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Cell Injury, Repair, Aging and Apoptosis

Anti-Fas Gene Therapy Prevents Doxorubicin-Induced Acute Cardiotoxicity through Mechanisms Independent of Apoptosis

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Activation of Fas signaling is a key mediator of doxorubicin cardiotoxicity, which involves both cardiomyocyte apoptosis and myocardial inflammation. In this study, acute cardiotoxicity was induced in mice by doxorubicin, and some mice simultaneously received an intramuscular injection of adenoviral vector encoding mouse soluble Fas (sFas) gene (Ad.CAG-sFas), an inhibitor of Fas/Fas ligand interaction. Two weeks later, left ventricular dilatation and dysfunction were apparent in the LacZ-treated control group, but both were significantly mitigated in the sFas-treated group. The *in situ* nick-end labeling-positive rate were similar in the two groups, and although electron microscopy revealed cardiomyocyte degeneration, no apoptotic structural features and no activation of caspases were detected, suggesting an insignificant role of apoptosis in this model. Instead, sFas treatment reversed doxorubicin-induced down-regulation of GATA-4 and attenuated ubiquitination of myosin heavy chain and troponin I to preserve these sarcomeric proteins. In addition, doxorubicin-induced significant leukocyte infiltration, fibrosis, and oxidative damage to the myocardium, all of which were largely reversed by sFas treatment. sFas treatment also suppressed doxorubicin-induced p53 overexpression, phosphorylation of c-Jun N-terminal kinase, c-Jun, and inhibitor of nuclear factor-κB, as well as production of cyclooxygenase-2 and monocyte chemoattractant protein-1, and it restored extracellular signal-regulated kinase activation. Therefore, sFas gene therapy prevents the progression of doxorubicin-induced acute cardiotoxicity, with accompanying attenuation of the cardiomyocyte degeneration, inflammation, fibrosis, and oxidative damage caused by Fas signaling. ***(Am J Pathol 2010, 176:687–698; DOI: 10.2353/ajpath.2010.090222)***

The antineoplastic drug doxorubicin (adriamycin) is effective in the treatment of a broad range of hematogenous and solid human malignancies, but its clinical use is limited by its dose-dependent side effects: irreversible degenerative cardiomyopathy and congestive heart failure.[1–3] The efficacy of doxorubicin against cancer has prompted a search to find treatments that reduce or prevent its cardiac side effects.[3,4] So far, however, the ability of these treatments to protect the heart from doxorubicin has been varied and limited.

The interaction of Fas with Fas ligand is an important trigger for apoptosis in many cell types, particularly cells related to the immune system.[5] Moreover, it has recently come to light that the Fas/Fas ligand interaction plays an important role in the development and progression of doxorubicin cardiomyopathy. Nakamura et al showed that in a rat doxorubicin cardiomyopathy model, myocardial Fas expression and cardiomyocyte apoptosis were concomitantly increased and that a neutralizing antibody against Fas ligand attenuated both, leading to improvement in cardiac function.[6] In addition, Yamaoka et al

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showed that Fas/Fas ligand interaction increases the susceptibility of cultured neonatal cardiomyocytes to doxorubicin-induced apoptosis.[7] Conversely, treatment with doxorubicin up-regulates expression of both Fas ligand and Fas in various organs, including the heart.[6,8] On the other hand, cardiomyocytes are reportedly very insensitive to Fas stimulation,[9,10] and one recent study reported that doxorubicin-induced cardiomyocyte apoptosis is independent of Fas signaling.[11] It is noteworthy in that regard that there is as yet no *in vivo* morphological evidence of the involvement of cardiomyocyte apoptosis in doxorubicin cardiotoxicity, despite numerous biochemical findings indicative of apoptosis (eg, DNA fragmentation, caspase activation).[12,13] In fact, we and others have never detected apoptotic cardiomyocytes in some *in vivo* models of doxorubicin cardiotoxicity.[14,15] Thus, the role of Fas-dependent cardiomyocyte apoptosis, or any other form of apoptosis, remains controversial in the pathogenesis of doxorubicin cardiotoxicity.

Recent studies indicate that Fas signaling also exerts biological effects unrelated to apoptosis, such as induction of inflammation and fibrosis,[16] generation of reactive oxygen species,[17] acceleration of proliferation/differentiation,[18] and induction of hypertrophy.[19] Indeed, its proinflammatory and hypertrophic effects have been noted in both heart and cardiomyocytes.[19,20] We therefore hypothesized that Fas signaling might contribute to the pathogenesis of doxorubicin cardiotoxicity through mechanisms unrelated to induction of cardiomyocyte apoptosis. To test that idea, we examined the efficacy of gene therapy using an adenoviral vector expressing soluble Fas (sFas), an inhibitor of Fas/Fas ligand interaction, on cardiac function and morphology in our mouse model of doxorubicin-induced acute cardiotoxicity where the role of apoptosis seems insignificant[15] and investigated the specific mechanisms involved in the observed effects.

Materials and Methods

Recombinant Adenoviral Vectors

A replication-incompetent adenoviral vector that ubiquitously and strongly expresses a chimeric fusion protein comprised of the extracellular region of mouse Fas and the Fc region of human IgG_1 (mFas-Fc), ie, soluble Fas (sFas), was generated as follows. The adenoviral vector plasmid pAd-sFas, which includes the cytomegalovirus immediate early enhancer, a modified chicken β-actin promoter, rabbit β-globin polyA (CAG), and sFas cDNA (Ad.CAG-sFas) was constructed using *in vitro* ligation as described previously.[21] Plasmid pFAS-FcII was generously provided by Dr. S. Nagata (Osaka University Graduate School of Medicine).[22] Control Ad-LacZ (Ad.CAG-LacZ) was prepared as described previously.[23]

Experimental Protocols

This study was approved by our Institutional Animal Research Committee. Cardiotoxicity was induced in 10-week-old male C57BL/6J mice (Japan SLC) with a single intraperitoneal injection of doxorubicin hydrochloride (Kyowa Hakko) at a dose of 15 mg/kg in saline (n = 20).

Just after the injection of doxorubicin, the sFas gene or LacZ gene was systemically delivered to mice by injection of Ad.CAG-sFas or Ad.CAG-LacZ (1×10^9 pfu/mouse) into the hindlimb muscles (n = 10 each). In sham-treated mice (n = 18), the same volume of saline (n = 10) or Ad.CAG-sFas (n = 8) was injected in a similar manner.

Measurement of the sFas Level in Plasma

The plasma concentration of sFas was measured 1 and 2 weeks after injection of Ad.CAG-sFas or Ad.CAG-LacZ (n = 3 each) by detecting human IgG-Fc using an enzyme-linked immunosorbent assay kit (Institute of Immunology) as previously reported.[24]

Physiological Studies

Physiological studies (echocardiography and cardiac catheterization) were performed as described previously with modifications.[15] Animals were anesthetized with halothane (induction, 2%; maintenance, 0.5%) in a mixture of N_2O and O_2 (0.5 l/min each) via a nasal mask. Echocardiograms were recorded before treatment and at sacrifice using an echocardiographic system (Vevo 770; VisualSonics) equipped with a 45-MHz imaging transducer. Following echocardiography, the right carotid artery was cannulated with a micromanometer-tipped catheter (SPR 671; Millar Instruments) that was advanced into the aorta and then into the left ventricle (LV) to record pressure and maximal and minimal dP/dt ($\pm$dP/dt).

Histological Analysis

Once the physiological measurements were complete, all mice were sacrificed, and the hearts were removed. Randomly chosen six hearts from each group served for histological analyses. The heart was cut in two by making a transverse slice between the atrioventricular groove and the apex. The basal specimens were fixed in 10% buffered formalin, embedded in paraffin, cut into 4-μm-thick sections and stained with hematoxylin and eosin, Masson's trichrome and Sirius red F3BA (0.1% solution in saturated aqueous picric acid, Sigma-Aldrich). Quantitative assessments, including fibrosis area, cardiomyocyte size, and immunopositive cell number, were performed in 20 randomly chosen high power fields (HPFs, $\times$400) in each section using a multipurpose color image processor (LUZEX F, Nireco). The cardiomyocyte size was expressed as the transverse diameter of myocytes cut at the level of the nucleus.

Immunohistochemistry

After deparaffinization, the 4-μm-thick sections were incubated with a primary antibody against panleukocyte antigen (CD45; PharMingen), guanine derivative 8-hydroxy-2′-deoxyguanosine (8-OHdG; Japan Institute of

The Control of Aging), or 4-hydroxyl-2-nonenal (4-HNE; NOF corporation). A Vectastain Elite ABC system (Vector Laboratories) was then used to immunostain the sections; diaminobenzidine served as the chromogen, and the nuclei were counterstained with hematoxylin.

In Situ *Nick End-Labeling (TUNEL) and DNA Gel Electrophoresis*

TUNEL assays were performed with sections using an ApopTag kit (Intergene) principally according to the supplier's instruction. Mammary tissue from the mice served as a positive control. In addition, to evaluate apoptosis of cardiomyocytes, we performed double immunofluorescence for myoglobin combined with TUNEL. Tissue sections were first stained with Fluorescein-FragEL (Oncogene) and then labeled with anti-myoglobin antibody (DAKO) followed by Alexa 568. Nuclei were stained with Hoechst 33342. Immunofluorescence preparations were observed under a confocal microscope (LSM510, Zeiss). Extraction of DNA from apical halves of cardiac tissue (n = 3 each per group) and its subsequent electrophoresis were performed as described previously.[25]

Electron Microscopy

Apical halves of the hearts (n = 3 from each group) were minced and fixed in phosphate-buffered 2.5% glutaraldehyde solution (pH 7.4) overnight, postfixed with 1% osmium tetroxide for 1 hour, dehydrated through a graded ethanol series and embedded in Epon medium. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in an electron microscope (H700; Hitachi).

