第2章　新しい神経治療法

1. RNAiの神経疾患への応用

1.1 はじめに

RNA干渉（RNAi）はいかなる遺伝子に対してもデザインすることができ、その標的遺伝子の発現抑制効果は他の核酸医薬であるアンチセンス核酸の$10^{3\sim7}$倍、リボザイムの$10^{2\sim5}$倍（自験）高いといわれている。しかもその配列特異性も高く1塩基の違いの認識も可能であり、医療分野におけるその臨床応用については発見当初から大きく期待されていた。それは、RNAiライブラリを始めとする創薬におけるツールといった側面と、short interfering RNA（siRNA）を直接核酸医薬として疾患に適応するという2つの方面から行われている。ここでは、すでにウイルス性疾患、遺伝性疾患、悪性腫瘍などで急速に進んでいるsiRNAの核酸医薬としての開発の研究現状と問題点について、神経疾患を中心に概説したい。

1.2 siRNAの特異性

1.2.1 変異遺伝子特異的なsiRNA

遺伝性疾患や癌遺伝子をsiRNAで治療しようとした場合、変異遺伝子のみを選択的に発現抑制して、野生型には作用しないことが望ましい。siRNAと基質RNAとの特異性については、一般に4塩基以上ミスマッチがあった場合でsiRNAの切断活性はおおむね消失するが、1～2塩基のミスマッチによる切断効率の低下は完全ではなく、ミスマッチの位置によってその効果は異なる。当初は5′端から9、10、11塩基目の中央部位の変異が失活化に最も有効とされた[1]。5′側は基質との結合よりRISCとのかかわりから基質を切断するルーラー（物差し）効果があるといわれ[2]、3′側よりのミスマッチほうがより失活効果が強い場合が多い[3]。現在のところsiRNAの5′端から9～16塩基目にミスマッチをデザインすると変異遺伝子の識別が最もよいと考えられている。

われわれも変異G93A SOD1RNAにおいて野生型のSOD1を切断しないsiRNAを作製する際、最も有効なミスマッチの位置を検討した結果、類似の結果を得た（図2.1.1）。

1.2.2 off-target効果などの副反応

siRNAを臨床応用する際にも、ライブラリーを用いた遺伝子探索をする際にも、off-target効果、すなわちターゲットとした遺伝子以外に、用いた19塩基のsiRNAの配列に部分的にホモロジーのある別の遺伝子の発現を抑えてしまういわゆる交叉反応が報告されている[4]。全般にその特異性はアンチセンスなどに

a

5th	5-GAUG**C**UGUGGCCGAUGGUG TT-3
7th	5-AAGAUG**C**UGUGGCCGAUGU TT-3
9th	5-CAAAGAUG**C**UGUGGCCGAU TT-3
10th	5-ACAAAGAUG**C**UGUGGCCGA TT-3
13th	5-CTGACAAAGAUG**C**UGUGGC TT-3
16th	5-TGACTGACAAAGAUG**C**UGU TT-3

b

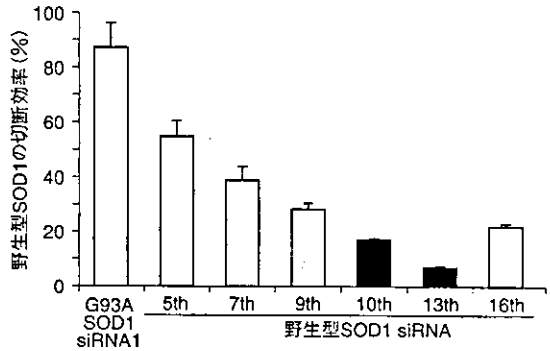

図2.1.1 siRNAへの標的遺伝子とのミスマッチ変異挿入位置によるsiRNA効果への影響

a：家族性筋萎縮性側索硬化症の遺伝子変異であるG93ASOD1（点変異G→C、太字で示す）を標的としたG93AsiRNAのデザイン。

b：G93AsiRNAの5′側から、10～13番目の塩基に変異部位を置いた場合、野生型SOD1の切断効率が最も低下する。

比較してかなり高いが、それでも多くの遺伝子の発現が少なからず影響を受ける可能性がある。Jacksonらの検討[4]で、通常19塩基中15塩基以上で、最低では11塩基のホモロジーのある遺伝子においても影響があったと報告された。その場合は上述のようにホモロジーのあるsiRNAの部位はセンス配列の中央部や3′側にある場合が多い。さらに稀ではあるが、アンチセンス配列でもその影響が出る場合もあり得るという。今後このoff-target効果の評価とその回避は重要な問題である。

また、通常の19塩基長のshort-hairpin型のsiRNA発現ベクターの発現によって、動物細胞でPKRの活性化などのインターフェロン反応が実は起こっていて、非特異的な蛋白合成と停止とRNA変性が起こり得るという報告がされ、これもその程度によっては今後問題になるかもしれない[5]。

1.3 疾患への遺伝子治療

1.3.1 ウイルス性、免疫性疾患

RNAiの本来の生理学的役割の1つとして細胞に感染したウイルスの蛋白合成を阻害する作用が考えられ、siRNAの発見以来、ウイルスゲノム遺伝子やウイルスmRNAを標的とした研究が急速に進んでいる。現在まで、エイズウイルス（HIV）[6]、C型[7]・B型肝炎ウイルス、ポリオウイルス[8]、インフルエンザウイルス、西ナイルウイルスで培養細胞レベルではあるが各ウイルスのレプリコンを用いるなどで有効なsiRNAが報告されている。ここでは、われわれが作製したC型肝炎ウイルス（HCV）に対するsiRNAについて[7]紹介する。

HCV遺伝子は9600塩基からなるプラス1本鎖RNAで、5′と3′非翻訳領域（UTR）に挟まれた翻訳領域（ORF）からなる。5′側の341塩基のUTRは複雑なRNA構造のIRES（internal ribosome entry site）を含み、HCV RNAはキャップ非依存的にこの5′IRESにより翻訳される。（図2.1.2a）。

HCVは1本鎖RNAウイルスであるがゆえ、プルーフリーディング機能がなく、ウイルス複製時にORFに変異を起こしやすくquasispeciesと呼ばれている。このため慢性のウイルス感染においては、siRNAによる治療をする際、siRNAの効果からすり抜け現象が予想される。そこで、われわれはHCVの遺伝子型にかかわらず保存されている5′UTR IRESにsiRNAのターゲットを絞ってデザインした。

図2.1.2bにわれわれの5′UTR IRESに対してデザインしたsiRNAの効果を示す。ヒト肝細胞癌株Huh-7細胞に導入したHCV遺伝子が自己複製するHCVレプリコンシステムにおいて、siRNA#5が著明にHCV遺伝子増殖を抑制した。このウイルス遺伝子の変異に対して、上記のように変異のない保存されたウイルス遺伝子領域を使うことや、複数のsiRNAを使用する方法および、長いhairpin発現ベクターを作製する解

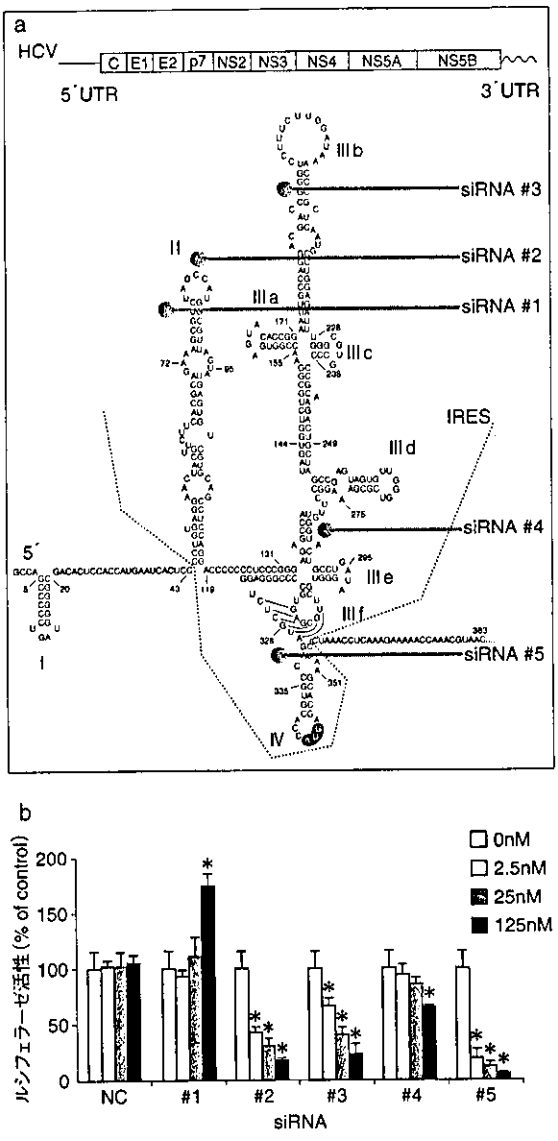

図2.1.2 HCV遺伝子とsiRNA（文献7より改変転載）

a：HCVの遺伝子構造と、HCV 5′UTR領域（IRES）のRNAの2次構造とsiRNAのターゲット部位。

b：siRNAのHCVレプリコンへのHCV遺伝子増殖抑制効果。siRNA#5がコントロールに比較して125 nMのsiRNA濃度では97 %のルシフェラーゼ活性の抑制が達せられ、2.5 nMの非常な低濃度siRNAでも約80 %の抑制がみられた。

決方法も考えられている。

また、ウイルス遺伝子そのものを標的とするのではなく、ウイルス増殖に必要な宿主側の内在性遺伝子を標的にする方法も考えられている。HIV感染における TSG101[9] や NF κ B p65[10] サブユニットなどを siRNA で発現を抑制し、HIV ウイルス増殖を抑制したとの報告もある。

さらに、CD4 や CCR5 などの HIV-1 感染におけるリンパ球側に内在するウイルス受容体を標的としてその発現を抑制する方法も成果があり、注目されている[11]。$CD34^+$造血幹細胞に CCR5 に対する siRNA をレンチウイルスで安定発現させたところ、正常に分化して *in vitro* でマクロファージに、*in vivo* で T リンパ球になり、その両者ともに HIV ウイルスに抵抗性になったとの報告がされ、今後の臨床応用に期待が持たれている。

一方、IL-1 や TNF-α などの炎症性サイトカインの発現を抑制することにより、免疫性疾患の治療としての可能性や感染症の初期治療としての試みが報告されている[12]。

1.3.2 遺伝性疾患

遺伝性疾患でゲノム遺伝子変異が原因で発症する場合、遺伝子変異に起因する発症機序には変異のある遺伝子の遺伝子産物である蛋白の本来の持つ機能の消失または低下する場合（loss of function）と変異遺伝子や変異蛋白が新たに病的機能を獲得する場合（gain of function）の2つがあることが知られている。遺伝子変異が常染色体にある場合、対立する2つのアリルの双方に遺伝子変異があって初めて発症する常染色体劣性遺伝形式の疾患の多くは loss of function をその機序とし、一方のアリルのみで発症する常染色体優性遺伝形式の疾患の多くの場合は gain of function と考え

