Delivery of Hypoxia Inducible Heme Oxygenase-1 Gene Using Dexamethasone Conjugated Polyethylenimine for Protection of Cardiomyocytes under Hypoxia

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Heme oxygenase-1 (HO-1) is an anti-inflammatory and anti-apoptotic protein and has been applied to various gene therapy researches. However, constitutive expression of HO-1 may induce deleterious side effects. In this research, hypoxia inducible HO-1 expression plasmid, pEpo-SV-HO-1, was constructed with the erythropoietin (epo) enhancer and simian virus 40 (SV40) promoter to avoid these unwanted side effects. Dexamethasone conjugated polyethylenimine (PEI-Dexa) was used as a gene carrier. It was previously reported that dexamethasone protected cardiomyocytes from apoptosis under hypoxia. In this research, PEI-Dexa reduced the caspase-3 level in hypoxic H9C2 cardiomyocytes as a derivative of dexamethasone, suggesting that PEI-Dexa is an anti-apoptotic reagent as well as a gene carrier. pEpo-SV-HO-1 was transfected to H9C2 cardiomyocytes using PEI-Dexa and the cells were incubated under normoxia or hypoxia. HO-1 expression was induced in the pEpo-SV-HO-1 transfected cells under hypoxia. In addition, cell viability under hypoxia was higher in the pEpo-SV-HO-1 transfected cells than the pEpo-SV-Luc transfected cells. Also, caspase-3 level was reduced in the pEpo-SV-HO-1 transfected cells under hypoxia. In addition to the anti-apoptotic effect of PEI-Dexa, hypoxia inducible HO-1 expression by pEpo-SV-HO-1 may be helpful to protect cardiomyocytes under hypoxia. Therefore, pEpo-SV-HO-1/PEI-Dexa complex may be useful for ischemic heart disease gene therapy.

Key Words: Dexamethasone, Cardiomyocytes, Heme oxygenase-1, Hypoxia, Polyethylenimine

Introduction

Heme oxygenase-1 (HO-1) is an enzyme involved in heme degradation.[1] HO-1 is induced in response to various cellular stresses such as inflammation and hypoxia.[2,3] HO-1 has anti-inflammatory and anti-apoptotic effects and has been used as a therapeutic protein.[4,5] It was reported that HO-1 was induced in the heart under ischemic condition and had a protective effect on cardiomyocytes.[6] Also, it protected cells from the angiotensin II-induced apoptosis by inhibiting the MAP kinase cascade.[1,7,8] Recently, it was reported that HO-1 was induced in acute lung injury and protected cells from the damage by reactive oxygen radical species.[9-12] Due to this cytoprotective effect of HO-1, gene therapy with HO-1 gene has been suggested as a useful strategy for ischemic heart disease or stroke. Some gene therapy studies showed that HO-1 expression by gene delivery had cytoprotection effect in ischemic tissues.[13,14] However, constitutive expression of HO-1 may have deleterious effect such as tumor growth. Also, it was previously reported that constitutive over expression of HO-1 may induce kernicterus and tissue hypoxia, which were caused by over-production of by-products of heme degradation.[13,15] Therefore, HO-1 expression should be carefully modulated to avoid these side-effects. Previously, a tissue-specific promoter, the myosin light chain-2v (MLC-2v) promoter was employed to limit gene expression to cardiomyocytes.[13,14] In combination with the MLC-2v promoter, the oxygen dependent degradation domain (ODD) was applied to the HO-1 expression vector to promote degradation of HO-1 under normoxia. The ODD domain stabilized the HO-1 protein under hypoxia.

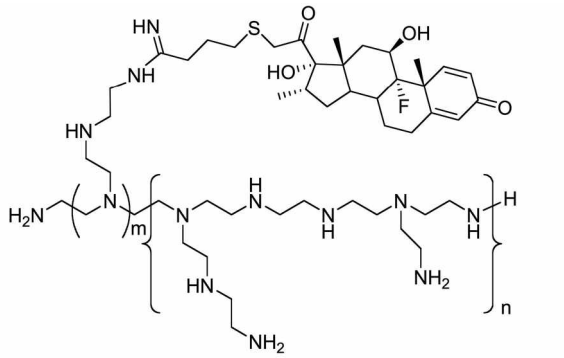

Figure 1. Structure of PEI-Dexa.