We performed a morphometric analysis under an electron microscope using the method previously described.[26] A uniform sampling of 20 electron micrographs, 10 with myofibrils oriented longitudinally and 10 with myofibrils sectioned transversely, was used for the morphometric assay of each group. Five random fields, micrographed at 10,000× from each of five tissue blocks were printed at a final magnification of 30,000× and analyzed on composite grids as described previously, to calculate the volume fraction of myofibrils and mitochondria within a cardiomyocyte.

Western Blotting

Samples of protein (100 μg) extracted from hearts (n = 4 from each group) were subjected to 14% polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes. The membranes were then probed using primary antibodies against Fas, Fas ligand (both from BD Transduction Laboratories/BD PharMingen), caspase-8, caspase-3 (both from Cell Signaling, Danvers, MA), Bcl-2, Bax (both from Santa Cruz), extracellular signal-regulated kinase (ERK), the phosphorylated (activated) form of ERK (p-ERK; both from Cell Signaling), c-Jun N-terminal kinase (JNK), the phosphorylated form of JNK (p-JNK; both from Santa Cruz), p38 mitogen-activated protein kinase (p38 MAPK), the phosphorylated form of p38 MAPK (p-p38; both from Sigma-Aldrich), c-Jun, the phosphorylated form of c-Jun (p-Jun; both from Cell Signaling), inhibitor of nuclear factor-κB (IκB), the phosphorylated form of IκB (p-IκB; both from Cell Signaling), GATA-4, myosin heavy chain (MHC), troponin I, p53, cyclooxygenase-2 (COX-2), monocyte chemoattractant protein-1 (MCP-1; all from Santa Cruz), transforming growth factor-β1 (TGF-β1; Promega) and 4-HNE (NOF Corporation), after which the blots were visualized using enhanced chemiluminescence (ECL; GE Healthcare UK Ltd.). α-Tubulin (antibody from Santa Cruz) served as the loading control.

Ubiquitination Assay Using Immunoprecipitation

An immunoprecipitation assay of the lysate of heart tissues was performed using anti-MHC or anti-troponin I antibody (Santa Cruz) with Dynabeads/protein A (Dynal; Invitrogen) according to the supplier's instruction. Subsequently, the isolated protein was analyzed by Western blotting using anti-ubiquitin antibody (Dako). Three hearts from each group were subjected to the assay.

Enzyme-Linked Immunosorbent Assay

The level of tumor necrosis factor-α (TNF-α) in myocardial tissue (n = 4 from each group) was quantified using enzyme-linked immunosorbent assay kits (R&D Systems) according to the supplier's instructions.

Statistical Analysis

Values are shown as means ± SEM The significance of differences between groups was evaluated using one-way analysis of variance with a post hoc Newman-Keuls multiple comparisons test. Values of $P < 0.05$ were considered significant.

Results

Effects of sFas on Doxorubicin-Induced Changes in Cardiac Structure and Function

In the sFas gene-delivered mice, the plasma level of exogenous sFas reached 76 ± 8.0 μg/ml 1 week after injection. It steeply decreased to 3.2 ± 0.18 μg/ml 2 weeks after injection when the experiments ended; these levels might be sufficiently high when considering that in humans, the normal level of plasma sFas is approximately 2 ng/ml.[27] Exogenous sFas was not detected in the plasma of mice that received LacZ gene at any time point.

Two weeks after doxorubicin administration, all of the mice of each group remained alive. Echocardiography and cardiac catheterization performed at that time showed that mice receiving doxorubicin and LacZ gene

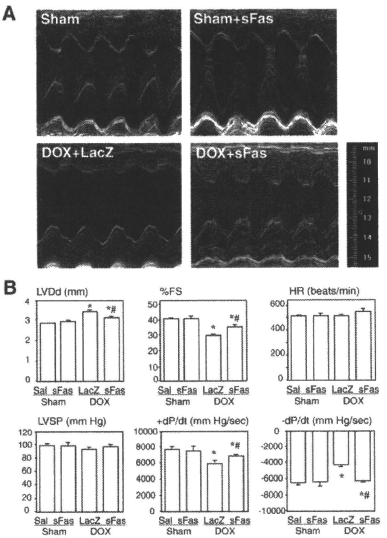

Figure 1. Effects of sFas gene delivery on LV geometry and function evaluated with echocardiography and cardiac catheterization 2 weeks after doxorubicin injection. **A:** Representative M-mode echocardiograms at the level of the ventricles in each group. **B:** Comparison of hemodynamic parameters among the groups. LVDd, left ventricular end-diastolic dimension; %FS, %fractional shortening; LVSP, left ventricular peak systolic pressure. Sal, saline treatment; sFas, sFas gene transfer; LacZ, LacZ gene transfer; DOX, doxorubicin treatment. $^{*}P < 0.05$ versus the saline-treated sham group; $^{\#}P < 0.05$ versus the doxorubicin-treated group with LacZ gene transfer.

had substantial deterioration of cardiac function characterized by enlargement of the LV cavity, increased LV diameter, reduced LV fractional shortening and reduced ±dP/dt, as compared with sham animals (Figure 1, A and B). Treatment with sFas gene significantly attenuated the doxorubicin-induced impairment of cardiac function but showed no influence on cardiac geometry and function in the sham-treated mice.

We observed no significant difference in the heart weight-to-body weight ratios among the groups (Table 1). On the other hand, examination of transverse sections of hearts stained with hematoxylin-eosin showed that cardiomyocytes were severely degenerative from the group receiving doxorubicin plus LacZ; they contained many vacuoles and the sarcomeres were poorly stained with eosin (Figure 2A). The transverse diameters of cardiomyocytes from this group were significantly smaller than in the sham group. The sFas treatment improved the doxorubicin-induced degenerative findings in cardiomyocytes (Figure 2A) but exerted no effect against this apparent cardiomyocyte atrophy (Table 1).

Table 1. Morphometric Data from Hearts

	Sham (n = 10)	DOX + LacZ (n = 10)	DOX + sFas (n = 10)
Heart weight (mg)	95 ± 2.1	81 ± 4.3*	83 ± 3.1*
Body weight (g)	26 ± 0.4	23 ± 0.7*	22 ± 0.7*
Heart to body weight ratio (mg/g)	3.7 ± 0.06	3.6 ± 0.13	3.7 ± 0.17
Size of myocyte (μm)	14.0 ± 0.2	12.3 ± 0.1*	12.6 ± 0.3*
$CD45^{+}$ cells (/HPF)	4.4 ± 0.28	7.6 ± 0.41*	4.8 ± 0.34*†
% fibrosis	0.46 ± 0.02	1.04 ± 0.07*	0.73 ± 0.06*†
8-OHdG^{+} cells (/HPF)	1.5 ± 0.1	16 ± 0.2*	4.5 ± 0.1*†

DOX, doxorubicin.
$^{*}P < 0.05$ versus the sham group.
$^{\dagger}P < 0.05$ versus the LacZ-treated group.

Effects of sFas on Doxorubicin-Induced Inflammatory Responses, Fibrosis, and Oxidative Damage

Immunohistochemical analysis revealed that doxorubicin induced significant infiltration of the myocardium by CD45-positive leukocytes (Figure 2A) and that sFas attenuated this effect, significantly reducing doxorubicin-induced CD45-positive leukocyte infiltration (Table 1). When we assessed cardiac fibrosis using Sirius red-stained sections (Figure 2A), we found that the amount of fibrosis was significantly greater in the group receiving doxorubicin plus LacZ than in the sham group, and that this effect, too, was significantly reduced by sFas treatment (Table 1). The 8-OHdG is a commonly used marker of oxidative damage to DNA.[28] We found that the prevalence of 8-OHdG-positive cardiomyocytes was markedly increased in the group receiving doxorubicin plus LacZ and that such oxidative damage was markedly attenuated in the sFas-treated mice (Figure 2A and Table 1). 4-HNE, a marker of oxidative damage to cell membranes,[29] is an α,β-unsaturated aldehyde that can be formed by peroxidation of ω6-unsaturated fatty acids such as linoleic and arachidonic acids.[30] It has been proposed that 4-HNE exerts a variety of cytotoxic, genotoxic, and mutagenic effects as well as inhibitory effects on cell proliferation because its facile reactivity with biological molecules, particularly with proteins. We similarly noted strong immunolabeling of 4-HNE in the hearts of doxorubicin-treated mice, and the labeling was substantially weaker in mice receiving the sFas gene therapy (Figure 2A). This finding was subsequently confirmed by Western blotting of 4-HNE (Figure 2B).

Western blot analyses revealed up-regulation of two inflammatory mediators cyclooxygenase-2 and MCP-1 in the doxorubicin-treated hearts, which was reversed by the sFas gene therapy (Figure 3A). On the other hand, tissue levels of TGF-β1 and TNF-α were unaffected by doxorubicin (Figure 3, A and B).