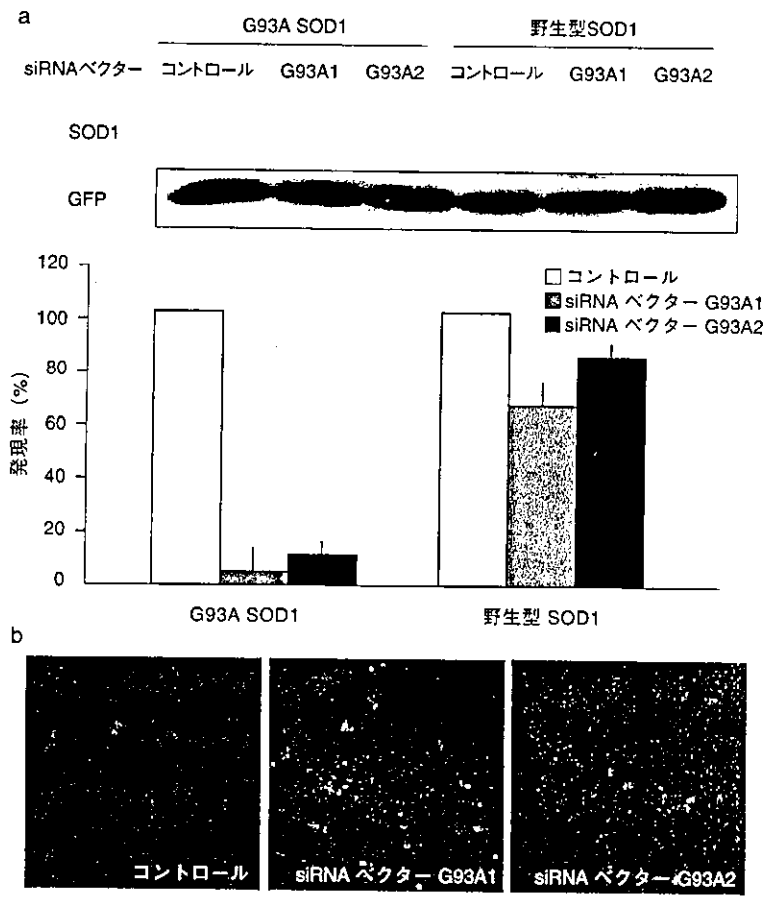

図 2.1.3 変異 SOD1 に特異的に作用する siRNA（文献 13 より改変転載、口絵 7 参照）

a：293T 細胞に G93A または野生型 SOD1 発現ベクターと siRNAG93A1、2 を共発現させ、野生型および変異 SOD1 の発現をウェスタンブロットした。siRNAG93A1、2 はともに G93ASOD1 の発現を著明に抑制し、野生型 SOD1 の発現はほとんど抑制しなかった。

b：GFP をタグに、SOD1 の発現を蛍光顕微鏡にて撮影した。

られている。常染色体劣性遺伝形式は、遺伝子産物である蛋白自体がまったく発現しないか（null変異）、発現しても発現蛋白すべてが変異体であるため、その機能が低下または消失していることが多い。一方、常染色体優性遺伝の場合は野生型のアリルからは原則として正常個体の半分の量の正常の蛋白は発現しているので、本来の蛋白の機能の影響は少ないかまったくなく、変異アリルから発現した変異蛋白が何らかの正常と異なった機能（gain of adverse function）や毒性（gain of toxic function）を新たに獲得することにより疾患が発症することが想定されている。

gain of toxic functionが強く想定されている神経変性疾患の1つとしてCu/Zn superoxide dismutase（SOD1）遺伝子変異による筋萎縮性側索硬化症（ALS）が知られている。常染色体優性遺伝形式をとる家族性ALSの1部の原因遺伝子がSOD1であることが判明した当初はSOD1が代表的なradical scavengerの1つであることから、SOD1酵素活性低下（loss of

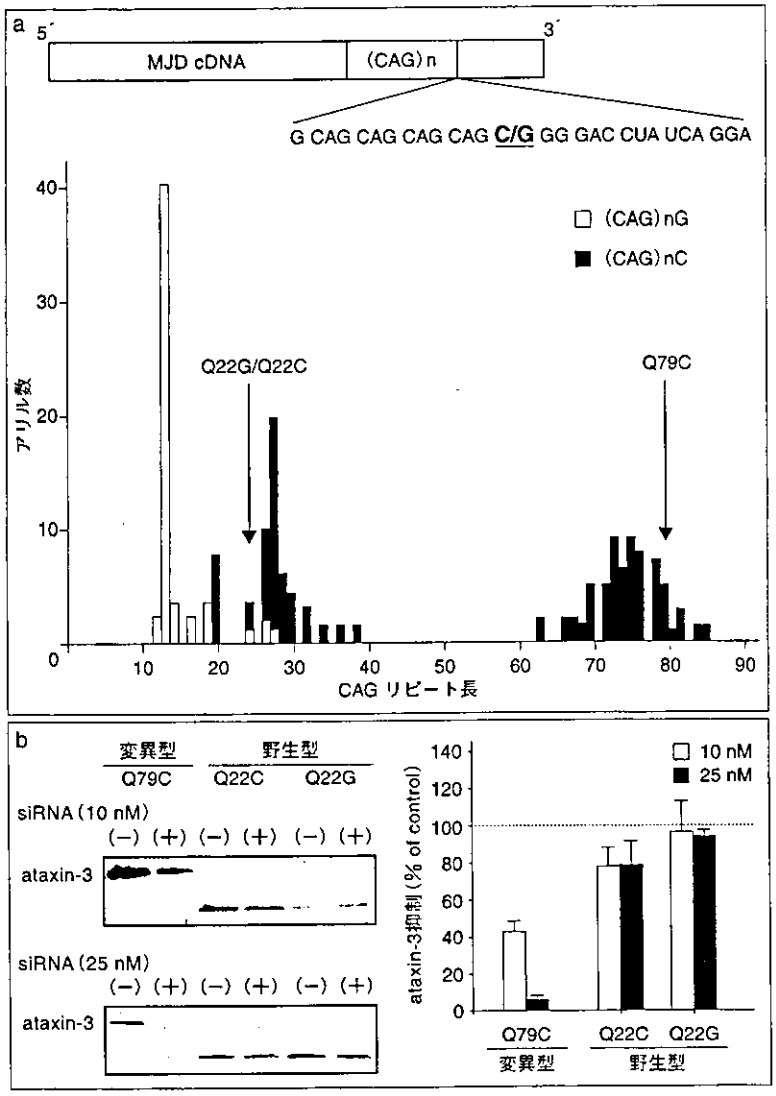

図2.1.4 MJD RNAに対する配列変異アリル特異的な1次配列非依存的なsiRNAの切断（文献16より改変転載）

a：Machado-Joseph（MJD）病遺伝子はMJD遺伝子内のCAGリピートの伸長によって発症する。CAGリピートの後にはG/C polymorphismがあり、伸長したCAGリピートを持つ変異アリルはすべてGで、正常アリルではG/Cが同頻度でみられる。

b：われわれのデザインしたMJD siRNAはこの1塩基の差を認識して変異アリル（Q79C）を切断し、正常アリル（Q22G）は切断しなかった。加えて、驚いたことにこのMJD siRNAはQ79Cと標的配列のまったく同じのもう1つの正常アリル（Q22C）もわずかにしか切断しなかった。この原因としてMJD mRNAの2次構造の変化やRNA結合蛋白の存在がその活性に影響したことが考えられた。

function）がALS発症機序と疑われたが、①SOD1をノックアウトしても前角細胞障害が起こらず、②患者遺伝子変異のほとんどが1塩基置換により1つのアミノ酸の置換が生じるmissense変異で、null変異がみつからない、③患者の赤血球中SOD1活性低下の程度と症状の重症度が相関しない、④変異SOD1を過剰発現したトランスジェニックマウスのSOD1活性自体は正常以上にある（G93Aなど）にもかかわらず運動神経が細胞変性を示す、などの根拠により、現在は変異SOD1が何らかの毒性を獲得する（gain of toxic function）ことがその機序と考えられている。各種ポリグルタミン病、APP、PS1遺伝子変異によるアルツハイマー病、alpha-synuclein変異によるパーキンソン病など常染色体優性遺伝形式を示す主要な神経変性疾患の多くがこのようなgain of toxic functionが原因と考えられている。それぞれの疾患においてそのgain of toxic functionの機序について必ずしも明らかになっていないが、このような疾患の治療を考えた場合、変異した蛋白の発現を抑制する方法があれば、その機序のいかんにかかわらず発症、進行を防止することが期待できるわけである。

さらにこれらの優性遺伝疾患の治療は、正常アリルの発現を損なわずに、変異アリルの発現のみを抑制することが望ましい。例えばSCA6はその原因遺伝子カルシウム1Aチャネルのノックアウトマウスは胎生死亡となることが知られており、正常アリルの発現抑制は新たな神経症状をきたす可能性が高い。

上述のように、変異が1塩基の違いである点変異でも正常アリルと変異アリルの配列の差を認識して変異アリルのみを切断できるsiRNAの作製は可能である。図2.1.3に家族性筋萎縮性側索硬化症の原因遺伝子であるSOD1の点変異G93Aを選択的に切断して正常配列にはほとんど影響しないsiRNAの例を示す[13]。同様の報告は捻転ジストニア[14]やfrontotemporal dementia[15]で報告されている。ポリグルタミン病のように、繰り返し配列の長さが変わることが変異である場合は、この伸長した繰り返し配列そのものに対するsiRNAのデザインをすることは難しいと考えられていた。Machado-Joseph病（SCA3）の場合、CAGリピートの直下の下流にC/GのpolymorphismがあるこのpolymorphismはCAGリピートの繰り返し配列の長さと関連しており、長い繰り返しを持つ病的アリルはすべてCだが、短い繰り返しを持つ正常アリルでは約半数の例でGである（図2.1.4a）。そこでわれわれはこのC/GのpolymorphismのSNPを標的としてsiRNAを設計して、病的アリルに特異的なsiRNAを作製した。ところが驚いたことにこのsiRNAはpolymorphismが変異アリルと同じCである短いCAGリピートの正常アリルもあまり切断しなかった（図2.1.4b）[16]。この機序は不明だが、CAGリピート長の変化に伴うRNAの2次構造の変化やMJD RNAのpolymorphism付近に結合するRNA結合蛋白の結合度の変化によって、siRNAの標的配列へのアクセスに差異が生じるためかもしれない。結果として、すべてのMDJ患者において、変異アリル特異的なsiRNAが作製できた。

