cardiomyocytes in a PGE₂ receptor (EP) specific manner. To examine the cardiomyocyte-targeted delivery of the PGE₂-Fas siRNA/poly(DAH/CBA) polyplex, cellular uptake experiments were conducted using rat cardiomyocytes (H9C2 cells). It has been known that the four subtypes of the PGE₂ receptor (EP1-4) show different expression patterns in different cell lines [12]. Therefore, we first estimated the expression level of EP1-4 in H9C2 cells by RT-PCR analysis with four different primer sets (Fig. 4a). The RT-PCR result showed that H9C2 cells expressed four types of EPs and that expression of EP4 was more than twofold higher compared with EP1-3. EP4 has been reported to be the most abundantly expressed EP subtype in the myocardium of several species [15]. Using a fluorescently labeled Fas siRNA (Cy3-Fas siRNA), the cellular uptake of PGE₂-Fas siRNA/poly(DAH/CBA) polyplex was estimated in H9C2 cells by flow cytometry (Fig. 4b). The cells were transfected by Fas siRNA alone, Fas siRNA polyplex, PGE₂-Fas siRNA polyplex, or mock-transfected with 11, 52, 86, or 3% efficiency (% cell count in the M region), respectively. The result agreed with that of our confocal microscopy studies in which the PGE₂-Fas siRNA polyplex was much more efficiently taken up by H9C2 cells compared to naked Fas siRNA or Fas siRNA polyplex (data not shown). These results demonstrate that the enhancement in cellular uptake of PGE₂-Fas siRNA/poly(DAH/CBA) polyplexes was induced via the PGE₂ ligand-receptor interaction. The dependence of PGE₂-siRNA polyplex delivery on the PGE₂ receptor-mediated internalization was further examined in a PGE₂ receptor competition assay (Fig. 4b). H9C2 cells transfected by PGE₂-Fas siRNA polyplexes were pre-incubated in medium containing 0.1 mM of PGE₂. The proportion of the transfected cells (% cell count in the M region) declined from 86% of the total cell population with the PGE₂-Fas siRNA polyplex to 30% with the Fas siRNA polyplex when excess PGE₂ was added. As the cellular uptake of PGE₂-Fas siRNA polyplexes was markedly suppressed by competition with free PGE₂, the cardiomyocyte targeting is the function of PGE₂ receptor-mediated internalization.

3.5. Cardiomyocyte-targeted Fas inhibition and anti-apoptosis activity of PGE₂-Fas siRNA/poly(DAH/CBA) polyplex

The cardiomyocyte-targeted Fas gene silencing and anti-apoptotic effects of PGE₂-Fas siRNA/poly(DAH/CBA) polyplexes were evaluated in H9C2 cells after exposure to hypoxia (Fig. 5). The amount of Fas mRNA was dramatically increased in H9C2 cells under hypoxic compared with normoxic culture conditions, as assessed by semi-quantitative RT-PCR analysis (Fig. 5a). It has been demonstrated that Fas upregulation under hypoxia is a widespread phenomenon in apoptotic cells [24]. The observed enhancement of Fas mRNA expression was reduced by Fas siRNA treatment. PGE₂-Fas siRNA polyplexes led to a more pronounced Fas downregulation in H9C2 cells than unmodified Fas siRNA polyplexes. This augmentation of Fas gene silencing appears to be due to the targeted delivery of PGE₂-Fas siRNA polyplexes into cardiomyocytes. As Fas plays a potent role in regulating apoptosis, we hypothesized that silencing Fas gene expression would inhibit cardiomyocyte apoptosis. Apoptosis of H9C2 cells was evaluated using propidium iodide (PI) flow cytometric assay (Fig. 5b and c). The cells cultured under normoxic conditions displayed no sub G₀/G₁ peak (a characteristic of apoptotic cells), whereas