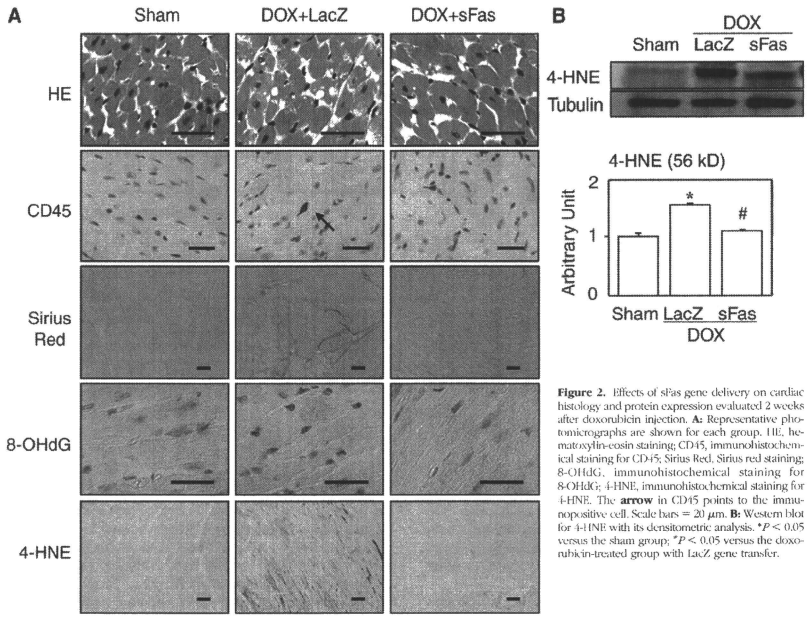

Figure 2. Effects of sFas gene delivery on cardiac histology and protein expression evaluated 2 weeks after doxorubicin injection. **A:** Representative photomicrographs are shown for each group. HE, hematoxylin-eosin staining; CD45, immunohistochemical staining for CD45; Sirius Red, Sirius red staining; 8-OHdG, immunohistochemical staining for 8-OHdG; 4-HNE, immunohistochemical staining for 4-HNE. The **arrow** in CD45 points to the immunopositive cell. Scale bars = 20 μm. **B:** Western blot for 4-HNE with its densitometric analysis. $^{*}P < 0.05$ versus the sham group; $^{\#}P < 0.05$ versus the doxorubicin-treated group with LacZ gene transfer.

Apoptosis in Hearts Showing Acute Doxorubicin Cardiotoxicity

Cardiac expression of both Fas and Fas ligand were found to be up-regulated by treatment with doxorubicin, and this effect was reversed by sFas gene therapy (Figure 4A). The TUNEL-positive cardiomyocytes and nonmyocytes were observed in each group, though in rare instances (Figure 4B). The prevalence of TUNEL-positivity in cardiomyocytes were significantly greater in the groups treated with doxorubicin, irrespective of whether the mice also received sFas gene, and sFas treatment had no significant effect on the prevalence. We failed to detect the ladder pattern of fragmented DNA characteristic of apoptosis in any groups (data not shown). Moreover, we detected no cleaved (activated) caspase-3 or caspase-8 in any of the groups, and the levels of both procaspase-8 and −3 were unchanged between the groups (Figure 4C). Levels of two apoptosis-regulating proteins, Bcl-2 and Bax, were not affected either by doxorubicin or the gene therapy (Figure 4D).

Electron microscopy revealed degenerative changes in cardiomyocytes from doxorubicin-treated mice, including myofibrillar derangement, disruption and loss and proliferation of subcellular organelles such as mitochondria (Figure 5A). Morphometric analysis at the electron microscopic level revealed that the %volume comprised of myofibrils in a cardiomyocyte cell area became significantly smaller by doxorubicin (43 ± 1.2% versus 54 ± 1.3%in sham, $P < 0.05$); this reduction was restored by the sFas gene therapy (52 ± 2.0%) (Figure 5A). However, no typically apoptotic cells were observed. Thus, an anti-degenerative effect, but not an anti-apoptotic effect, appears to contribute significantly to the beneficial effects of sFas gene therapy on cardiac structure and function in the present model of doxorubicin cardiotoxicity.

Effects of sFas on Doxorubicin-Induced Cardiomyocyte Degeneration

Although apoptotic features were not seen, doxorubicin-induced cardiotoxicity was accompanied by severe degenerative changes to the cardiomyocytes. These changes included myofibrillar derangement and disruption with increased numbers of subcellular organelles such as mitochondria (Figure 5A), which are all consistent with previously described findings.[15,31] We also noted that levels of GATA-4, a key transcriptional factor regulating expression of cardiac sarcomeric proteins,[32,33] were significantly diminished in the doxorubicin-treated hearts (Figure 5B), which is also consistent with earlier

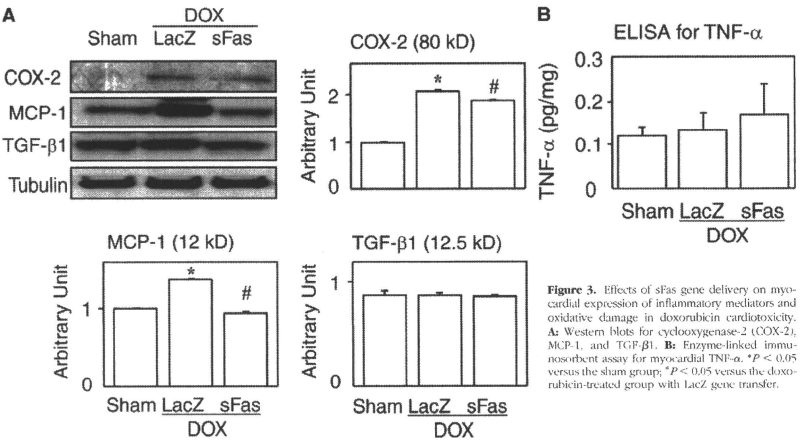

Figure 3. Effects of sFas gene delivery on myocardial expression of inflammatory mediators and oxidative damage in doxorubicin cardiotoxicity. **A:** Western blots for cyclooxygenase-2 (COX-2), MCP-1, and TGF-β1. **B:** Enzyme-linked immunosorbent assay for myocardial TNF-α. *$P < 0.05$ versus the sham group; #$P < 0.05$ versus the doxorubicin-treated group with LacZ gene transfer.

reports.[34] Notably, GATA-4 expression was significantly restored by sFas treatment (Figure 5B). Likewise, the levels of two sarcomeric proteins, myosin heavy chain and troponin I, which were significantly reduced by doxorubicin, were also significantly reversed by sFas gene treatment (Figure 5B).

A recent study reported that doxorubicin treatment reduces cardiac mass via p53-dependent inhibition of mammalian target of rapamycin (mTOR), which is the major contributor to acute doxorubicin cardiotoxicity.[35] Our Western blot showed a marked up-regulation of p53 in the heart by doxorubicin treatment consistent with the report[35] and also revealed a significant attenuation of p53 expression in the sFas-treated group (Figure 6A). The ubiquitination assay with immunoprecipitation revealed that doxorubicin increased polyubiquitinated myosin heavy chain and troponin I, and that the sFas treatment significantly attenuated this increase (Figure 6B). Collectively, these findings suggest that Fas signaling mediates doxorubicin-induced sarcomeric disintegration by down-regulation of GATA-4, a transcriptional factor for myosin heavy chain and troponin I, by ubiquitin-dependent degradation of those sarcomeric proteins that are in parallel with p53 expression, or by both. Although the sFas treatment exerted no protective effect against doxorubicin-induced atrophy, it appears to qualitatively improve cardiomyocyte structure and function possibly through restoration of the proportion of sarcomeric proteins in the cytoplasm.

Molecular Signaling Downstream of Fas

Our histological analysis revealed that inhibition of Fas signaling with sFas treatment attenuated doxorubicin-induced myocardial inflammation and fibrosis as well as cardiomyocyte degeneration. It is known that in addition to its apoptotic signaling, Fas stimulation also evokes inflammatory signaling through activation of downstream mediators such as JNK and nuclear factor-κB (NF-κB).[16,36] Activation of JNK and c-Jun, as indicated by expression of the respective phosphorylated forms, was stimulated by doxorubicin, and this effect was attenuated by the sFas treatment (Figure 7A) while p38 MAPK activation was suppressed by doxorubicin, which sFas treatment did not affect (Figure 7B). The phosphorylated level of IκB activation was augmented by doxorubicin, and this, too, was significantly attenuated by sFas treatment (Figure 7C), suggesting that sFas gene therapy suppresses doxorubicin-induced NF-κB activation. Consistent with previous reports,[15,37] activity of ERK was suppressed by doxorubicin and interestingly, this was restored by sFas treatment (Figure 7D).