1.4 siRNAの *in vivo* へのデリバリー

McCaffrey[17]らはマウスの尾静脈から10～50 mgのNS5Bに対する合成siRNAやsiRNA発現ベクターを体重の5～10％の大量のPBS溶液で5～7秒の短時間で注入するハイドロダイナミックス導入法で、マウスの肝細胞にsiRNAの導入に成功した。さらに最近このハイドロダイナミックス導入法で導入されたFas[18]やcaspase 8に対する合成siRNA（2′-ACEで化学的に修飾したsiRNAでその安定性の上昇を図っている）で、マウスに誘発された劇症肝炎による死亡率を低下させたとの報告がされた。このハイドロダイナミックス導入法をそのまま臨床応用することは難しいが、siRNAが*in vivo*で有効に作用することを示した重要な報告である。

長期の抑制効果にはウイルスベクターが必要となる。hairpin型siRNA発現ベクターコンストラクトをアデノウイルス[19]やレンチウイルス[20]、レトロウイルス[21]、アデノ随伴ウイルス[22]などのウイルスベクターに組み込んで作製したsiRNA発現ウイルスベクターを用いての、*in vivo*の細胞へのsiRNA導入が次々と報告されている。特に最近開発されたアデノ随伴ウイルスの新しい血清型8型（AAV-8）は非常に高い遺伝子導入効率があり、期待されている。

1.5 おわりに

siRNAの核酸医薬としての臨床応用の研究には、off-target effectなど安全性の問題やsilencingなど効果の持続の問題、血液脳関門を越えるデリバリー方法など解決すべき課題はまだ多くある。しかし、siRNAの遺伝子抑制効果は顕著で、その機序は急速に解明され、基礎研究は爆発的に進んでいる。したがって非常に近い将来、難治性疾患での新しい治療法の開発にsiRNAの利用が突破口になることに十分に期待したい。

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Down regulation of DJ-1 enhances cell death by oxidative stress, ER stress, and proteasome inhibition

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Abstract

Mutations in DJ-1 gene have been linked to autosomal recessive early onset parkinsonism (AR-EOP). Although the mechanism of neuronal cell death due to DJ-1 mutation has not been fully elucidated, loss of DJ-1 function was considered to cause the phenotype. Here, we demonstrated that the down regulation of endogenous DJ-1 of the neuronal cell line by siRNA enhanced the cell death which was induced by oxidative stress, ER stress, and proteasome inhibition, but not by pro-apoptotic stimulus. The cell death with hydrogen peroxide was dramatically rescued by over-expression of wild-type DJ-1, but not by that of L166P mutant DJ-1. Furthermore, DJ-1 rescued the cell death caused by over-expression of Pael receptor, which was a substrate of Parkin, another gene product for autosomal recessive juvenile parkinsonism. These results suggest that loss of protective activity of DJ-1 from neuro-toxicity induced by these stresses contributes to neuronal cell death in AR-EOP with mutant DJ-1.

Keywords: DJ-1; Park 7; Autosomal recessive early onset parkinsonism; Parkinson's disease; Oxidative stress; Hydrogen peroxide; ER stress; Proteasome inhibition

Although most patients with Parkinson's disease (PD) are sporadic, some of the juvenile PD patients with autosomal recessive inheritance (AR-JP) have mutations in Parkin gene (PARK2) [1]. Recently, DJ-1 has been reported as the second causative gene for autosomal recessive early-onset Parkinsonism (AR-EOP) (PARK7) [2]. Since the inheritance is autosomal recessive and the mutations in the DJ-1 gene include a large deletion, the mutations cause Parkinsonism, probably through a loss of DJ-1 protein or function [2]. DJ-1 first identified as an oncogene [3] and later was also found to be a hydrogen peroxide-responsive protein, suggesting that it may function as an antioxidant [4]. Furthermore, DJ-1 was sumoylated through binding to the SUMO-1 ligase PIAS that modulates the activity of transcription factors [5]. Here, we examined the effect of down regulation or over-expression of DJ-1 on the cell death after the oxidative-, ER-stress, apoptotic stimulation, and proteasome inhibition.

Methods

Plasmid constructs, cell culture, transfection, Western blotting. The constructions of expression plasmids of human DJ-1 were previously reported [5]. The coding region of human DJ-1 cDNA was subcloned into pEGFP-N1 (Clontech) (pEGFP-DJ-1). To make L166P mutant DJ-1 expression vector, thymin was changed to cytosine at position 497 from ORF start in of pEGFP-DJ-1 using the QuickChange site-directed mutagenesis system (Stratagene). siRNA-expressing vector was constructed by a previously reported method [6]. For targeting mouse DJ-1 (GTGATTCC TGTGGATGTCATG), or human DJ-1 (GGTCATTACACCTAC TCTGAGAATCGT), the loop sequence (TTCAAGAGA) flanked

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by the sense and antisense siRNA sequence was inserted immediately downstream of U6 promoter in pUC19. As negative controls, siRNA-expressing vectors expressing shuffled siRNA sequence were used. A mouse Neuro2a cell and a human embryonic kidney cell line 293T cells (293T) were maintained in Dulbecco's supplemented with 10% fetal bovine serum at 37 °C under 5% CO_2. These siRNA-expression vectors were transfected to these cells with Lipofectamine Plus (Invitrogen). Forty-eight hours after transfection, cells were harvested by TNG buffer (50 mM Tris–HCl, 150 mM NaCl, and 1% Triton X-100) with protease inhibitor cocktail (Roche), separated on 15% SDS–polyacrylamide gels, and transferred onto polyvinylidene difluoride membrane (Bio-Rad). The membrane was probed with anti-human DJ-1 polyclonal antibodies [3], or anti-mouse DJ-1 monoclonal [7], and visualized by using enhanced chemiluminescence detection kit (ECL; Amersham–Pharmacia Biotech).

Assessment of cell death. In order to assess the effect of down regulation or over-expression of DJ-1 by siRNA on the cell death induced by various stresses, Neuro2a or 293T cells in 24-well culture plates were transfected with DJ-1 siRNA vector (1 μg/well), or pEGFP-DJ-1 (0.5 μg/well) with Lipofectamine Plus. At 24 h after transfection of pEGFP-DJ-1, and 48 h after transfection of DJ-1 siRNA vector, various stresses were given to the cells. After 24 h after the stresses cell death was assessed with trypan blue exclusion method, and measurement of cytoplasmic lactate dehydrogenase (LDH) activity with the Cytotox 96 nonradioactive cytotoxicity assay (Promega). In addition, cells with nuclear condensation were counted under a fluorescence microscope in 15–30 min after application of 1.0 mM Hoechst dye (33258).

Results

Both siRNA vectors reduced expression level of endogenous mouse DJ-1 in Neuro2a cells or endogenous human DJ-1 in 293T cells by more than 90% on band intensity of Western blotting at 48 h after the transfection (Fig. 1A). Without stresses, down regulation of DJ-1 or over-expression of wild-type and mutant DJ-1 alone was confirmed to have no effect on cell death by LDH assay (data not shown).

After down regulation of endogenous DJ-1, Neuro2a cells were much more susceptible to the oxidative stress with 0.2 mM H_2O_2 (Fig. 1B). This cell death is apoptotic, because it showed nuclear fragmentation and condensation by Hoechst dye staining, which was also increased after down regulation of DJ-1 (Fig. 1C). In contrast, the cell death induced by proapoptotic stimulus, 0.1 mM staurosporin, was not influenced by down regulation of DJ-1 (Fig. 1D).

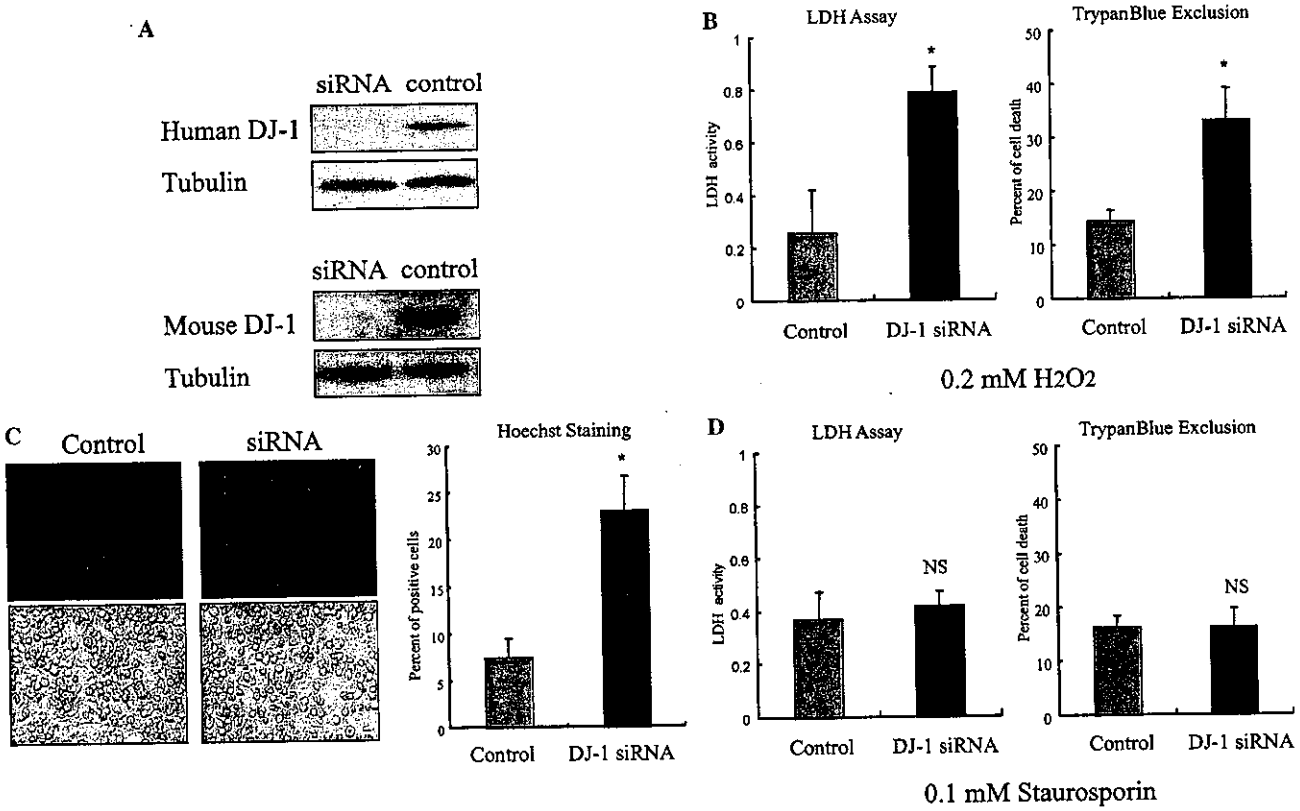

Fig. 1. Effect of down regulation of endogenous DJ-1 on cell death induced by oxidative stress with hydrogen peroxide (H_2O_2). (A) Down regulation of endogenous DJ-1 with siRNA-expressing vector on Western blotting after 48 h after transfection. One microgram of the siRNA-expressing vector for human or mouse was transfected to 293T or Neuro2a cells in 24 well, respectively. (B) Down regulation of endogenous DJ-1 increased cell death of Neuro2a induced with 0.1 mM H_2O_2. $p < 0.01$. (C) Nuclear condensation of Neuro2a cells by Hoechst dye staining was increased by down regulation of endogenous DJ-1 after exposure to H_2O_2. The lower panels were pictures under light field. $p < 0.01$. (D) Down regulation of endogenous DJ-1 increased cell death of Neuro2a did not influence the apoptosis induced by 0.1 mM staurosporin. NS, not significant.