the cells under hypoxic conditions had a large population of sub G_0/G_1 cells ($63.5 \pm 5.7\%$). Indeed, treatment with Fas siRNA abolished hypoxia-induced apoptosis in H9C2 cells. By using the PGE₂-modified Fas siRNA polyplex, the apoptotic cell population was reduced more significantly ($24.6 \pm 3.9\%$), compared to the unmodified Fas siRNA polyplex ($35.3 \pm 2.2\%$). In contrast, PGE₂-GFP siRNA polyplexes showed no significant change in the apoptotic cell population ($62.5 \pm 5.0\%$) caused by hypoxic stress. These results demonstrate that the Fas gene silencing by the PGE₂-Fas siRNA/poly(DAH/CBA) polyplex provides a putative mechanism of the anti-apoptotic effect in H9C2 cells.

3.6. Interferon- α response of PGE₂-Fas siRNA/poly(DAH/CBA) polyplex

Recent studies have suggested that some duplex siRNA sequences have off-target effects and toxicities associated with immune stimulation [21,30,31]. There has been considerable controversy over the potential of siRNA-induced interferon response. Particularly at high concentration, an immunogenic siRNA sequence can induce an interferon response [32]. Since it is uncertain that the immunostimulatory activity of siRNA is caused by a specific sequence, single or double strand, or delivery vehicle, the immunogenicity of siRNA delivery systems should be examined prior to *in vivo* application. We, therefore, monitored the interferon- α (IFN- α) response of PGE₂-Fas siRNA/poly(DAH/CBA) polyplexes in human peripheral blood mononuclear cells (PBMCs) (Fig. 6). Synthetic double-stranded RNA poly(I:C), a known potential immunostimulant, was used as a positive control to induce IFN- α [21]. PBMCs did respond strongly to poly(I:C) and produced 286.5 ± 70.1 pg/ml of IFN- α when formulated with poly(DAH/CBA). However, we observed no detectable IFN- α induction in PBMCs treated with the Fas siRNA formulations. The PGE₂-Fas siRNA/poly(DAH/CBA) polyplex delivery system, therefore, does not have an immunostimulatory effect that might contribute to cardiomyocyte protection. Thus, the PGE₂-Fas siRNA/poly(DAH/CBA) polyplexes can be safely used for cardiomyocyte-targeted Fas siRNA delivery to prevent cardiomyocyte apoptosis after hypoxic injury.

4. Conclusions

Cardiomyocyte apoptosis is the leading cause of heart failure after myocardial infarction. It has been reported that many apoptosis-related genes including Fas/Fas ligand are overexpressed in apoptotic cardiomyocytes [28]. Efficient Fas gene silencing by RNAi has significantly promoted anti-apoptosis in many cell types. For successful *in vivo* application of siRNA therapeutics, it is necessary to develop a cardiomyocyte-targeted siRNA delivery system with a high transfection efficiency. From these requirements for siRNA delivery and targeting, we proposed the PGE₂-Fas siRNA/poly(DAH/CBA) polyplex as a cardiomyocyte-targeted siRNA delivery system. The PGE₂-Fas siRNA/poly(DAH/CBA) polyplexes were taken up by cardiomyocytes in a PGE₂ receptor specific manner, showed significant Fas gene silencing, and thereby ultimately inhibited cardiomyocyte apoptosis in H9C2 cells. The results of this study suggest that cardiomyocyte-targeted Fas siRNA delivery using the PGE₂-Fas siRNA/poly(DAH/CBA) polyplex is a promising approach to inhibit apoptosis in the treatment of cardiovascular disease.

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Fig. 1.

The selection of the rat Fas siRNA target sequence. (a) Four siRNA sequences directed against rat Fas mRNA. The upper and lower oligonucleic acids are sense and antisense strands, respectively. (b) The expression of Fas mRNA in H9C2 cells treated with Fas siRNAs. The level of Fas mRNA was monitored by RT-PCR. The housekeeping gene GAPDH was used as an internal control. The intensity of the band representing Fas mRNA was normalized to the intensity of the bands for GAPDH and expressed as a ratio of relative band intensity (indicated below each sample). The density value of each gel band was measured using Image J analysis software. The untreated control group was arbitrarily assigned a relative value of 1.0.