Discussion

Fas Signal Inhibition and Apoptosis in Acute Doxorubicin Cardiotoxicity

The present study has shown that inhibition of Fas signaling significantly attenuates progression of acute doxorubicin cardiotoxicity. Nakamura et al previously used a neutralizing antibody against Fas ligand to demonstrate the beneficial effect of Fas inhibition in doxorubicin cardiotoxicity.[6] We have now confirmed and extended those findings by demonstrating for the first time the efficacy of anti-Fas gene therapy in the treatment of acute doxorubicin cardiotoxicity. More important, however, may be the difference in the phenotypes of the affected hearts in the two studies. In their model, Nakamura et al noted aug-

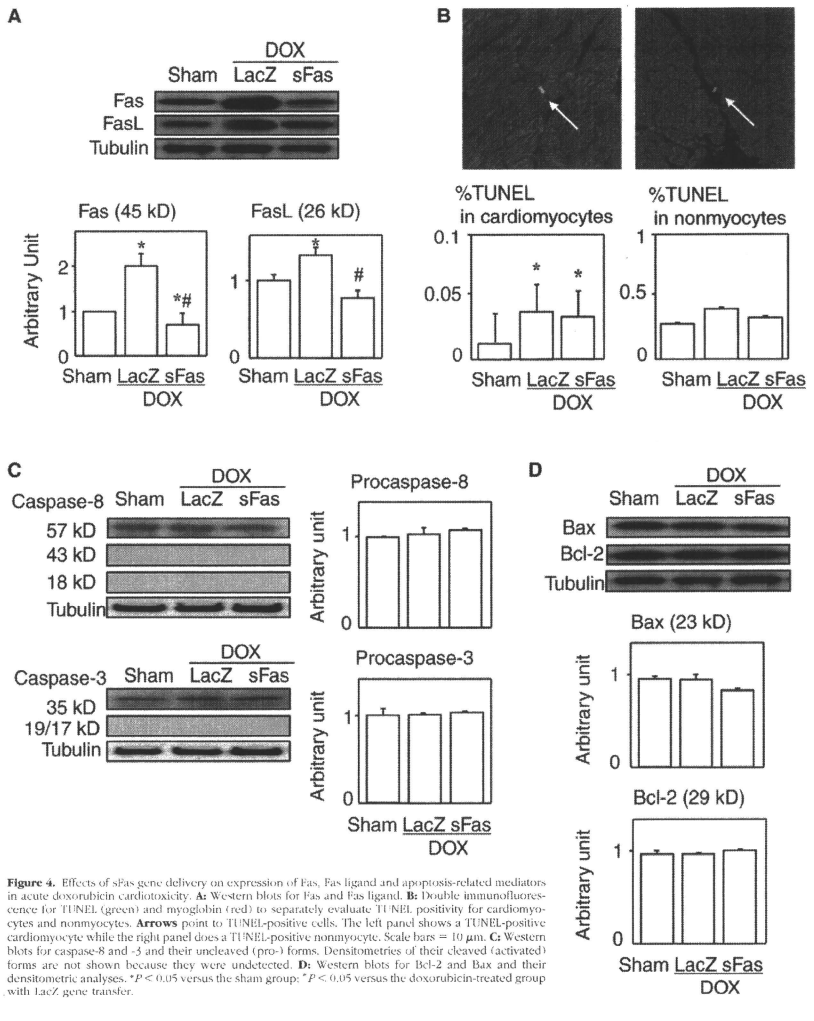

Figure 4. Effects of sFas gene delivery on expression of Fas, Fas ligand and apoptosis-related mediators in acute doxorubicin cardiotoxicity. **A:** Western blots for Fas and Fas ligand. **B:** Double immunofluorescence for TUNEL (green) and myoglobin (red) to separately evaluate TUNEL positivity for cardiomyocytes and nonmyocytes. **Arrows** point to TUNEL-positive cells. The left panel shows a TUNEL-positive cardiomyocyte while the right panel does a TUNEL-positive nonmyocyte. Scale bars = 10 μm. **C:** Western blots for caspase-8 and -3 and their uncleaved (pro-) forms. Densitometries of their cleaved (activated) forms are not shown because they were undetected. **D:** Western blots for Bcl-2 and Bax and their densitometric analyses. $^{*}P < 0.05$ versus the sham group; $^{\#}P < 0.05$ versus the doxorubicin-treated group with LacZ gene transfer.

mented cardiac apoptosis (indicated by TUNEL and DNA ladder) that was significantly suppressed by Fas signal inhibition.[6] By contrast, we found that inhibiting Fas signaling had no effect on the prevalence of TUNEL positivity, activation of caspase-8 and −3, or expression of apoptosis-related proteins Bcl-2 and Bax. The discrepancy in apoptosis between two studies may be most likely explained by the fact that Nakamura et al did not see apoptotic bodies until week 9 of doxorubicin treatment.[6] That is, different from the study by Nakamura et al using chronic model, the current study is basically a model of acute doxorubicin cardiotoxicity. It is known

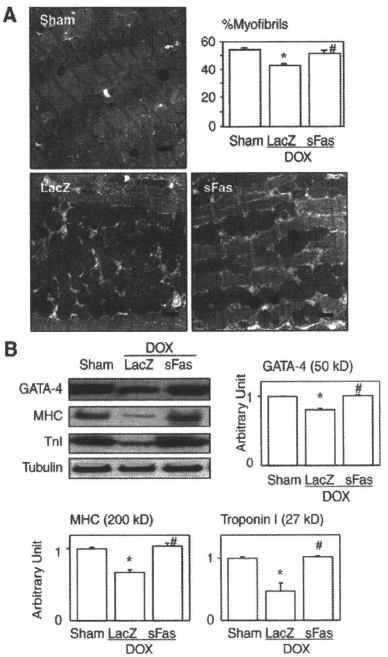

Figure 5. Effects of sFas gene delivery on cardiomyocyte ultrastructure and expression of GATA-4 and sarcomeric proteins in doxorubicin cardiotoxicity. **A:** Electron microphotographs. Doxorubicin treatment induced marked degenerative changes, and the damage was significantly attenuated by sFas gene delivery. Scale bars = 1 μm. Graph showing the percent volume comprised of myofibrils assessed by ultrastructural morphometry. $^{*}P < 0.05$ versus the sham group; $^{\#}P < 0.05$ versus the doxorubicin-treated group with LacZ gene transfer. **B:** Western blots for GATA-4, MHC, and troponin I. $^{*}P < 0.05$ versus the sham group; $^{\#}P < 0.05$ versus the doxorubicin-treated group with LacZ gene transfer.

clinically that doxorubicin occasionally produces an acute cardiotoxicity while the drug causes a chronic cardiomyopathy when given repeatedly.[1–4] Acute injuries occur immediately after treatment and may cause transient arrhythmia, pericarditis, myocarditis, and acute failure of the left ventricle. Chronic effects depend on the cumulative dose and result in dilated cardiomyopathy-like congestive heart failure. The relevance and relationship of the findings of acute effects of doxorubicin involving non-apoptotic pathways induced by Fas signaling to chronic effects of Fas signaling to apoptosis in chronic doxorubicin cardiomyopathy is unclear. Therefore, apoptosis may be a phenomenon specifically related to chronic treatment and cardiomyopathy, but not to the relatively short-term (2 weeks) toxicity of doxorubicin like the present study.

Fas Signaling Inhibition Attenuates Inflammation, Fibrosis and Oxidative Damage in Acute Doxorubicin Cardiotoxicity

The interaction of Fas and Fas ligand activates a well-known pathway via which apoptotic signals are transduced in a variety of cell types.[5] However, recent studies have shown that Fas signaling also mediates a number of biological effects unrelated to apoptosis,[36] including induction of inflammation and fibrosis,[16] generation of reactive oxygen species,[17] acceleration of proliferation/differentiation,[18] and induction of hypertrophy.[19] Consistent with those findings, transgenic mice overexpressing Fas ligand in their hearts showed inflammation and fibrosis but not apoptosis, while development of cardiac hypertrophy induced by pressure overload was shown to be Fas signal-dependent.[20] In the present study, inhibition of Fas signaling attenuated the myocardial inflammation and fibrosis characteristic of doxorubicin cardiotoxicity. This was accompanied by suppression of the activities of c-Jun and NF-κB (two inflammation-related transcription factors) and by down-regulation of cyclooxygenase-2 and MCP-1 (two inflammatory mediators), all of which were activated or augmented in hearts affected by doxorubicin. Although earlier studies reported TNF-α and TGF-β1 to be potent stimulators of inflammation and fibrosis in the failing heart,[38,39] their involvement in doxorubicin-induced cardiotoxicity was challenged in the recent reports.[15,37] Consistent with the latter, we found no significant doxorubicin- or sFas-induced changes in the expression of TNF-α and TGF-β1 despite augmented infiltration of inflammatory cells in the doxorubicin-treated hearts, which are the major source of the cytokines. The discrepancy may be partly explained by the action of doxorubicin that directly down-regulates transcription of TNF-α as previously reported.[37] Effect of doxorubicin on TGF-β1, on the other hand, should be elucidated in future. In addition, the diminished production of 8-OHdG (a marker of oxidative DNA damage) and 4-HNE (a marker of oxidative damage of plasma membrane) indicates that inhibition of Fas signaling attenuates doxorubicin-induced oxidative damage to the heart.