Furthermore, the cell death of Neuro2a cells induced by oxidative stress with 0.4 mM H_2O_2 was dramatically rescued by over-expression of wild-type DJ-1, but not by that of L166P mutant DJ-1 (Figs. 2A and B). The expressions of wild type and mutant DJ-1 proteins were confirmed by GFP-fluorescence (data not shown). In contrast, over-expression of DJ-1 did not influence the cell death caused by 0.2 mM staurosporin (Fig. 2C).

ER stress was given to the cells with tunicamycin and thapsigargin. ER stress-induced cell deaths of Neuro2a cells were also enhanced by down regulation of endogenous DJ-1 expression by the siRNA (Fig. 3A). At higher concentration of tunicamycin (5.0 μg/ml) and thapsigargin (5.0 μM), the induced cell death was decreased by over-expression of wild-type DJ-1, but not by that of L166P mutant DJ-1 (Fig. 3B, left). This rescue effect of DJ-1 overexpression on ER stress-induced cell death, however, is much less than that on oxidative stress-induced cell death. Unexpectedly, at lower concentration of tunicamycin (1.0 μg/ml) and thapsigargin (2.5 μM), over-expression of L166P DJ-1 seems to have a toxic effect (Fig. 3B, right).

Proteasome inhibition was made with lactacystin. The cell death induced by lactacystin was enhanced after down regulation of endogenous DJ-1 of Neuro2a cells by siRNA (Fig. 4A). At higher concentration of lactacystin (50 μM) the induced cell death was decreased by over-expression of wild-type DJ-1, but not by that of L166P mutant DJ-1. In contrast, at higher concentration of lactacystin (20 μM) the over-expression of L166P DJ-1 seems to be mildly toxic to the cells (Fig. 4B).

Putative G protein-coupled transmembrane polypeptide receptor (Pael R) was identified as an interacting protein of Parkin, another gene product of AR-JP [16]. Cell death of Neuro2a cells induced by over-expression of Pael R was rescued by co-expressed DJ-1 (Fig. 5).

All above results were confirmed by three independent experiments. The similar results were also obtained with 293T cells except for experiments with lower concentration of tunicamycin, thapsigargin, and lactacystin (data not shown).

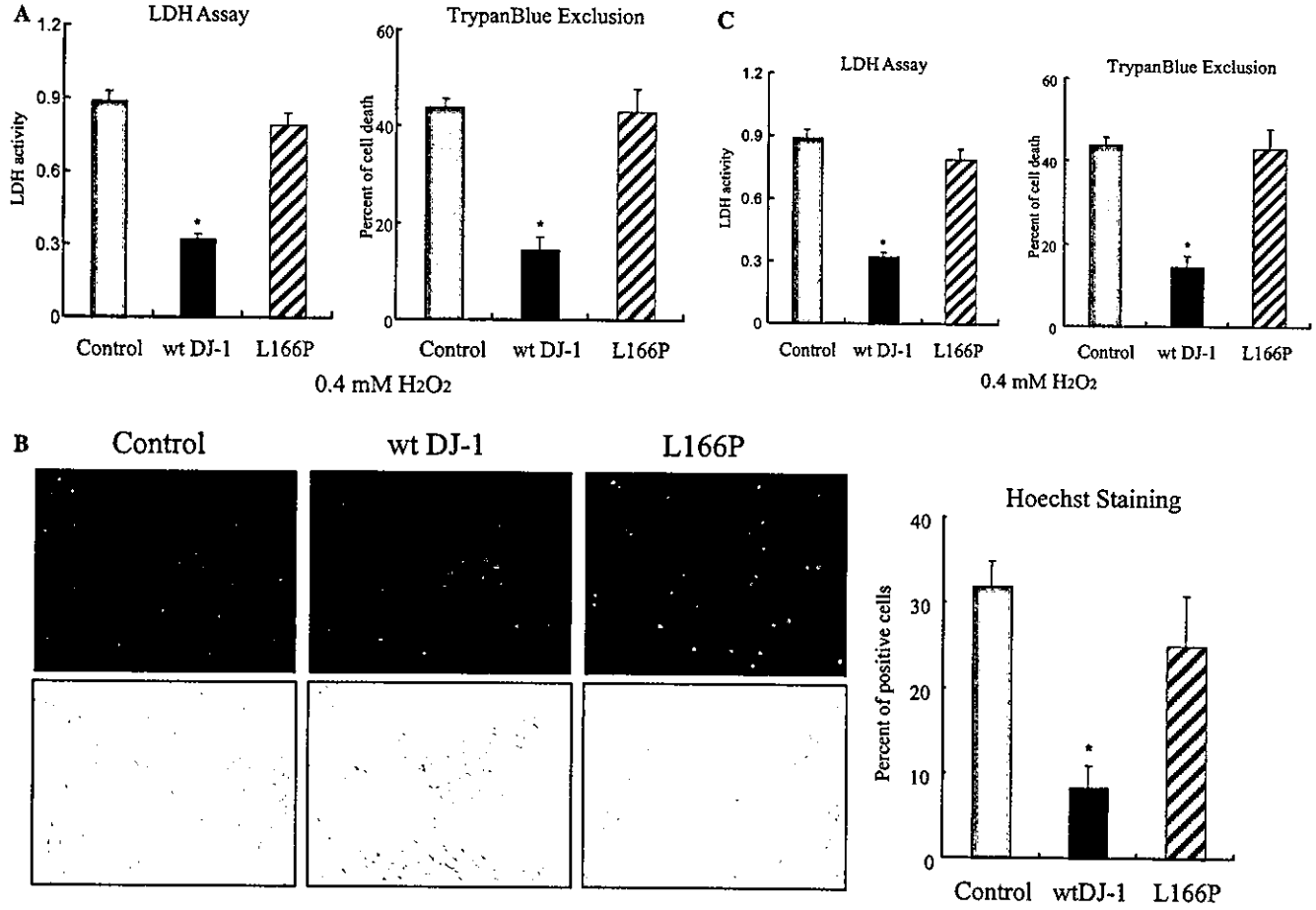

Fig. 2. Effect of DJ-1 over-expression on cell death induced by oxidative stress with H_2O_2. (A) The cell death of Neuro2a induced by 0.4 mM H_2O_2 was rescued by over-expression of wild-type DJ-1, not by that of L166P mutant DJ-1. $p < 0.001$. (B) Nuclear condensation of Neuro2a cells by Hoechst dye staining was much reduced by over-expression of wild-type DJ-1, not by that of L166P mutant DJ-1. The lower panels were pictures under light field. $p < 0.001$. (C) Over-expression of wild-type DJ-1 did not influence the apoptosis of Neuro2a induced by 0.2 mM staurosporin. NS, not significant.

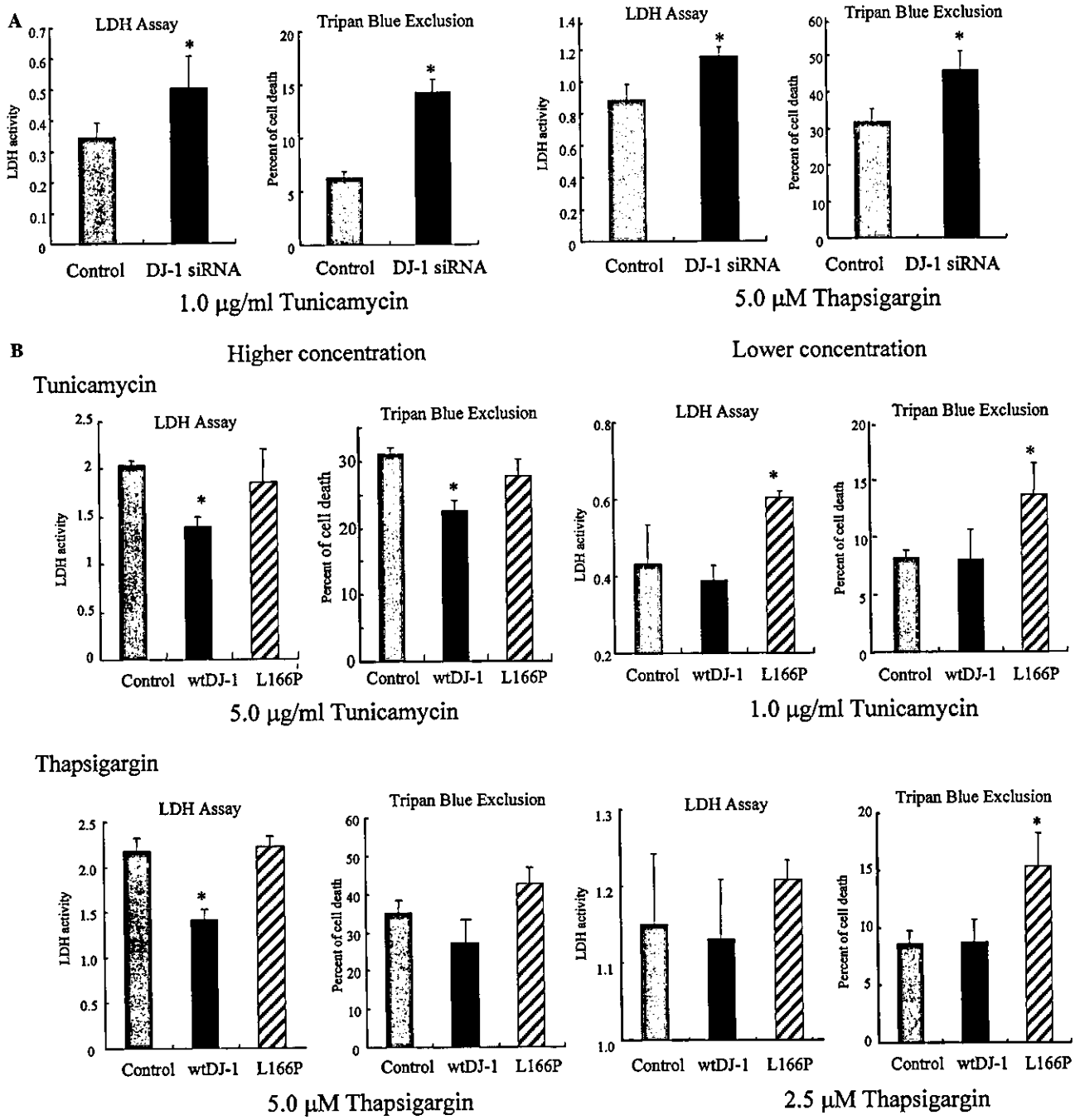

Fig. 3. Effect of DJ-1 down regulation or over-expression on cell death induced by ER stress. (A) Effect of down regulation of endogenous DJ-1 on cell death induced by ER stress. Knockdown of endogenous DJ-1 by siRNA increased cell death of Neuro2a induced by 1.0 μg/ml tunicamycin ($p < 0.01$) and that by 5.0 μM thapsigargin ($p < 0.05$). (B) Effect of DJ-1 over-expression on cell death induced by ER stress. Left panels: at higher concentration of tunicamycin (5.0 μg/ml, $p < 0.01$) and thapsigargin (5.0 μM, $p < 0.05$ only for LDH assay) cell death was decreased by over-expression of wild-type DJ-1, not by that of L166P DJ-1. Right panels: at lower concentration of tunicamycin (1.0 μg/ml, $p < 0.01$) and thapsigargin (2.5 μM, $p < 0.05$ only for trypan blue exclusion), over-expression of L166P DJ-1 tends to have toxic effect.