Dexamethasone is a potent glucocorticoid with anti-inflammatory activity. Dexamethasone was conjugated to low molecular weight polyethylenimine (PEI2K, 2 kDa).[16] High molecular weight polyethylenimine (PEI25K, 25 kDa) has higher transfection efficiency than PEI2K. However, PEI25K is not clinically applicable, since it is highly toxic to cells. On the contrary, PEI2K is not toxic to cells, although its transfection efficiency was much lower than PEI25K. In our previous report, the transfection efficiency of PEI2K was improved by the conjugation of dexamethasone (Fig. 1).[16] Dexamethasone binds to a glucocorticoid receptor after cellular entry, and the receptor/dexamethasone complex subsequently translocates it into the nucleus.[17,18] Therefore, the nuclear trafficking of dexamethasone may facilitate the nuclear entry of PEI-Dexa/DNA complexes, which in turn, increases the expression of the transfected gene. One of the important biological activities of dexamethasone is the cyto-

protective effect of cardiomyocytes from apoptosis.[19] In the previous studies, it was shown that the treatment with dexamethasone reduced apoptosis of cardiomyocytes under oxidative stress.[19-21] Therefore, dexamethasone conjugated polymer may also have an anti-apoptotic effect in hypoxic cardiomyocytes as a derivative of dexamethasone. Indeed, PEI-Dexa had anti-apoptotic effect in cardiomyocytes in the presence of H_2O_2.[22] However, it was reported that dexamethasone might reduce the HO-1 promoter activity, reducing the endogenous HO-1 expression level.[23] This possible drawback of dexamethasone-conjugated polymer can be compensated by delivery of exogenous HO-1 gene. In this study, hypoxia inducible HO-1 was constructed to avoid the deleterious side effects caused by non-specific expression. Previously, our group developed various hypoxia regulatory expression systems for transcriptional, post-transcriptional, or post-translational regulation.[24-27] The erythropoietin (epo) enhancer and simian virus 40 (SV40) promoter system can induce gene transcription specifically under hypoxia.[28] Therefore, a hypoxia specific transcriptional regulatory HO-1 expression plasmid, pEpo-SV-HO-1 was constructed with the epo enhancer and SV40 promoter system. pEpo-SV-HO-1 was transfected into H9C2 cardiomyocytes and anti-apoptotic effect of PEI-Dexa and pEpo-SV-HO-1 under hypoxia were evaluated. The results suggest that PEI-Dexa/hypoxia inducible HO-1 gene complex can protect cardiomyocytes under hypoxia and may be useful for ischemic heart disease gene therapy.

Materials and Methods

Synthesis of PEI-Dexa. PEI-Dexa was synthesized as previously described.[16] Low molecular weight PEI (PEI2K, 2 kDa) was dissolved in 1.8 mL anhydrous dimethyl sulfoxide (DMSO) with 2-fold molar excess Traut's reagent and dexamethasone-21-mesylate. To minimize the cross-linking side reaction, anhydrous DMSO was used, and humidity was mitigated during the reaction. The reaction was allowed to proceed for 4 hrs at room temperature and was quenched by the addition of an excess amount of cold ethyl acetate. The precipitated product was solubilized in water and dialyzed for 1 day against pure water using a dialysis membrane (MWCO 1,000). The mixture was further freeze-dried, and a white product was obtained (60%, yield).