Attenuation of Cardiomyocyte Degeneration by Fas Signaling Inhibition in Acute Doxorubicin Cardiotoxicity

Interestingly, we could not confirm the hypertrophic effect of Fas in our present model. In contrast to the compensatory cardiomyocyte hypertrophy seen in response to pressure overload,[20] doxorubicin exerted an atrophic effect on cardiomyocytes that was unaffected by Fas inhibition with sFas treatment. Nonetheless, Fas inhibition did attenuate the degenerative changes to cardiomyocytes. Cardiomyocytes affected by doxorubicin cardiotoxicity show severe degenerative changes at the subcellular level, including myofibrillar derangement, disruption and loss and proliferation of subcellular organelles such as mitochondria.[15,31] Such myofibrillar degeneration was re-

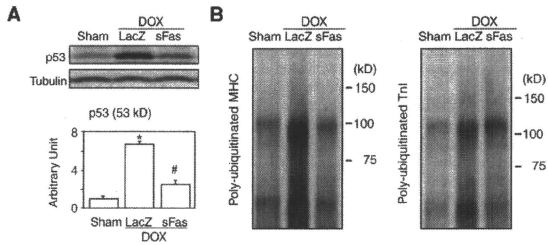

Figure 6. Effects of sFas gene delivery on expression of p53 and ubiquitination of sarcomeric proteins in acute doxorubicin cardiotoxicity. **A:** Western blots for p53. $^{*}P < 0.05$ versus the sham group; $^{\#}P < 0.05$ versus the doxorubicin-treated group with LacZ gene transfer. **B:** Immunoprecipitation and Western blots for polyubiquitinated myosin heavy chain (MHC) and troponin I (TnI).

portedly associated with doxorubicin-induced down-regulation of GATA-4; Kim et al[40] reported doxorubicin down-regulates GATA-4 expression at the gene transcriptional level and we and others,[15,34] and the present study, too, confirmed the doxorubicin-induced decrease in protein expression of GATA-4. Because GATA-4 is a key transcriptional factor regulating cardiac expression of sarcomeric proteins (eg, myosin heavy chain and troponin I),[32,33] it seems plausible that its down-regulation underlies the observed sarcomeric disintegration. Indeed, we not only confirmed that there was a significant reduction in GATA-4 levels in our present model, but we also noted that this reduction was significantly reversed by Fas inhibition, and that expression of myosin heavy chain and troponin I changed in accordance with the GATA-4 level. However, decrease in GATA-4 expression by doxorubicin was indeed significant but only approximately 20% in the present study, which may not be sufficient to hamper synthesis of sarcomeric proteins. A recent study reported that doxorubicin treatment mediates p53-dependent inhibition of mTOR.[35] mTOR is a serine/threonine protein kinase that regulates protein translation and cell growth.[41] In the present study, we found that Fas inhibition significantly attenuates doxoru-

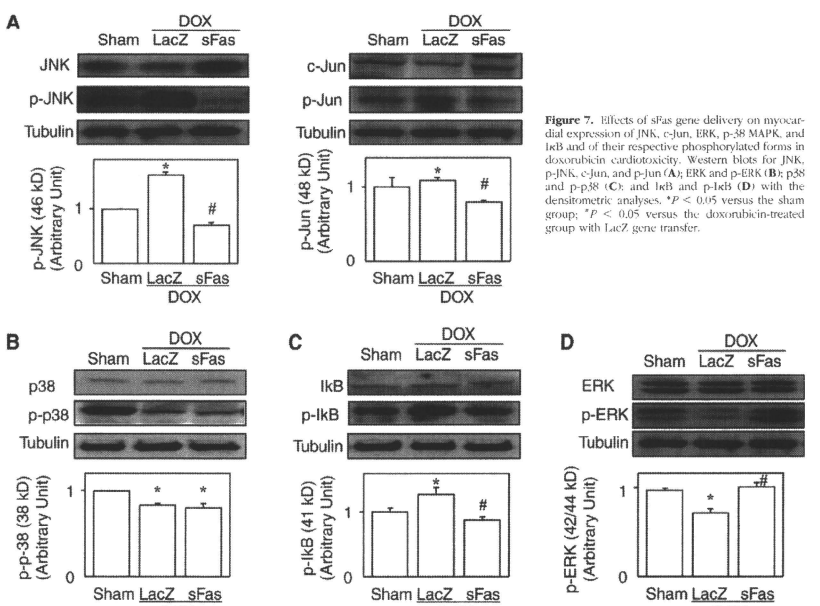

Figure 7. Effects of sFas gene delivery on myocardial expression of JNK, c-Jun, ERK, p-38 MAPK, and IκB and of their respective phosphorylated forms in doxorubicin cardiotoxicity. Western blots for JNK, p-JNK, c-Jun, and p-Jun (**A**); ERK and p-ERK (**B**); p38 and p-p38 (**C**); and IκB and p-IκB (**D**) with the densitometric analyses. $^{*}P < 0.05$ versus the sham group; $^{\#}P < 0.05$ versus the doxorubicin-treated group with LacZ gene transfer.

bicin-induced overexpression of p53. Since molecular pathways that inhibit protein synthesis in doxorubicin cardiotoxicity seem very complicated as discussed here, further studies are warranted to elucidate the whole picture of them.

Not only protein synthesis but also protein degradation is an important regulator of sarcomeric protein volume. For example, activation of the ubiquitin-proteasome system has been reported in doxorubicin-treated cardiomyocytes.[42] In the present study, we found that doxorubicin increased ubiquitination of sarcomeric proteins (ie, myosin heavy chain and troponin I) and that the increased ubiquitination was reversed by Fas inhibition. In addition, doxorubicin was also reported to badly affect actin assembly.[43] Collectively, we suggest that improvement of sarcomeric integrity by the sFas gene therapy is, at least in part, attributable to decreased GATA-4-mediated synthesis, increased degradation through ubiquitination, or both in the present acute doxorubicin cardiotoxicity model.

One would expect that restoration of sarcomeric proteins by Fas inhibition should return the cardiomyocyte size to normal. But, Fas inhibition did not affect doxorubicin-induced atrophy of cardiomyocytes. On the other hand, Fas inhibition made the proportion of sarcomeres within the cytoplasm return to normal as shown by our electron microscopic morphometry. These indicate that Fas inhibition made the proliferated nonsarcomeric constituents (eg, mitochondria) be replaced with sarcomeres to bring about a qualitative improvement of the cardiomyocytes. Also, the heart weight reduction was not affected by Fas inhibition. If the heart weight returned to normal in the sFas-treated group, the heart to body weight ratio should, curiously enough, have been significantly greater than that of the sham group because the body weight in the sFas-treated group was markedly diminished by doxorubicin. Thus, the heart weight in the sFas-treated group might have not needed to return normal as far as cardiac function was actually restored although our data suggest that mass of sarcomeres is indeed increased by Fas inhibition.

Molecular Signaling in Acute Doxorubicin Cardiotoxicity Affected by Fas Inhibition

In the present study, we found that the Fas inhibition with sFas treatment significantly affected several transcriptional factors of which expression or activation was altered by doxorubicin. p53 is one of the most extensively characterized tumor suppressor proteins and is a master regulator with pleiotropic effects on metabolism, antioxidant defense, genomic stability, senescence and cell death.[44] It is well known that doxorubicin-induced p53 up-regulates Fas.[45] Fas signals include JNK and NF-κB activation and reactive oxygen species generation.[17] These activated signals subsequently evoke COX-2 and MCP-1-involved inflammation and also augment oxidative stress, both of which may again stimulate p53. The sFas gene therapy might have interrupted such a vicious feedback loop.

The effect of doxorubicin on ERK/MAPK activation has been studied previously, but the results appear to be conflicting. However, an overall consensus may be that ERK is activated during earlier phase (hours to 5 days) but is inactivated during later phase (2 to 3 weeks) after treatment with doxorubicin.[15,37,46] Activity of GATA-4 transcription factor is subjected to regulation not only at the expression level but also through posttranscriptional modification of GATA-4 proteins.[47] For instance, Liang et al[48] reported that activated ERK (p-ERK) phosphorylates GATA-4 to enhance its DNA binding and transcriptional activation. Another study using isolated rat heart subjected to excessive LV wall stress (induced by balloon inflation) showed an involvement of MAPK (p38 and ERKs) in activation of GATA-4 binding to DNA.[49]

In addition, p53 and protein degradation through the ubiquitin-proteasome system interact each other.[50,51] Activated ERK again negatively regulates ubiquitin-proteasome system as well as autophagy to inhibit protein degradation.[52,53] Collectively, we speculate that sFas exerts its cardioprotective effects via inhibition of Fas signals that evoke inflammation, fibrosis, and reactive oxygen species production which reproduce oxidative stress to activate p53 and inactivate ERK; diminished ERK decelerates GATA-4-dependent sarcomeric protein synthesis and promotes ubiquitin-dependent sarcomeric protein degradation while increased p53 not only activates Fas signaling but also promotes ubiquitin-dependent sarcomeric protein degradation. That said, it remains unknown how Fas signaling affects GATA-4 expression and ERK activation and whether the inflammation and cardiomyocyte degeneration are associated with one another. Further study will be needed to fully clarify the extra-apoptotic effects of Fas signaling in the heart.

Conclusion

In conclusion, we found that sFas gene therapy prevents the progression of doxorubicin-induced acute cardiotoxicity, accompanying attenuation of the cardiomyocyte degeneration, inflammation and oxidative damage caused by Fas signaling. These findings not only provide novel mechanistic insight into the pathogenesis of acute doxorubicin cardiotoxicity but also suggest that anti-Fas gene therapy is a potentially useful approach to preventing or ameliorating acute doxorubicin cardiotoxicity. However, safety of anti-Fas strategies or a virus-mediated gene therapy has not been confirmed in humans. These issues should be resolved before clinical application of the anti-Fas gene therapy.