Discussion

Homozygous deletion, missense mutations (L166P, M26I), and compound heterozygous mutation in DJ-1 gene have been reported to be directly pathogenic for the phenotype of AR-EOP [2,8,9]. A homozygous 14 kb large deletion removing exons 1–5 [2], and a compound heterozygous mutation leading to the frameshift in the exon 1 and the splice error in exon 7 [8], both are predicted to result in a loss of functional protein. Recently, L166P DJ-1 protein proved to be unstable and rapidly degraded when expressed in

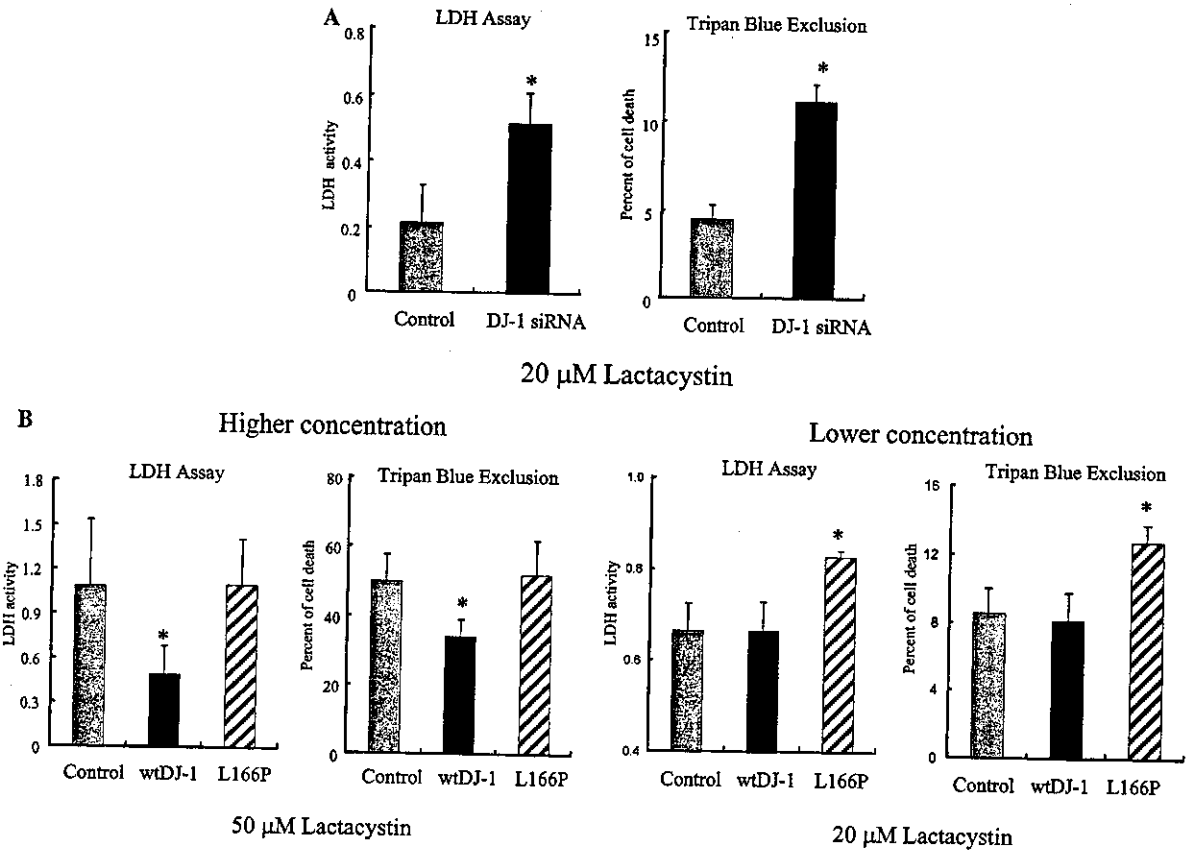

Fig. 4. Effect of DJ-1 down regulation or over-expression on cell death induced by proteasome inhibition with lactacystin. (A) Knockdown of endogenous DJ-1 by siRNA increased cell death of Neuro2a induced with 20 μM lactacystin ($p < 0.01$). (B) Effect of DJ-1 over-expression on cell death induced by lactacystin. Left: at higher concentration of lactacystin (50 μM), cell death was decreased by over-expression of wild-type DJ-1 ($p < 0.01$), not by that of L166P DJ-1. Right: at lower concentration of lactacystin (20 μM), over-expression of L166P DJ-1 tends to have a toxic effect. $p < 0.05$.

culture cells, and the level of L166P DJ-1 protein in patient's lymphoblasts, actually, was very low as compared to the wild type protein [10,11]. Therefore, in at least AR-EOP with mutant DJ-1, loss or reduction of DJ-1 protein function has been considered to cause the phenotype.

The pathophysiology of PD has not been well known, but oxidative stress is suggested to participate in the process of dopaminergic neuronal cell death that undergoes selective degeneration in PD (see recent review, [12]). Ferrous iron (Fe^{2+}) level is elevated and glutathione level is decreased in the substantia nigra, the dopamine-containing region of the brain, in patients with the disorder [13,14]. Accessible iron can react with H_2O_2 produced during oxidative deamination of dopamine to generate hydroxyl radicals (·OH) that can damage proteins, nucleic acids, and membrane phospholipids, leading to cellular degeneration [15]. We showed that neuronal cell death induced by H_2O_2 was enhanced by down regulation of endogenous DJ-1 and was dramatically rescued by over-expression of wild-type DJ-1 but not by that of L166P mutant DJ-1. These results suggest that DJ-1 works as a powerful antioxidant and that loss of its activity is related to dopaminergic neuronal cell death in patients with DJ-1 mutation.

There are several pieces of evidences suggesting that cell death in PD is related to ER-stress and proteasome inhibition. ER-stress maker protein, BiP, was up-regulated in AR-JP (PARK2) brain tissue compared with controls [16]. Parkin protein is E2-dependent E3 ubiquitin–protein ligase and its mutant lost the E3 activity [17]. Over-expression of mutant alpha-synuclein, causative mutation for autosomal dominant PD, produced a proteasome inhibition and sensitized the culture cells to toxicity induced by lactacystin [18,19]. Antisense knockdown of Parkin, another causative gene for autosomal recessive Parkinsonism, increased sensitivity to proteasome inhibitors [20]. Over-expression of Parkin, but not its mutant, specifically suppressed ER-stress-induced cell death [17].

Similar to the results with Parkin, in this study, the cell deaths of Neuro2a induced by ER-stress and proteasome inhibition were enhanced by down regulation of endogenous DJ-1 and slightly suppressed by DJ-1 over-expression. These results suggest that loss of DJ-1 protein activity makes neurons to be vulnerable to ER-stress or

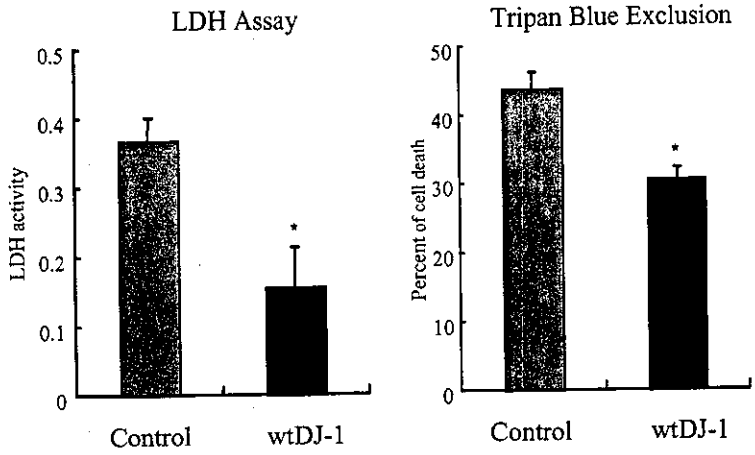

Fig. 5. Effect of DJ-1 over-expression on cell death induced by Pael R over-expression. Cell death was analyzed after co-transfection of 0.3 μg of Pael-R-expressing vector and 0.6 μg wild-type DJ-1 expressing vector (Neuro2a cells in 24 well). Over-expression of DJ-1 rescued cell death induced by Pael R expression ($p < 0.01$). In mock transfection with 0.3 μg pcDNA3.1 (Invitrogen) and 0.6 μg pEGFP-N1, the background cell death showed less than 0.06 in LDH activity and less than 4% in trypan blue exclusion method (data not shown).

proteasome inhibition. The protective activity of DJ-1 from ER stress might be attributed, at least in part, to anti-oxidant activity of DJ-1, because ER stress leads to accumulate endogenous peroxides and promotes oxidative stress [21]. Unexpectedly, at lower concentration of tunicamycin, thapsigargin, and lactacystin, over-expression of L166P mutant DJ-1 not only lost protective activity to these stresses, but also seemed to have a toxic effect on the cells. This mechanism is not known, but an aberrant DJ-1 protein might act in a dominant manner, because heterozygous missense mutations were found in sporadic Parkinson's disease [8,9]. However, further studies are needed to make clear the pathological mechanism of missense mutant DJ-1 under ER-stress and proteasome inhibition.

Pael R is a substrate of Parkin, E3 ubiquitin–protein ligase. Over-expression of parkin, but not mutant, reduced accumulation of insoluble Pael R and suppressed Pael-R-induced cell death. Furthermore, Pael R in insoluble fraction was actually increased in AR-JP brains compared with normal controls. The accumulation of unfolded Pael R is therefore suspected to be causative in AR-JP [16]. Here, DJ-1 over-expression rescues Pael-R-induced cell death. This result also supports that DJ-1 is related to neuronal cell death in patients with Parkinsonism.