Preparation of Plasmid DNA. pEpo-SV-Luc was constructed previously.[28] The human heme oxygenase-1 cDNA (Genebank accession number: NM002133) was cloned by RT-PCR using total RNA from 293 cells as a template. The sequences of the primers are as follows: forward primer, 5'-CCCA AGCTTATGGAGCGTCCGCAACCCG-3', backward primer, 5'-GCTCTAGAGCATTCACATGGCATAAAGC-3'. HinD III and XbaI sites were incorporated into the forward and backward primers, respectively, for cloning convenience (The enzyme sites are underlined). The amplified HO-1 cDNA was inserted into pEpo-SV-Luc (pGL3-promoter, Promega, Madison, WI) at the place of the luciferase cDNA, resulting in the construction of pEpo-SV-HO-1. The construction of the plasmids was confirmed by restriction enzyme analysis and direct sequencing.

Plasmid DNAs (pDNAs) were transformed in *Escherichia Coli* DH5α and amplified in Terrific Broth media at 37 °C overnight at 220 rpm. The plasmid was purified using the Maxi plasmid purification kit (Qiagen, Valencia, CA).

Cell Culture and Transfection. H9C2 rat cardiomyocytes were purchased from Korean Cell Line Bank (Seoul, Korea). The cells were maintained in DMEM supplemented with 10% FBS in a 5% CO_2 incubator. For the transfection assays, the cell was seeded at a density of 1×10^5 cells/well in 6-well flat-bottomed microassay plates (Falcon Co., Becton Dickenson, Franklin Lakes, NJ) 24 hrs before the transfection. PEI2K/pDNA and PEI-Dexa/plasmid complex was prepared at an 8/1 weight ratio, based on the previous report.[16] Transfection was performed as described in the previous reports.[29-35] Before transfection, the medium was replaced with 2 mL of fresh DMEM with or without FBS. Then, the polymer/pDNA complexes were added to the cells. The amount of plasmid was fixed at 2 μg/well. The cells were then incubated for 4 hrs at 37 °C in a 5% CO_2 incubator. After 4 hrs, the transfection mixtures were removed, and 2 mL of fresh DMEM medium containing FBS was added. The cells were incubated for an additional 44 hrs at 37 °C under the hypoxia condition (1% O_2) or normoxia condition (5% CO_2).

Enzyme-Linked Immunosorbent Assay (ELISA) of HO-1. ELISA was performed using a human HO-1 ELISA Kit (Assay Designs, Ann Arbor, MI) to measure the human heme oxygenase-1 in cell lysates. Briefly, the cells were harvested and lysated using extraction reagent (Assay Designs, Ann Arbor, MI). Fifty microliters of the samples were added to the designated wells. One hundred microliters of anti-human HO-1 rabbit polyclonal antibody was added to each well, and the plate was incubated at room temperature for 1 hour. After washing, 100 μL of the horseradish peroxidase conjugated to anti-rabbit IgG was added into each well, and the plate was incubated at room temperature for 30 min. After washing, 100 μL of the stabilized tetramethylbenzidine substrate was added to each well, and the plate was incubated at room temperature for 15 min. After incubation, the stop solution was added to the wells, and the absorbance was measured at 450 nm.

Apoptosis Assay. Apoptosis of the transfected cells was measured using Caspase-Glo 3/7 Assay reagent (Promega, Madison, WI). For transfection, the H9C2 cells were seeded at a density of 5×10^3 cells/well in 96-well flat-bottomed microassay plates (Falcon Co., Becton Dickenson, Franklin Lakes, NJ) 24 hrs before the transfection. PEI2K/pDNA and PEI-Dexa/pDNA complexes were prepared at an 8/1 weight ratio. Before transfection, the medium was replaced with 200 μL of fresh medium without FBS. Then, polymer/pDNA complexes were added to the cells. The amount of pDNA was fixed at a 0.2 μg/well. The cells were then incubated for 4 hrs at 37 °C in a 5% CO_2 incubator. After 4 hrs, the transfection mixtures were removed, and 100 μL of fresh DMEM medium containing FBS was added. The cells were incubated for an additional 44 hrs at 37 °C under the normoxia (20% oxygen) or hypoxia condition (1% oxygen). After incubation, 50 μL of Caspase-Glo reagent was added to each well, and samples are incubated at room temperature for 1 hr. The luminescence of each sample was measured in terms of relative light units

(RLU), using a 96-well plate luminometer (Berthold Detection System GmbH, Pforzheim, Germany).