Fig. 2. Schematic representation of the PGE₂-Fas siRNA/poly(DAH/CBA) polyplex delivery system. Fas siRNA no. 1 with amino group at 3'-end of sense strand was conjugated to PGE₂. The right panel shows the RP-HPLC profiles for PGE₂-Fas siRNA (red), Fas siRNA (green), PGE₂ (blue), and baseline (black). Reducible cationic copolymer poly(DAH/CBA) was condensed with PGE₂-Fas siRNA conjugate to form a nanosize polyplex. The degradation of poly(DAH/CBA) in a cytosolic reducing environment triggers intracellular siRNA release from the condensed polymers.

Fig. 3.

(a) Particle size and (b) gel retardation assay of PGE₂-siRNA/poly(DAH/CBA) polyplexes prepared at various N/P ratios from 1 to 30. The polyplexes were incubated in the medium containing 10 mM GSH for 0 and 18 h prior to agarose gel electrophoresis. The particle size distributions of the polyplexes at an N/P ratio of 5 and 20 are presented in the left and right inserted panels in (a), respectively.

Fig. 4.

Cardiomyocyte-targeted Fas siRNA delivery via PGE₂ receptor-mediated endocytosis. (a) The expression level of four PGE₂ receptors in H9C2 cells. PGE₂ receptor has four subtypes: EP1, EP2, EP3, and EP4. Lane M, DNA size marker. The intensity of the band representing EP mRNA was normalized to the intensity of the bands for GAPDH and expressed as a ratio of relative band intensity (indicated below each sample). (b) The cellular uptake of naked Fas siRNA, Fas siRNA/poly(DAH/CBA) polyplex, and PGE₂-Fas siRNA/poly(DAH/CBA) polyplex by H9C2 cells. For the PGE₂ receptor competition assay, cells were transfected with PGE₂-Fas siRNA/poly(DAH/CBA) polyplex in the medium containing 0.1 mM PGE₂. M means a gated area [fluorescence intensity (arbitrary unit): 10¹-10⁴].

Fig. 5.

Cardiomyocyte-targeted Fas gene silencing and anti-apoptosis effect of the PGE₂-Fas siRNA/poly(DAH/CBA) polyplex delivery system. (a) The expression level of Fas mRNA in H9C2 cells transfected by Fas siRNA formulations under normoxic or hypoxic conditions. The Fas mRNA level was monitored by RT-PCR analysis. The intensity of the band representing Fas mRNA was normalized to the intensity of the bands for GAPDH (internal control) and expressed as a ratio of relative band intensity (indicated below each sample). The untreated-hypoxia group was arbitrarily assigned a relative value of 1.0. (b) The representative DNA histograms of H9C2 cells treated with various siRNA formulations under normoxic or hypoxic conditions. Apoptotic cells were counted by PI staining flow cytometric analysis. (c) The graph

shows the results of PI-stained apoptotic H9C2 cells transfected with various siRNA formulations under normoxic or hypoxic conditions. Cells were gated and analyzed for the hypodiploid fraction (sub G_0/G_1). The values are represented as the mean $\pm$ standard deviation (SD) of triplicate experiments. Asterisk indicates significantly different from Fas siRNA/poly (DAH/CBA) polyplex at $p < 0.05$.

Fig. 6.

Interferon- α response induced by varying Fas siRNA formulations in PBMCs. IFN- α release from PBMCs was determined after 24 h of stimulation with naked Fas siRNA, PGE₂-Fas siRNA, poly(DAH/CBA) alone, Fas siRNA polyplex, PGE₂-Fas siRNA polyplex, poly(I:C) polyplex, and poly(I:C) alone (nucleic acid: 3 μ g/ml, N/P ratio: 20). Asterisk indicates significantly different from untreated control group at $p < 0.01$.