In conclusion, our results suggest DJ-1 functions in protecting neuron from oxidative stress and ER stress, and that loss of these activities in mutant DJ-1 contributes to the pathophysiology in AR-EOP.

Acknowledgment

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siRNA-based inhibition specific for mutant SOD1 with single nucleotide alternation in familial ALS, compared with ribozyme and DNA enzyme☆,☆☆

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Abstract

In many of autosomal dominant diseases such as familial amyotrophic lateral sclerosis (ALS) with SOD1 mutation, a missense point mutation may induce the disease by its gain of adverse property. Reduction of such a mutant protein expression is expected to improve the disease phenotype. Duplex of 21-nt RNA, known as siRNA, has recently emerged as a powerful tool to silence gene, but the sequence specificity and efficacies have not been fully studied in comparison with ribozyme and DNA enzyme. We could make the siRNA which recognized even a single nucleotide alternation and selectively suppress G93A SOD1 expression leaving wild-type SOD1 intact. In mammalian cells, the siRNA much more efficiently suppressed the expression of mutant SOD1 than ribozyme or DNA enzyme. Furthermore, these siRNAs could suppress cell death of Neuro2a induced by over-expression of mutant SOD1s with stress of proteasome inhibition. Our results support the feasibility of utilizing siRNA-based gene therapy of familial ALS with mutant SOD1.

Amyotrophic lateral sclerosis (ALS) is characterized by the degeneration of "lower motor neurons" in the spinal cord and brainstem, and degeneration of the descending motor pathway in the corticospinal tracts. Although most cases of ALS are sporadic and have an unknown etiology, 5–10% of ALS cases are familial, and of these, approximately 20% are due to missense, point mutations in the gene encoding Cu,Zn-superoxide dismutase (SOD1) [1]. Recent studies with transgenic mice and cell culture models of ALS with SOD1 mutations indicated that SOD1 mutations induce the disease by its toxic property, not by a loss of the SOD1 activity [2,3]. Similar 'gain of toxic function' of mutant protein is predicted to cause cell death in other autosomal dominant neurodegenerative diseases with a missense point mutation, such as familial Alzheimer's disease, prion disease, and Parkinson's disease. In all these familial diseases, one rational approach to therapy is to develop a method to specifically eliminate the aberrant protein.

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing, initiated by double-stranded RNA (dsRNA). This has a multi-step process that involves generation of 21–23 nt small interfering RNA (siRNA), resulting in degradation of the homologous RNA [4]. In mammalian cells, however, this provokes a strong cytotoxic response, leading to the

☆ *Abbreviations:* ALS, amyotrophic lateral sclerosis; SOD, superoxide dismutase; siRNA, small interfering RNA; dsRNA, double-stranded RNA; rAAVs, recombinant adeno-associated viruses.

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non-specific degradation of RNA transcripts and a general shut down of host cell protein translation [5,6]. This problem has been recently overcome by use of in vitro-synthesized siRNA, which is long enough to mediate gene-specific suppression, but short enough to evade adverse effects of long dsRNA [5].

Ribozymes and DNA enzyme are also small RNA/DNA molecules that possess sequence-specific RNA cleavage activity. Ribozymes occur naturally, but can also be created artificially to target specific sequences in *cis* or *trans* (i.e., on the same molecule or on a different molecule). They recognize and cleave a specific target sequence motif, 5′-NUX (where N is any nucleotide; X is A, C, or U) [7]. DNA enzyme or deoxyribozyme was derived by in vitro selection from a combinatorial library of DNA sequence and has a potential to cleave RNA at any purine (A, G)–pyrimidine (C, U) junction [8]. Both of ribozyme and DNA enzyme consist of a Mg^{2+}-dependent catalytic domain flanked by two substrate-binding arms and cleave a specific phosphodiester linkage of target RNA without recruiting endogenous nuclease. Ribozymes and DNA enzymes also can discriminate even a single nucleotide mismatch and have been successfully used to target and destroy specific RNAs. However, there was no report in which the specificity and efficiency of siRNA were directly compared with those of ribozyme and DNA enzyme.

In the present report, we have engineered siRNAs and DNA-based expressing siRNAs for mutant and wild-type SOD1s, to evaluate their efficiencies in comparison with ribozyme and DNA enzyme to decrease the expression of mutant SOD1 protein as a potential method for gene therapy of familial ALS.

Materials and methods

siRNA and DNA-vector based expressing siRNA preparation. The targeted region of siRNA was the coding sequence of SOD1 cDNA. The 5′ or 3′ UTRs and regions nearby the start codon are avoided, as these may be richer in regulatory protein binding sites. siRNA sequences of the form AA N_{19-20} and CA N_{19-20} with GC content less than 70% were selected from this region [9]. Next, the nucleotide preference after AA/CA is G or A, because it is required for efficient RNA polymerase initiation. Selected 19- or 20-nucleotide RNAs followed by TT or TdG were chemically synthesized and gel-purified. Synthesized single strand oligonucleotides were annealed at 95 °C for 1 min followed by slow cooling in the annealing buffer (PBS, pH 6.8, 2 mM $MgCl_2$).

siRNA-expressing vectors were made by modified reported methods using stem-loop type of siRNA [10,11]. The stem-loop type vector contains siRNA hairpin which contains 20–21 nt sense and antisense sequences of siRNA, connected by 3′ end of the sense strand and 5′ end of the antisense strand by a 9-nt loop sequence. Inserts containing the 9-nt loop sequence (TTCAAGAGA) flanked by sense and antisense siRNA sequences were made by PCR. These were inserted immediately downstream of U6 promotor in pUC19 [12].

Construction of plasmids encoding the substrate RNA and ribozymes. For target coding sequence cDNA clones of the human wild-type SOD1 and mutant A4V (kindly provided by T. Usdin) were used. Full-length human SOD1 cDNA was subcloned in the *Eco*RI and *Xba*I sites of pcDNA3 (Invitrogen). G93A, G93R, G93S, and G93C constructs were created using the QuikChange site-directed mutagenesis system from Stratagene. GFP–SOD1 fusion clones were constructed in *Eco*RI and *Sal*I sites of pEGFP-C2 (Clontech, Palo Alto, CA) using PCR method.

For a target site of hammerhead ribozyme, 5′-NUX (N = any nucleotide; X = A, U, or C) sequence [7] was selected. For the selection of DNA enzyme, purine–pyrimidine or AG [8] sequence was searched. DNA enzymes were chemically synthesized. Ribozyme coding sequences were made from two complementary synthetic DNA oligonucleotides flanked by *Xba*I and *Eco*RI restriction sites. These oligonucleotides were annealed and ligated into pcDNA3(−) (Invitrogen). Each ribozyme was followed by internally cleaving hairpin ribozyme [13].

Cell culture and transfection. Cells from the human embryonic kidney cell line 293T (293T) and mouse Neuro2a were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum with 1% penicillin/streptomycin. Transient transfection using Lipofectamine Plus reagent (Life Technologies, Rockville, MD) was carried out in 24-well plates with cells reaching 40–60% confluence. Mutant or wild-type SOD1 expression plasmid (0.25 μg) and 2.5–125 nM (0.07–3.5 μg) siRNA, 0.5–2.0 μg DNA-vector-based expressing siRNAs, 10 μg DNA enzyme, or 1.0–2.0 μg ribozyme expression plasmid were transfected together with 0.05 μg GFP expression plasmid (pEGFP-C2; Clontech) to monitor for transfection efficiency. The medium for DNA enzyme was changed with fresh medium containing 1 μg DNA enzyme at 24 h after transfection. For control transfection of the same volume of siRNA, DNA enzyme for unrelated Machado-Joseph disease gene or empty pcDNA3 was used.

For fluorescence analysis, 0.25 μg GFP fused-mutant or wild-type SOD1 expression plasmids and 25 nM siRNA were transfected with 0.1 μg DsRed expression vector (pDsRed2; Clontech) to monitor for transfection efficiency. Cell was visualized with fluorescence microscopy (Nikon, Tokyo).

In vitro transcription and cleavage reactions by ribozyme and DNA enzyme. Plasmids containing target SOD1 sequences were linearized with *Xba*I, and plasmids containing ribozyme sequences with *Eco*RI. In vitro transcription was performed with 10 μg linear DNA template and T7 RNA polymerase using RiboMAX (Promega) and labeled by incorporation of [α-^{32}P]uridine triphosphate. After digesting DNA with DNase I, the transcripts were purified.

For standard cleavage reactions in vitro, 8 μM SOD1 substrate RNA and 40 μM ribozyme or 40 μM DNA enzyme were mixed in a 10 μl reaction buffer containing 20 mM $MgCl_2$ and 40 mM Tris–HCl (pH 7.5). The mixture was incubated at 37 °C for 1–4 h and stopped by addition of 50 mM EDTA, and then an equal volume of 10 M urea and 0.02% bromphenol blue. The cleavage products were denatured at 90 °C for 2 min and electrophoresed in 6% polyacrylamide–7 M urea gel in Tris–borate EDTA buffer.

Western blot analysis. At 24 or 48 h after transfection, cells were harvested by gentle scraping in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Triton X, and a proteinase inhibitor cocktail, Complete (Roche)). Equal amounts of total cellular protein were mixed with 5× Laemmli sample buffer, denatured at 95 °C for 5 min, and separated on 15% SDS–polyacrylamide gels. Protein was transferred electrophoretically to polyvinylidene difluoride membranes, immunoblotted with anti-SOD1 polyclonal antibody S-100 (Stressgen Biotechnologies) and anti-GFP monoclonal antibody (Clontech), and detected using enhanced chemiluminescence (Amersham–Pharmacia Biotech).

All experiments of Western blotting were separately performed at least three times.

Cell-toxicity assay. Neuro2a cells in 24-well culture plates were co-transfected with mutant or wild-type SOD1 expression plasmid (1.0 μg/well), 25 nM siRNA (or 1.0 μg expressing siRNA vector), and pCMV-β-gal (50 ng/well) (Clontech). The medium was replaced with that containing 2 mM dibutyryl cyclic-AMP 3 h after transfection to

differentiate the cells. At 24 h after transfection, proteasome inhibitor, lactacystin (20 μM), was added to the medium. At 48 h, the cells were harvested and assayed with β-galactosidase assay kit (Promega). The LacZ expression levels correlate with cellular viability [14]. The percent viability was calculated in comparison with control lysates without lactacystin. Statistical significance was evaluated by single factor ANOVA (analysis of variance) or two-way ANOVA followed by Scheffe's method.