Cell Viability Assay. The evaluation of cell viability was performed by the MTT assay.[36-39] H9C2 cells were seeded at a density of 1.3×10^4 per well in 24-well plates and incubated for 24 hrs before transfection. PEI2K/pDNA and PEI-Dexa/pDNA complexes were prepared at an 8/1 weight ratio. The amount of pDNA was fixed at 0.25 μg/well. The medium was replaced with fresh DMEM medium without FBS before transfection, and polymer/pDNA complex was added to the cells. After incubation at 37 °C for 4 hrs, the transfection mixture was replaced with 500 μL of fresh DMEM medium supplemented with 10% FBS, and the cells were incubated for The cells were incubated for an additional 44 hrs at 37 °C under the hypoxia condition (1% O_2) or normoxia condition (5% CO_2). MTT solution in PBS was added. The cells were incubated for an additional 4 hrs at 37 °C, at which point, MTT-containing medium was aspirated off, and 750 μL of DMSO was added to dissolve the formazan crystal formed by live cells. Absorbance was measured at 570 nm. The cell viability (%) was calculated according to the following equation:

Cell viability (%) = $OD_{570(sample)}/OD_{570(control)} \times 100$, where the $OD_{570(sample)}$ represents the measurement from the well treated with polymer/plasmid DNA complex and the $OD_{570(control)}$ represents the measurements from the wells treated with 5% glucose.

Statistical Analysis. Results were reported as mean ± standard deviation. The comparison of luciferase activity or HO-1 concentration was made by Student's *t*-test. A *P* value less than 0.05 was considered statistically significant.

Results

Anti-apoptotic effect of PEI-Dexa. To evaluate cytoprotective effect of PEI-Dexa, PEI-Dexa/pEpo-SV-Luc complex was transfected into H9C2 cells. After transfection, the cells were incubated under hypoxic condition. The apoptotic level of the cells was measured by caspase-3 assay. The results showed that PEI2K/pEpo-SV-Luc complex did not have significant effect on the caspase-3 activity compared with the control (Fig. 2). However, capase-3 activity in the cells transfected with PEI-Dexa/pEpo-SV-Luc was lower than that in the cells transfected with PEI2K/pEpo-SV-Luc complex (Fig. 2). These results suggest that the dexamethasone conjugated to PEI2K had anti-apoptotic effect.

Construction of pEpo-SV-HO-1 and hypoxia inducible HO-1 expression. For hypoxia inducible gene expression, transcriptional regulation plasmid of the HO-1 gene was constructed with the epo enhancer and SV40 promoter (Fig. 3). The HO-1 cDNA was inserted at the place of the luciferase gene in pEpo-SV-Luc. The construction of the plasmid was confirmed by DNA sequencing.

pEpo-SV-HO-1 was transfected into H9C2 cells using PEI-Dexa as a gene carrier. pEpo-SV-Luc was also transfected as a control. After the transfection, the cells were incubated under normoxia or hypoxia. After 24 hrs of transfection, the cell extracts were subjected to ELISA. As a result, the pEpo-SV-Luc transfected cells showed basal level of endogenous

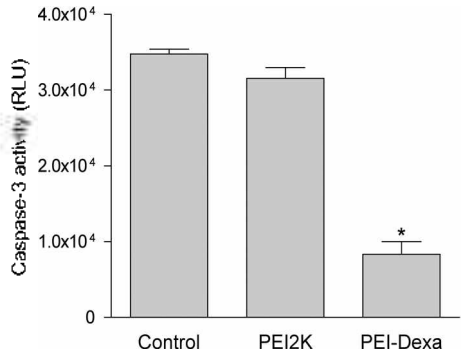

Figure 2. Caspase-3 activity after transfection of PEI-Dexa/pEpo-SV-Luc complex. PEI2K/pEpo-SV-Luc or PEI-Dexa/pEpo-SV-Luc complexes were prepared and transfected to H9C2 cells. The cells were incubated under hypoxia for 44 hrs. Apoptosis level was measured by Caspase-3 activity. The data is expressed as the mean values (± standard deviation) of quadruplicated experiments. *P < 0.05 as compared with PEI-Dexa/pSV-Luc.