Acknowledgment

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Expert Opinion

Leptin gene therapy in the fight against diabetes

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Importance of the field: The incidence of diabetes is increasing worldwide, yet current treatments are not always effective for all patient or disease types.
Areas covered in this review: Here, we summarize the biologic and clinical roles of leptin in diabetes, and discuss candidate viral vectors that may be employed in the clinical use of central leptin gene therapy for diabetes.
What the reader will gain: We discuss how studies on leptin, a regulator of the insulin–glucose axis, have significantly advanced our understanding of the roles of energy homeostasis and insulin resistance in the pathogeneses of metabolic syndrome and diabetes. Recent studies have demonstrated the long-term therapeutic effects of central leptin gene therapy in obesity and diabetes via decreased insulin resistance and increased glucose metabolism. Many of these studies have employed viral vectors, which afford high *in vivo* gene transduction efficiencies compared with non-viral vectors.
Take home message: Adeno-associated viral vectors are particularly well suited for central leptin gene therapy owing to their low toxicity and ability to drive transgene expression for extended periods.

Keywords: adeno-associated viral vector, diabetes, gene therapy, leptin, viral vector

Expert Opin. Biol. Ther. [Early Online]

1. Introduction

Diabetes is a genetic disorder in which environmental factors precipitate phenotypic expression of the disease. The young and adult patient populations with obesity-related diabetes have recently been increasing at an alarming pace worldwide [1]. Current treatments for diabetes aim to establish glycemic control, and several medications that lower blood glucose levels, including insulin, have been developed. Although some of these medications are useful for certain patient types with diabetes, there is significant room for improvement. For instance, repeated daily administration of medications can be troublesome for patients, and some glucose-lowering medications are ineffective for certain diabetes types. Thus, novel therapeutic strategies, including those that provide prolonged effects after each application, are necessary to treat patients with diabetes more effectively.

Adipocyte-derived leptin is a pleiotropic hormone that peripherally and centrally regulates food intake and energy homeostasis [2-4]. Central administration of leptin inhibits appetitive drive, body weight gain and adiposity, and stimulates nonthermogenic energy expenditure via brown adipose tissue [3,5-7]. Several studies have suggested that leptin also contributes to hypothalamic control of the insulin–glucose axis. For example adeno-associated viral vector (AAV)-mediated central leptin gene therapy via an intracerebroventricular (i.c.v.) route was recently demonstrated to increase hypothalamic leptin levels to ameliorate hyperglycemia and hyperphagia in diabetic, insulin-deficient nonobese Akita mice and leptin-deficient obese *ob/ob* mice and also to promote survival in insulin-deficient mice studied by Kojima *et al.* [8,9]. The technique aided by AAV produces a robust and durable central leptin supply, which reinstates euglycemia and energy homeostasis for

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Article highlights.

- The biologic and clinical roles of leptin in diabetes.
- Central leptin gene therapy provides long-term therapeutic effects.
- Characteristic features of the representative viral vectors.
- Adeno-associated viral vectors may be suitable for central leptin gene therapy.
- Possible adverse effects of future leptin gene therapy are discussed.

This box summarizes key points contained in the article.

an extended period in the absence of insulin and is expected to be an effective therapeutic strategy for type 1 and type 2 diabetes. Here, we discuss diabetes, leptin and gene therapy vectors, including the potential of central leptin gene therapy as a substitute for conventional insulin therapy.

2. Diabetes

Diabetes is a chronic illness that requires continuous medical care and patient education on self-management to prevent acute complications and reduce the risk of long-term morbidity and mortality [10]. Diabetes afflicts an estimated 6% of the adult population in the Western hemisphere, and is increasingly problematic as the Eastern hemisphere adopts Western life-style approaches [11]. The WHO estimates that 170 million people worldwide have diabetes, and that the population will increase to 366 million by 2030 [12]. The rapid increasing of obesity is the most important pathogenic factor in diabetes [11].

2.1 Classification and clinical manifestations

Diabetes can be categorized into four clinical classes. Type 1 diabetes is a chronic autoimmune disorder characterized by destruction of insulin-producing pancreatic β cells [13]. Type 2 diabetes results from a progressive defect in insulin secretion on a background of insulin resistance; symptoms can manifest at any age. The disorder is influenced by a persistent metabolic imbalance that is engendered by a range of internal and external environmental factors, including diet and other lifestyle choices [1,14-17]. Type 2 diabetes is characterized by a range of metabolic disturbances – for example chronic hyperglycemia, insulin insensitivity in fat and muscle cells, hepatic glucose production in the prandial state, and decreased β cell effectiveness, which disrupts the first-phase insulin response to nutrient ingestion [12]. Most diabetes patients suffer from either type 1 or type 2 diabetes, with type 2 insulin-resistant diabetes accounting for 90 – 95% of all diabetes cases [11]. Another type of diabetes is caused by specific disorders, such as diseases of the exocrine pancreas (e.g., cystic fibrosis), or genetic defects in β cell function or insulin signaling. Finally, gestational diabetes mellitus (diabetes diagnosed during pregnancy) is generally classified as an independent diabetes type [10].

2.2 Current treatments

In addition to symptomatic treatment of disease complications, current diabetes therapy attempts to supplement insulin, increase insulin sensitivity, reduce excessive hepatic glucose production. and/or enhance glucose-stimulated insulin secretion [11]. Medications include recombinant insulin, oral anti-diabetes drugs, anti-hypertensive medications, and anti-dyslipidemic agents [12]. Diabetes is accompanied by hypertension, cardiovascular disease and microvascular disorders, which complicate treatment and can result in blindness, non-traumatic limb amputation, and renal failure [10]. Clinical studies have demonstrated that glycemic control is crucial for avoiding and/or delaying the onset of diabetes complications. The American Diabetes Association (ADA) and European Association for the Study of Diabetes (EASD) have established < 7.0% glycosylated hemoglobin (HbA_{1c}) as a treatment goal for adults with diabetes [18]. Although conventional treatments, such as diet, exercise and insulin replacement regimens, can effectively lower HbA_{1c} levels in diabetes patients, none address obesity or insulin resistance, or maintain normoglycemia permanently. Therefore, novel therapeutic strategies that persistently normalize the abnormal phenotypes of diabetes are eagerly awaited.

3. Leptin

3.1 Biology of leptin

Leptin is a 167 amino-acid protein product of the obese (*ob*) gene, which was identified in 1994 based on the morbid obesity phenotype that results from its absence [19-21]. Leptin is secreted from adipocytes in white adipose tissue, stomach, placenta, and mammary glands [19,22-25]. It acts primarily on the hypothalamus to modulate food intake and energy expenditure. The leptin receptor (OB-R) is alternatively spliced into six isoforms (OB-Ra, OB-Rb, OB-Rc, OB-Rd, OB-Re and OB-Rf) [3,26,27]. The short isoforms contain a C-terminally truncated intracellular domain (OB-Ra, OB-Rc, OB-Rd, OB-Rf) and may transport leptin through physiological barriers. OB-Ra is expressed in the endothelium of the vasculature and epithelium of the choroid plexus of the circumventricular organs, where it transports leptin across the blood-brain barrier. The long isoform (OB-Rb), which contains an extensive C-terminal intracellular domain, is abundantly expressed in hypothalamic neurons, including the hypothalamic arcuate nucleus (ARC), dorsomedial hypothalamic nucleus, paraventricular nucleus (PVN), ventromedial hypothalamic nucleus (VMH), and lateral hypothalamic nucleus of the CNS as described by Friedman and Halaas in 1998 [3]. OB-Rb activates JAK/signal transducer and activator of transcription signaling, affecting expression of hypothalamic neuropeptides. The soluble isoform (OB-Re), which contains only the extracellular domain, is a serum

leptin-binding protein. Leptin circulates as a free form or OB-Re–bound forum, and the sum of the two is generally measured as the total leptin level.

The synthesis and secretion of leptin are markedly increased in obese subjects [28]. Notably, leptin replacement either peripherally or centrally in leptin-deficient subjects [3,29-33], or selective introduction of the leptin receptor into the hypothalamus of OB-Rb mutant rats has been shown to normalize food intake and body weight [34]. Taken together, these results highlight the important metabolic roles of adipose tissue.

3.2 Leptin and diabetes

A number of studies in mice, rats, and humans have demonstrated that leptin is a key hormone in the regulation of not only food intake and energy expenditure but also insulin secretion and glucose metabolism via hypothalamic control of pancreatic insulin–glucose homeostasis [2-6,28,35-43]. Insulin is secreted from pancreatic β cells to stimulate adipogenesis and increase fat deposition in adipocytes [2,44-49]. Recent studies have shown that leptin inhibits episodic and postprandial insulin hypersecretion from the pancreas and modulates insulin activity in adipocytes, myocytes, and hepatocytes. C57BL/6H *ob/ob* mice, which carry a mutation in the obese gene, are obese and diabetic, and show reduced activity, metabolism, and body temperature [42]. Injections of recombinant leptin in C57BL/6H *ob/ob* and wide-type mice reduced body weight, the percentage of body fat, food intake and serum concentrations of glucose and insulin [20,21,42]. Furthermore, white and brown adipose tissues completely disappeared for an extended period in transgenic skinny mice that overexpressed leptin in the liver [28]. Glucose metabolism in the skinny mice increased in concert with an activation of insulin signaling in skeletal muscle and liver. Moreover, the livers of these mice were small and showed decreased stores of glycogen and lipid. Similar effects were found after acute intracerebroventricular infusion of leptin, which led to a significant increase in glucose turnover without altering the plasma insulin concentration [41]. Central administration of leptin has been shown to increase glucose turnover and uptake, and to decrease hepatic glycogen storage, suggesting that the effects of leptin on glucose metabolism are mediated largely via central mechanisms [8,40]. Leptin increases glucose uptake in peripheral tissues in humans independently of weight loss, which may be beneficial to some patients with type 1 or type 2 diabetes [2,3,49]. Together, these studies suggest a number of potential pathophysiologic and therapeutic roles for leptin in diabetes [13,34].