Results

Suppression effect of siRNA, DNA enzyme, and ribozyme specific for mutant SOD1 at codon 93

siRNA and expressing siRNA for G93A SOD1

Two siRNAs targeting G93A SOD1 corresponding to regions 277–297 (siRNA G93A.1) and 275–294 (siRNA G93A.2) were designed (Fig. 1A). Transfection of both siRNAs into mammalian cells could effectively reduce G93A SOD1 expression. Western blot analysis revealed that both siRNA G93A.1 and 2 reduced the expression of G93A SOD1 protein by about 90% when expression efficiency was adjusted with co-transfected GFP (Fig. 1B). The suppression of G93A SOD1 protein by siRNA G93A.1 increased in a dose-dependent manner when the amount of siRNA was changed from 2.5 to 125 nM (Fig. 1C). These siRNAs recognized only one nucleotide alteration, because they suppressed wild-type SOD1 protein much less than G93A SOD1—especially siRNA G93A.2, for which the reduction of wild type was only 1.8% (Fig. 1B). These suppression effects were confirmed by the reduction in GFP fluorescence when siRNA was co-transfected with a GFP-fused SOD1 plasmid (Fig. 1D) using DsRed fluorescence as a control for transfection efficiency (data not shown).

All these results were similarly confirmed in both 293T and Neuro2a cells.

DNA enzyme and ribozyme

We can design the G93A-specific DNA enzyme (Dz G93A) which cleaves G93A mutant SOD1 RNA, but not wild-type SOD1 RNA. Because the wild-type purine-purine sequence at G93A site (GGT) that is not recognized by the DNA enzyme is mutated to a

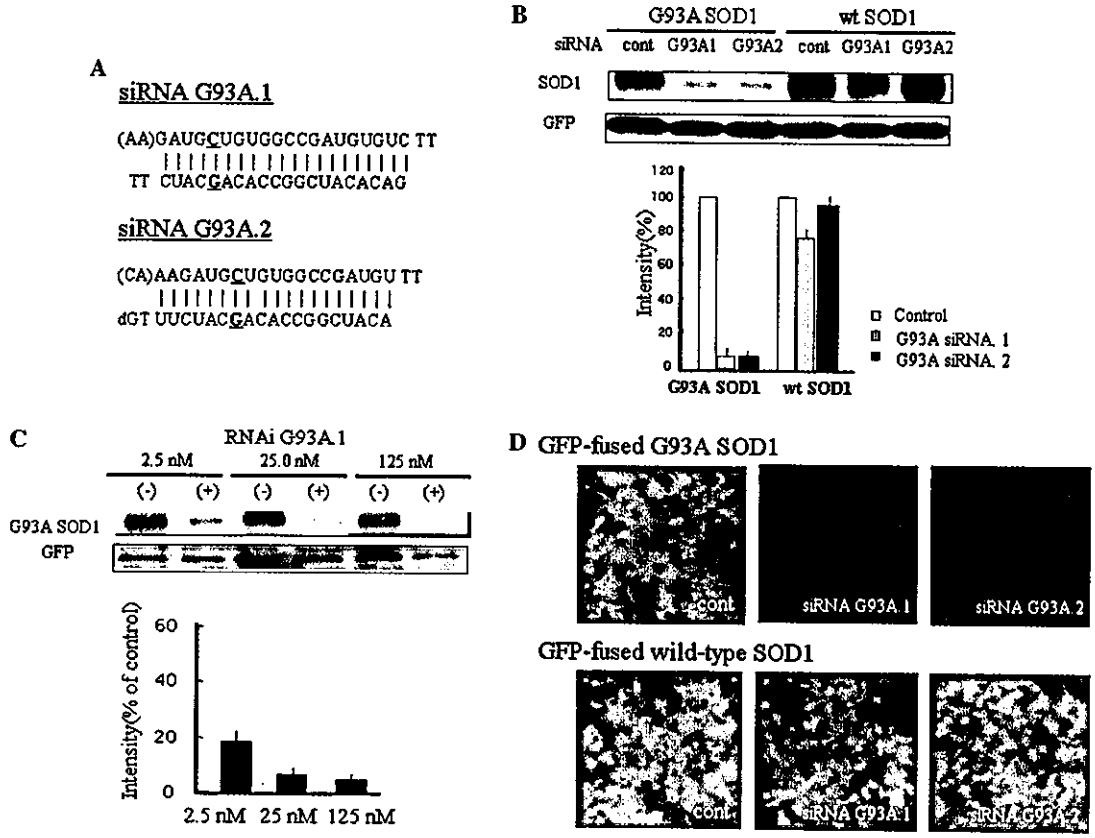

Fig. 1. siRNAs specific for G93A SOD1. (A) Sequences of siRNA G93A.1 and G93A.2. Underlined characters indicate mutations. (B) Effect of siRNA G93A.1 and 2 on G93A and wild-type SOD1 proteins (with no tag) expressed in 293T cells as detected by Western blotting with anti-SOD1 antibody. Expression level of target protein was adjusted by level of co-transfected GFP expression (B). Data are at 48 h after transfections. (C) Dose-dependent effect of suppression of siRNA G93A.1. Figure shows the percentages of band intensity with siRNA G93A.1 with respect to that with each mock transfection. (D) Effect of siRNA G93A.1 and 2 on fluorescence of GFP-fused SOD1s. Expression level of target protein was adjusted by level of co-transfected DsRed fluorescence (data not shown). Values are means and SEM.

purine–pyrimidine (GCT) sequence that is cleaved by the DNA enzyme. The sequence of Dz G93A is shown in Fig. 2A. Cleavage of SOD1 mRNA was carried out by incubating in vitro transcribed mRNA with Dz G93A. The cleavage by Dz G93A resulted in two discrete products from G93A SOD1 mRNA in a sequence-specific manner (Fig. 2B, left). In contrast, Dz G93A produced no detectable cleavage of wild-type target mRNA. Because of the key role of Mg^{2+} in RNA structure and in the cleavage of phosphodiester bonds, cleavage of the mutant was also assessed in the absence of $MgCl_2$ and no cleavage was observed. Next, in order to optimize the arm length to get the most effective cleavage, it was varied from 6 to 12 bases. The maximum cleavage effect was observed when the arm length was 9 bases (Fig. 2B, right). For stabilization of DNA enzyme to degradation by DNAase in cells, the two nucleotides of DNA enzymes were modified with phosphorothioate at both their 5′ and 3′ ends. This modified Dz G93A was confirmed to remain active and specific for mutant mRNA in an in vitro cleavage assay (supplement figure).

For the design of ribozyme, G93A sequence at the mutation site does not follow NUX rule, but sequences of other reported mutations at the same codon, G93R, G93S, and G93C [15], can be targeted by ribozyme. AUX triplet of G93R, G93S, and G93C is cleaved by Rz G93X, but the corresponding triplet in the wild-type, AUG, does not follow the NUX rule (Fig. 2A). The sequences of Rz G93R, G93S, and G93C are shown in Fig. 2A. In vitro cleavage reactions demonstrated that Rz G93X could convert G93R, G93S, and G93C mRNAs to the expected two cleavage products, but that wild-type mRNA was, as expected, resistant to cleavage (Fig. 2C). The optimal arm length of Rz G93X was proved to be 8 for the maximum effect (data not shown).

Rz G93X or phosphorothioate-modified Dz G93A was co-transfected with plasmids encoding each mutant SOD1 to investigate their suppression effect of protein in

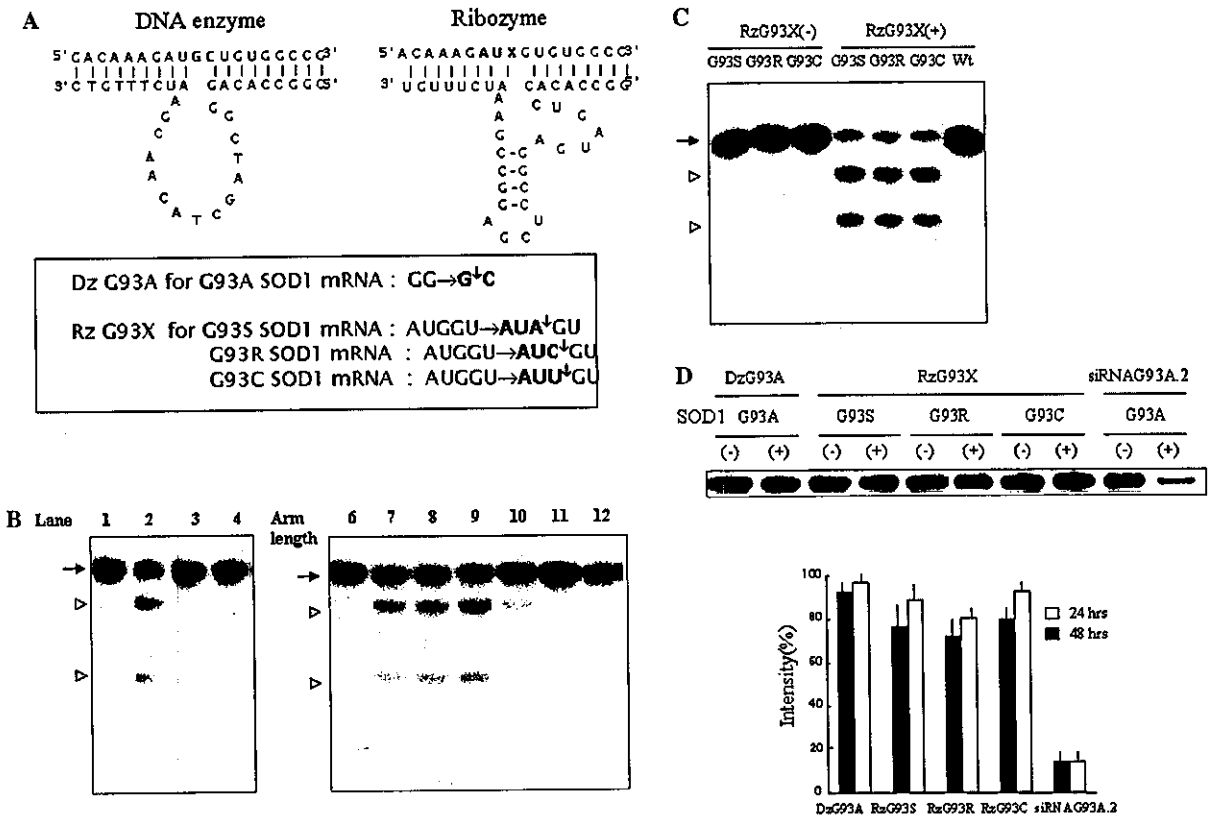

Fig. 2. DNA enzyme and ribozyme specific for mutant SOD1 at codon 93. (A) Sequences of DNA enzyme and ribozyme (upper panel) and target sequence of wild-type and mutant SOD1s. Bold characters are the target sequences. Vertical arrows indicate cleavage sites. (B) Sequence-specific (left) and arm length-dependent (right) cleavage of G93ASOD1 mRNA by Dz G93A. When [α-^{32}P]-labeled G93ASOD1 RNAs were incubated with unlabeled Dz G93A in buffer with 20 mM Mg^{2+} for 4 h, the target RNA was cleaved into two expected products in 4 h (lane 2) (lane 1, before reaction), but was not cleaved without Mg^{2+} in the reactions (lane 3). Dz G93A did not cleave wild-type SOD1 mRNA (lane 4). Cleavage by DzG93A was most effective when the arm length was nine nucleotides (right panel). Arrow, full-length SOD1 mRNA (765 nt); arrowheads, fragments (581 and 184 nt). (C) Sequence-specific cleavage of mutant SOD1 mRNAs by RzG93X in vitro. [α-^{32}P]-Labeled G93S, G93R, and G93C mRNAs were incubated with unlabeled Rz G93X for 4 h. RzG93X could cleave all three mutant mRNAs, but not wild-type mRNA. Arrow, full-length SOD1 mRNA (765 nt); arrowheads, fragments (581 and 184 nt). (D) Western blot analysis of the effect of Rz G93X, Dx G93A, and siRNA G93A on the expression of mutant SOD1 proteins in 293T cells. Blot is from 48 h after transfection. Figure shows the percentages of band intensity with Rz G93X, Dx G93A, and siRNA G93A (+) with respect to those with mock transfection (−). Values are means and SEM.