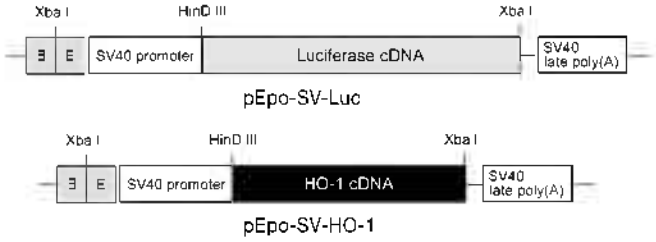

Figure 3. Structures of pEpo-SV-Luc and pEpo-SV-HO-1. E indicates the erythropoietin enhancer.

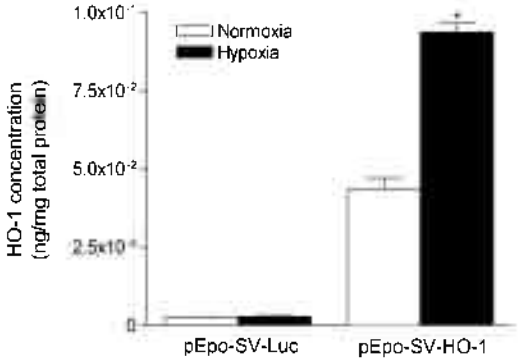

Figure 4. HO-1 expression in H9C2 cells under normal or hypoxic conditions. pEpo-SV-Luc and pEpo-SV-HO-1 were transfected into H9C2 cells using PEI-Dexa as a gene carrier. The cells were incubated under normal or hypoxic conditions for 44 hrs. HO-1 expression levels were measured by HO-1 ELISA. The data are expressed as the mean values (± standard deviation) of quadruplicated experiments. *P < 0.01 as compared with pEpo-SV-HO-1 under normoxia and pEpo-SV-Luc.

HO-1 protein (Fig. 4). However, the transfection of pEpo-SV-HO-1 elevated HO-1 level in the cells (Fig. 4). Especially, the pEpo-SV-HO-1 transfected cells under hypoxia showed approximately twice higher HO-1 expression than the cells under normoxia, suggesting that HO-1 expression under

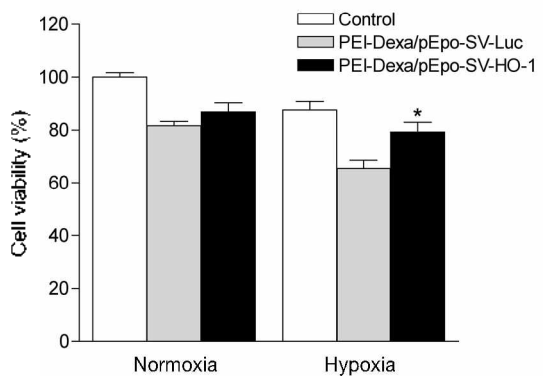

Figure 5. Viability of hypoxic H9C2 cells after transfection of PEI-Dexa/pEpo-SV-Luc or PEI/Dexa/pEpo-SV-HO-1 complex. pEpo-SV-Luc and pEpo-SV-HO-1 were transfected into H9C2 cells using PEI-Dexa as a gene carrier. The cells were incubated under normal or hypoxic conditions for 44 hrs. Cell viability was determined by MTT assay. The data is expressed as the mean values (± standard deviation) of quadruplicated experiments. *P < 0.05 as compared with PEI-Dexa/pEpo-SV-Luc under hypoxia.