4. Gene therapy

Gene therapy is a novel therapeutic strategy that introduces a specific gene into patients to treat their disease. To date, more than 1,500 clinical trial protocols have been approved around the world [50]. A key issue for successful gene therapy is the development of an appropriate vector for the target disease. General requirements for vectors that can be used to treat congenital or acquired diseases (outside of cancer, which is a somewhat special case) include high *in vivo* gene transduction efficiency in target tissues and/or cell types, a clinically acceptable safety profile, long-term expression of the transgene, and the ability to carry and transduce a full-length gene, including transcriptional control elements. Although ideal vectors have not yet been developed, recombinant viral vectors have been widely used for clinical trials owing to their high *in vivo* gene transduction efficiency.

4.1 Features of representative vectors and their potential for central leptin gene therapy

Gene therapy with leptin is a promising treatment strategy for diabetes. Here, we summarize characteristic features of representative vectors and discuss their potential utility for central leptin gene therapy (**Table 1**).

4.1.1 Adenoviral vector (ADV)

Adenovirus is double-stranded DNA virus with a 36 – 40 kb genome, which encodes more than 70 gene products. The viral genome contains five early transcription units (E1A, E1B, E2, E3 and E4), two early delayed (intermediate) units (pIX and IVa2), and five late units (L1 – L5), which encode structural proteins for the capsid and internal core. Inverted terminal repeats at the end of the viral chromosome function as replication origins. ADVs can be easily prepared to high titer and can transduce genetic material into a variety of tissues *in vivo*, such as heart, liver, kidney, CNS, and cancers, irrespective of whether the cells are dividing [45,51-59]. A particularly important clinical advantage of ADVs is the relatively low potential for carcinogenesis, which is largely due to the episomal nature of the transgene (i.e., the transgene is not integrated into the chromosome).

First-generation ADVs, which lack E1 and E3 regions are viral-replication defective, have been used clinically for *in vivo* gene therapy directed at a variety of diseases [60,61]. Although first-generation ADVs can package and transduce up to 7 kb of sequence, they produce only transient transgene expression over the course of weeks when they are administered to immunocompetent hosts, because even low levels of certain viral proteins transcribed from the vector induce cytotoxic immune responses. To address this issue, helper-dependent ADVs (HD-ADVs), were developed by deleting all viral coding sequences from the vector backbone. *In vivo* administration of HD-ADVs in rodents resulted in persistent transgene expression. An additional advantage of HD-ADVs is their ability to package up to 37 kb of transgene sequence. A comparative study of leptin gene therapy using HD-ADVs and first-generation ADVs demonstrated that HD-ADVs efficiently delivered the transgene, causing prolonged elevations of serum leptin levels and weight loss. Furthermore, the liver toxicity, inflammation and cellular infiltration observed with first-generation ADVs were

Table 1. Characteristic features of the representative viral vectors.

	ADV			AAV	RV	LV
	1G-ADV	HD-ADV	CRA			
Capacity for transgene	~ 7 kb	~ 37 kb	*	~ 4.5 kb	~ 7 kb	~ 7 kb
In vivo gene transduction efficiency	High	Medium	Very high	High	Very low	Medium
Integration into host genome (frequency)	Low	Low	Low	Low	Very high	Very high
Duration of transgene expression	Transient	Persistent	N.D.	Persistent	Persistent	Persistent
Cytotoxic immune response (severity)	Relatively high	Minimal	*	Medium	Minimal	Minimal
Titer	Very high	Medium	Very high	High	Low	Medium
Clinical protocols[‡] (total: 815)	387[§]			71	336	21
Possible usefulness for central leptin gene therapy	Medium	High	N.D.	Very high	Very low	N.D.

*Depends on CRA construction and/or a protocol for *in vivo* use.

[‡]Gene Therapy Clinical Trial Worldwide, 2010 (http://wiley.co.uk/genmed/clinical/)

[§]Total number of ADV clinical protocols.

1G-ADV: First generation ADV; AAV: Adeno-associated virus; ADV: Adenovirus; CRA: Conditionally replicating ADV; HD-ADV: Helper-dependent ADV; LV: Lentivirus, N.D.: Not determined; RV: Retrovirus.

significantly better with HD-ADVs [62]. Potential problems with the clinical use of HD-ADVs include a relatively low titer and possible contamination from the helper virus.

On the other hand, most clinical trials of ADV-based gene therapy have been conducted in the field of oncology, which can be used to formulate plans for ADV-based leptin gene therapy [63]. A major obstacle to cancer gene therapy is inefficient and nonspecific gene delivery to cancer cells, leading to unsatisfactory outcomes in clinical trials due to tumor recurrence from nontransduced cancer cells [64]. Conditionally replicating ADVs (CRAs), which selectively replicate in tumor cells but not in normal cells, may circumvent this problem to achieve efficient, tumor-specific gene delivery. Moreover, we have recently developed a novel method to construct m-CRAs, highly engineered CRAs that are regulated by multiple factors [63,64]. Although the utility of current CRAs for central leptin gene therapy is unknown, the lessons from ADVs may be useful in the development of ideal ADVs for central leptin gene therapy.

4.1.2 *Adeno-associated viral vector (AAV)*

AAVs are single-stranded DNA parvoviruses. They are not pathogenic and can efficiently infect both dividing and nondividing cells. Serotype 2 AAV is often used for gene therapy because wild-type serotype 2 AAV is not associated with any human diseases. AAV does not contain any genes that encode viral proteins, eliminating the risk of potentially undesirable immune responses. High titers of AAV ($> 10^{12} - 10^{13}$ particles/ml) can be stably prepared. Since the first AAV-based gene therapy trial in human patients was performed in 1995 [65], several additional clinical trials of AAV-mediated gene therapy have been initiated for cancer and other diseases. Long-term transgene expression, which is required for many gene therapy applications, has been observed after AAV-mediated *in vivo* gene transduction into lung, liver, muscle, heart, and brain [34,66-74]. New AAV serotypes have advanced our ability to target specific cell types [75], facilitating studies in a diverse range of disease models, including Parkinson's disease, Alzheimer's disease, hemophilia, diabetes, obesity, α1-antitrypsin deficiency, Canavan disease, cystic fibrosis, lysosomal storage diseases and Duchenne muscular dystrophy [76]. Some treatments have progressed to clinical use, and AAV is thought to be a promising viral vector for clinical gene therapy. A disadvantage of AAV is that the size of the packaged transgene is limited to less than 5 kb.

4.1.3 *Retroviral and lentiviral vectors (RV and LV)*

RV was used for the first clinical gene therapy trials in human patients. RV can accommodate up to 7 kb of transgene sequence and can integrate into the host genome, resulting in persistent transgene expression [52,77,78]. A disadvantage of this vector is the requirement for active host cell replication at the time of gene transduction, which is particularly problematic for *in vivo* neuronal gene therapy. To address this issue, recently developed LVs can transduce genes into nondividing cells, including neurons, after *in vivo* administration. Both RV and LV integrate the transgene into the host genome, a critical safety concern in clinical trials.

4.2 AAV-mediated central leptin gene therapy for diabetes

In summary, ADV and AAV have the advantage of high *in vivo* gene transduction efficiency and safety compared with RV and LV. Thus, we will focus on ADV- and AAV-mediated leptin gene therapy. In 1995, injections of recombinant leptin were shown to reduce body weight and fat deposition [21,42], through the direct activities of leptin on neuronal networks that control feeding and energy balance [20]. At that time, first-generation ADV were being developed and

used for gene therapy studies of many disease models. In 1996, obesity and diabetes in leptin-deficient *ob/ob* adult mice were treated effectively with tail-vein injections of ADV expressing mouse leptin [79]. ADV-mediated leptin gene therapy for *ob/ob* mice led to serum leptin levels that were 70 times higher than those observed in control C57BL/6J mice; the treated *ob/ob* mice demonstrated a rapid reduction in food consumption and marked weight loss. Notably, the elevated serum leptin levels in the treated animals did not persist beyond 2 – 3 weeks due to a cytotoxic immune response against proteins encoded by the first-generation ADV [80,81]. Interestingly, complete amelioration of the obese phenotype was achieved after leptin gene transfer in *ob/ob* mice despite the lack of persistent leptin gene expression. A rapid resumption of food intake to pretreatment values and gradual body weight gain were observed after the serum leptin concentrations fell to undetectable levels, indicating that treatment did not result in long-lasting adverse effects. The data also showed that hyperinsulinemia and insulin resistance were improved and fasting blood glucose was reduced after leptin gene transduction, which together eliminated the non-insulin-dependent diabetic phenotype in the *ob/ob* mice.