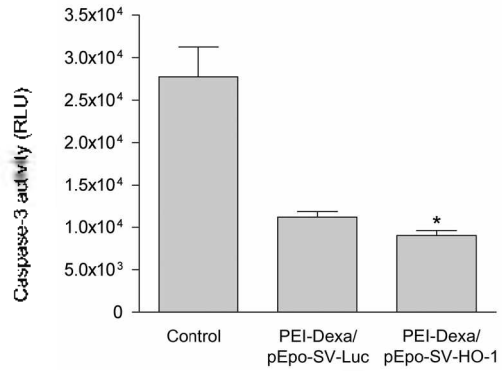

Figure 6. Caspase-3 activity after transfection of PEI-Dexa/pEpo-SV-HO-1 complex. PEI-Dexa/pEpo-SV-Luc or PEI-Dexa/pEpo-SV-HO-1 complexes were prepared and transfected to H9C2 cells. The cells were incubated under hypoxia for 44 hrs. Apoptosis level was measured by Caspase-3 activity. The data is expressed as the mean values (± standard deviation) of quadruplicated experiments. *P < 0.05 as compared with PEI-Dexa/pEpo-SV-Luc.

hypoxia was transcriptionally induced by the epo enhancer.

Effect of hypoxia inducible HO-1 expression. Under hypoxia, H9C2 cells are prone to apoptosis. The cell viability under hypoxia was evaluated by MTT assay. The cells were transfected with PEI-Dexa/pEpo-SV-Luc or PEI-Dexa/pEpo-SV-HO-1. The control cells were treated with only PBS. Due to the toxicity of polymeric carrier, PEI-Dexa, the cell viability of the PEI-Dexa/pEpo-SV-Luc treated cells was lower than that of the control (Fig. 5). Under normoxia, the cells treated with PEI-Dexa/pEpo-SV-HO-1 complex showed a slightly higher viability than the cells treated with PEI-Dexa/pEpo-SV-Luc, which was not statically significant (Fig. 5). However, the viability of the cells transfected with PEI-Dexa/pEpo-SV-HO-1 complex was significantly higher than that of the cells transfected with PEI-Dexa/pEpo-SV-Luc complex.

Apoptosis level of the cells were measured by caspase-3 assay. Fig. 6 showed that the cells transfected with PEI-Dexa/pEpo-SV-HO-1 complex had lower capase-3 level than the cells transfected with PEI-Dexa/pEpo-SV-Luc (Fig. 6). This suggests that apoptosis level was lower in the pEpo-SV-HO-1 transfected cells, compared with the pEpo-SV-Luc transfected cells.

Discussion

In this study, we evaluated the effect of hypoxia-inducible HO-1 gene delivery using PEI-Dexa. In combination with PEI-Dexa, HO-1 gene may have synergistic effect with PEI-Dexa for protection of cardiomyocytes under hypoxia. In this study, the hypoxia inducible HO-1 plasmid, pEpo-SV-HO-1 showed higher HO-1 expression in hypoxic H9C2 cells (Fig. 4) and protected the cells from apoptosis (Figs. 5 and 6).

Hypoxia inducible gene expression can be achieved at transcriptional, translational, or post-translational step. For translational regulation, hypoxia specific untranslated region (UTR) such as the epo 3'-UTR can be integrated into the therapeutic gene.[26] The epo RNA binding protein (ERBP) binds to the epo 3'-UTR and stabilizes the epo 3'-UTR linked mRNA under hypoxia. For post-translational regulation, the oxygen dependent degradation (ODD) domain can be integrated into therapeutic gene to produce a fusion protein.[27] Under normoxia, the fusion protein with the ODD domain is rapidly degraded via proteasome pathway, while it is stabilized under hypoxia. In this research, the epo enhancer increased HO-1 expression under hypoxia effectively (Fig. 4). The transcriptional, translational and post-translational regulatory mechanisms are independent of each other. Therefore, the combination with transcriptional, translational and post-translational regulations will further improve the HO-1 expression specificity and may be more useful for safe gene therapy.