Then, in 1997, a single intramuscular injection of AAV encoding mouse leptin (AAV-lep) to *ob/ob* mice was shown to prevent obesity and diabetes for more than six months by maintaining normal circulating levels of leptin (2 – 5 ng/ml) [31]. Although systemic injection of AAV-lep is effective for obesity and diabetes in *ob/ob* mice, enhancing ectopic leptin production in peripheral tissues (skeletal muscle or liver) is not an appropriate weight control strategy because of the pleiotropic effects of leptin [65,76,82,83]. Chronic increases in the peripheral leptin concentration after intraperitoneal or subcutaneous injection led to reduced food intake in lean and, to a lesser extent, in diet-induced-obese mice. Both groups, however, developed peripheral leptin resistance, which could be overcome by intracerebroventricular injection of a leptin dose that was 4,000-fold lower than the peripherally applied dose [32,84]. Peripheral but not central, leptin resistance in this murine model of obesity suggests that leptin resistance can be overcome by a central leptin supply. In addition, high levels of peripheral leptin in overweight individuals do not appear to cross the blood–brain barrier [85,86]. In accordance with the observation that the weight-reducing effects of leptin are predominantly mediated through the hypothalamus, AAV-mediated gene transduction and leptin overexpression in the hypothalamuses of normal rats suppressed age-related body weight gain and increases in fat mass, adiposity and serum insulin for more than six months without affecting food consumption was reported by Dhillon *et al.* in 2001 [87]. In normal rats, at six weeks after i.c.v. injection of AAV-leptin, which mediated leptin overexpression, the hypothalamus leptin mRNA levels were three times higher than those in control rats. As reported at two weeks post-injection, reduced serum leptin correlated with significant reductions in body weight due to a loss of fat depots with no change in lean mass. The expression of uncoupling protein-1 (UCP1) mRNA, a measure of thermogenic capacity, in brown adipose tissue doubled in the treatment group, indicating that thermogenic energy expenditure was augmented in the treated rats.

On the other hand, gene transduction therapy with AAV bearing leptin receptor (AAV-OB-Rb) was shown to improve energy balance and reproductive status in obese female Koletsky rats [34]. When AAV-OB-Rb was microinjected into the media preoptic area, PVN, VMH, ARC or dorsal vagal complex in the brainstem, all groups showed marked expression of human leptin receptor mRNA. Evaluation based on body weight, food intake and UCP-1 mRNA, leptin, insulin and glucose levels revealed that injections in the PVN were generally ineffective, whereas injections in the ARC were most effective. These results suggest that AAV can be used to achieve long-term expression of functional leptin receptors in the CNS, and that leptin acts at specific brain locations to affect food intake, energy expenditure, and reproduction. Intracerebroventricular AAV-mediated leptin gene therapy can produce a stable leptin supply in the hypothalamus, and the exogenous leptin is not transported to extra-hypothalamic sites [9,29,38,39,87] or the periphery [33,88]. Therefore, increasing the local supply of leptin via ADV- or AAV-mediated gene therapy may represent a novel therapeutic approach for diabetes patients [2,14,45,89-92].

Hyperinsulinemic, obese *ob/ob* mice (leptin mutants) and severely insulin-deficient, nonobese Akita mice (insulin 2 mutants) were used to examine the effects of intracerebroventricularly administered AAV-lep by Kalra and colleagues [88]. A key to the prevention of type 2 diabetes is suppression of hyperglycemia through decreased insulin resistance and increased glucose disposal [3,47]. AAV-leptin-treated *ob/ob* mice show elevated mRNA levels of GLUT1 and GLUT4, glucose transporters that play important roles in regulating glucose uptake in brown adipose tissue. This result may reflect increased glucose uptake in brown adipose tissue if glucose transporter expression is regulated solely through the hypothalamus.

One possible mechanism to improve glucose metabolism is related to central leptin-mediated increases in UCP1 expression and enhanced glucose uptake in brown adipose tissue. Increased UCP1 mRNA expression has been detected in wild-type, *ob/ob*, and Akita mice after treatment with AAV-lep. Studies of viral-vector-based central leptin gene therapy in insulin-deficient or leptin-deficient animal models have provided profound insights into the regulatory effects of leptin on glucose homeostasis, insulin resistance, food intake, and body weight. Data from a number of sources support central leptin gene therapy as a potential alternative to current insulin therapy for diabetic patients to ameliorate hyperglycemia and hyperphagia. Of particular interest is the observation that a single course of central leptin gene therapy effectively

and durably reinstates euglycemia and energy homeostasis in the absence of insulin. Thus, a local enhancement of the leptin supply in the hypothalamus offers an alternative anti-diabetic treatment paradigm for both type 1 and type 2 diabetes. Results that support treatment of type 1 diabetes include the observed amelioration of hyperglycemia and diabetes in Akita mice after a single intracerebroventricular injection of AAV-leptin, which enhanced the rate of glucose disposal and insulin sensitivity [88]. The key to preventing type 2 diabetes with leptin gene therapy is to suppress hyperglycemia, decrease insulin resistance, and increase glucose disposal [24,46,47,88]. Furthermore, central leptin gene therapy may also be associated with anti-obesity and anti-aging effects, as described by Kalra *et al.* [2,89,91].

4.3 Possible adverse effects

In most fields of biomedical research, including gene therapy, experimentally promising results have not always resulted in successful outcomes in actual clinical trials. Adverse effects, which are sometimes unpredictable or undetected in preclinical studies, are the most critical types of failure in clinical trials. One possible side effect may be caused by long-term leptin production, because the previously reported systems for administering central leptin gene therapy, which were described in the earlier subsections, involve the constitutive overexpression of leptin without native transcriptional regulation. Although the possibility of production of an antibody against leptin may be low or none, such aberrantly overexpressed leptin may have the possibility of side effects related to an imbalance of the leptin feedback regulatory loop. To avoid or reduce the possible side effects, the optimal dose of leptin-expressing vector should be carefully determined in preclinical studies. Other side effects may also be caused by viral vectors as follows.

Adverse events after RV-mediated gene therapy are mainly caused by insertional mutagenesis. The vector sequence of the mouse leukemia long-terminal repeat, when integrated into chromosome transactivated neighboring promoters, leading to aberrant gene expression and a growth advantage for the transduced cells in which the oncogene is abnormally activated, thereby resulting in the later onset of leukemia in patients [93]. Although, in theory, any integration vectors such as LV may carry a similar risk of insertional mutagenesis, it is quite uncertain that this side effect occurring after retroviral *ex vivo* gene therapy to hematopoietic cells would be similarly reproduced after *in vivo* local administration of LV, for example after LV-mediated leptin gene transduction into the CNS.

In contrast, recombinant ADV has a safety advantage due to the episomal nature of the transgene, leading to much lower risk of insertional mutagenesis, and the safety in the case of local injection of ADV has been well established by a number of clinical trials in human cancer patients. On the other hand, only a single case of the death of a patient after ADV-mediated gene therapy has been reported; however, this clinical trial was quite special, and several questions as to its suitability were raised from clinical and scientific viewpoints [94,95].

Infusion of a large amount of ADV into the right hepatic artery – that is a delivery system similar to systemic ADV administration – of a patient with congenital metabolic disease has been shown to result in lethal systemic inflammatory response syndrome [95]. It is uncertain the degree to which side effects related to the immune reaction are induced by local injection of a small amount of ADV, including HD-ADV, into the CNS of diabetic patients in clinical trials.

Compared with the first-generation ADV, recombinant AAV has safety advantages not only in terms of the episomal nature of the transgene, but also due to the reduced induction of cellular immunity. However, it must also be considered that a patient with rheumatoid arthritis died during an AAV-mediated gene therapy trial, which employed the intra-articular delivery of a TNF-α antagonist through the AAV delivery system [96]. Although a later investigation demonstrated that the patient's death was primarily the result of disseminated histoplasmosis as an opportunistic infection with subsequent bleeding complications and multiorgan failure, the contribution of an immune response to the AAV could not be evaluated [96]. In this regard, not only should the safety concerns associated with ADV be extensively examined in preclinical studies, but these concerns should also be carefully monitored in actual human clinical trials of AAV-mediated central leptin gene therapy.

5. Expert opinion

Ideal anti-diabetes therapies would allow safe and long-lasting glycemic control without perturbing physiological homeostasis. In addition to conventional insulin therapy, central leptin gene therapy may develop into an effective therapeutic strategy for diabetes patients. Particularly positive findings with leptin therapy include decreased body weight and food intake via activity in brown adipose tissue, whereas these parameters increase with insulin therapy. Moreover, the effects of leptin therapy result in improvements in insulin resistance. Therefore, central leptin gene therapy that results in local hypothalamic supplies of leptin is a promising approach for diabetes patients, and may even substitute for insulin therapy in the future, as a previous review also suggested [90].

A key issue in central leptin gene therapy is the development of appropriate vectors for human clinical trials. Vectors should result in high *in vivo* gene transduction efficiency, long-term transgene expression and minimal immunogenicity and toxicity. Although AAV is a promising vector for gene therapy, clinical evidence supporting its use is still embryonic. In practice, an unexpected death occurred in a human clinical trial that used AAV, although the possible contribution of an immune response to the AAV could not be evaluated [96,97]. This event, together with lessons from

clinical gene therapy trials for other diseases, suggests that extensive preclinical studies focusing on safety should be performed before testing central leptin gene therapy in human patients with diabetes.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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