Dexamethasone has an anti-inflammatory or anti-apoptotic effect in ischemic myocardium.[19-21] Recently, it was also reported that ischemic brain could be protected by the administration of dexamethasone.[40,41] Dexamethasone was conjugated to PEI2K to apply the anti-apoptotic effect of dexamethasone to ischemic heart disease therapy.[16] The conjugation of dexamethasone increased the transfection efficiency of PEI2K and PEI-Dexa has higher transfection efficiency than PEI2K. The efficiency of PEI-Dexa was comparable to that of PEI25K, one of the most efficient polymeric carriers. Also, PEI-Dexa was less toxic than PEI25K. These characteristics of PEI-Dexa suggest that PEI-Dexa is a useful gene carrier. Furthermore, dexamethasone segment of PEI-Dexa retains anti-apoptotic effect. In the previous report, we showed that PEI-Dexa protected cardiomyocytes in the presence of H_2O_2.[22] This anti-apoptotic effect of PEI-Dexa was confirmed in cardiomyocytes under hypoxia (Fig. 2). Therefore, PEI-Dexa is an efficient gene carrier with anti-apoptotic effect.

Previously, it was suggested that dexamethasone might reduce the HO-1 promoter activity, which resulted in the reduced level of endogenous HO-1.[23] This may hamper the anti-apoptotic effect dexamethasone in ischemic myocardium. The disadvantage of dexamethasone, reducing HO-1 level, may be compensated by the delivery of the HO-1 gene. As

shown in Fig. 2 and 6. capase-3 level was reduced by PEI-Dexa and further decreased by HO-1 gene. The cell viability under hypoxia was improved in the cells transfected with PEI-Dexa/pEpo-SV-HO-1 complex (Fig. 5).

As in Fig. 2, PEI-Dexa reduced caspase-3 level significantly compared with the control. However, the viability of the cells treated with PEI-Dexa was lower than the control in Fig. 5. These suggest that the cell death in the control was caused by necrosis as well as apoptosis. Although PEI-Dexa was less toxic than PEI25K, it still induces a certain level of toxicity to cells.[16,22] Even though PEI-Dexa reduced apoptosis level, the necrosis level might be higher in the PEI-Dexa treated cells than the control. The necrosis of the cells may be due to cationic charge of the polymer. This necrosis may be limited to early phase of transfection. It was previously suggested that cytotoxicity of PEI has two phases, immediate and delayed toxicities.[42] Immediate toxicity is mainly due to free PEI. Free PEI has higher charge density than PEI/DNA complex and aggregates at the surface of plasma membrane. The aggregation causes necrosis by the rupture of the membrane. At the delayed phase, toxicity is mainly due to the internalized PEI/DNA complex. After release of DNA, PEI binds to negatively charged molecules in the cells. This interaction may interrupt with normal cellular process and induce apoptosis. In the present study, MTT assay was performed at the early phase after 24 hrs of incubation. Although PEI-Dexa has anti-apoptotic effect, it may not be able to prevent necrosis caused by the immediate toxicity of free PEI-Dexa. In the previous study, we showed that PEI25K/DNA complex had high apoptosis level at the delayed phase, compared with free PEI25K.[43] We suggested that loss of gene expression in the transfected cells might be due to apoptosis of the transfected cells at the delayed phase.[43] Since PEI-Dexa may protect the cells from apoptosis at the delayed phase and reduce the loss of transfected cells, the gene expression may be more persistent in the cells transfected with PEI-Dexa than the cells transfected with PEI25K. It would be interesting to whether PEI-Dexa can increase gene expression duration compared with PEI25K.

In conclusion, PEI-Dexa reduced the caspase-3 level in the H9C2 cells under hypoxia. The anti-apoptotic effect was further increased by delivery of hypoxia inducible HO-1 plasmid, pEpo-SV-HO-1, using PEI-Dexa. Therefore, PEI-Dexa/pEpo-SV-HO-1 complex may be useful for protection of cardiomyocytes under hypoxia and gene therapy of ischemic diseases.

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