mammalian cells. The DNA enzyme at 1–10 μg had very little effect and 2 μg ribozymes suppressed expression of each mutant only to the extent of 30% in 24 h. In contrast, 2.5 nM siRNA G93A.2 (= 0.07 μg) suppressed the expression of G93A SOD1 by more than 80% as reflected in the band intensity on Western blotting both at 24 and 48 h after transfection (Fig. 2D). Thus, on a molarity comparison basis, siRNA was more effective than either ribozyme or DNA enzyme in suppressing mutant SOD1 expression.

Suppression effect of siRNA, DNA enzyme, and ribozyme for wild-type SOD1

Four siRNAs for wild-type SOD1 were designed (Fig. 3A). These siRNAs were named by the nucleotide number at the center position of each siRNA sequence towards the 5′ side of which the siRNA is presumed to cleave the substrate RNA [8,9]. siRNA396 most effectively suppressed SOD1 expression in 293T (Fig. 3B, upper) and Neuro2a cells (data not shown). In addition, this siRNA396 could suppress the expression of any mutant SOD1, such as G93A, A4V (Fig. 3B, lower), G85R or G93S (data not shown) with a similar degree of efficiency as wild-type SOD1. siRNA396 could reduce the expression of endogenous human SOD1 in 293T cells (Fig. 3C) and mouse SOD1 in N2a cells (data not shown). This reduction was most prominent at 24 h and expression returned to the normal level in 96 h.

For comparing the efficacy of siRNA, DNA enzymes, and ribozymes for wild-type SOD1, they were designed from the region between nucleotide number 382 and 419 of SOD1 ORF mRNA. The 382–419 region is presumed to be the biggest single-strand RNA region with minimal free energy (Fig. 4A, arrow), when the secondary structure of SOD1 mRNA is analyzed with the mFold program of Zuker (http://mfold.wustl.edu/~folder/rna/form1.cgi). Two siRNAs, siRNA396 and 412, three DNA enzymes, Dz394, Dz398, and Dz403, and one ribozyme, Rz405, were designed within this region (Fig. 4A). DNA enzyme and ribozyme were named by the nucleotide number on the 5′ side of the cleavage site. An in vitro cleavage assay revealed that more than 90% of the full-length mRNA was converted to the expected products by Dz394 and Rz405 after 4 h of incubation (Fig. 4B). However, in 293T and Neuro2a cells, Dz394 did not suppress the expression of SOD1 protein and Rz405 suppressed only about half of the expression. In contrast, siRNA396 suppressed the expression almost to the baseline level (Fig. 4C). As to any suppression effect on endogenous SOD1 of 293T cells, neither DNA enzyme nor ribozyme decreased the endogenous expression clearly (Fig. 4D).

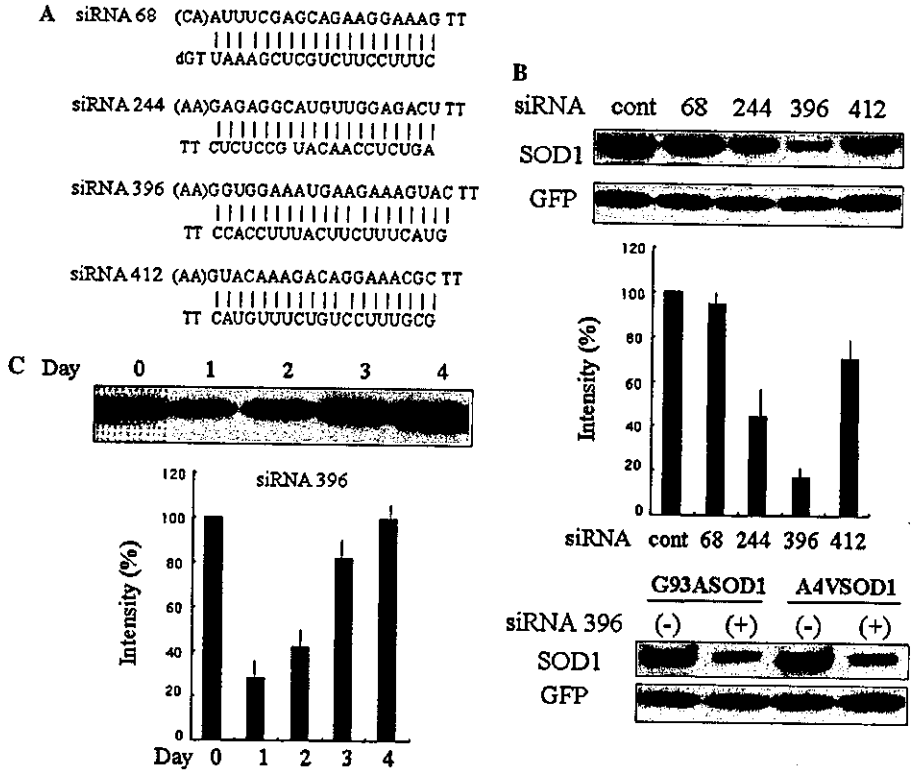

Fig. 3. siRNAs for wild-type SOD1. (A) Sequences of four siRNAs for wild-type SOD1. (B) Effect of four non-specific siRNAs on wild-type SOD1(with no tag) (upper panel) and mutant SOD1(with no tag) (lower panel) expressions in 293T cells as detected by Western blotting with anti-SOD1 antibody. Expression level of target protein was adjusted by level of co-transfected GFP expression. (C) Western blot analysis of the effect of siRNA 396 on endogenous wild-type SOD1 in 293T cells. Values are means and SEM.

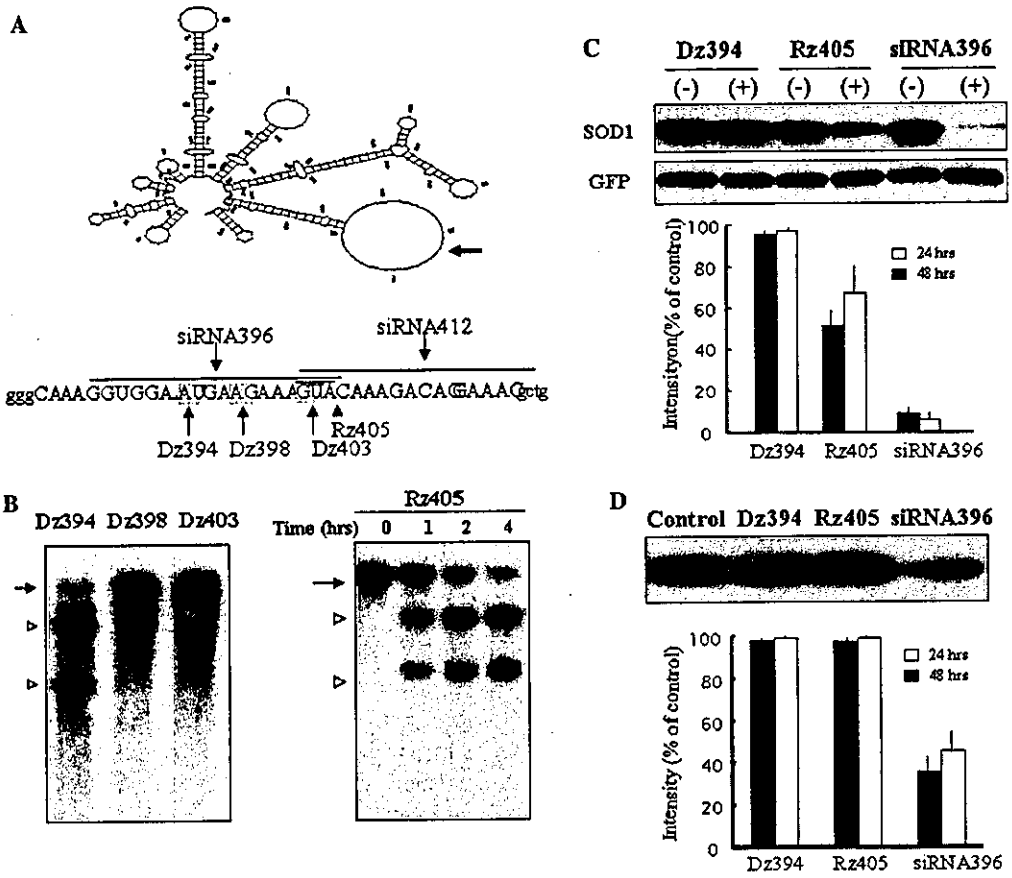

Fig. 4. DNA enzyme, ribozyme, and siRNA targeting predicted single-strand region 382–419 of wild-type SOD1 mRNA. (A) Secondary structure of the coding region of human SOD1 mRNA (upper panel). Arrow indicates the largest single-strand region (382–419) with minimal free energy. Locations of candidate cleavage site of DNA enzyme, ribozyme, and siRNA within region of 382–419 (lower panel). Upper-case letters of 382–419 sequences are included in the single-strand region and lower-case letters are in the stem region. (B) Sequence-specific cleavage of DNA enzymes (left) and Rz 405 (right). [α-^{32}P]-Labeled wild-type SOD1 RNA was incubated with DNA enzymes for 4 h. Dz 394 cleaved most of the target RNA into the two expected products, but other DNA enzymes did not (left). Arrow, full-length SOD1 mRNA (765 nt); arrowheads, fragments (446 and 319 nt). Rz 405 cleaved most of the target RNA in a time-dependent manner (right). Arrow, full-length SOD1 mRNA (765 nt); arrowheads, fragments (457 and 308 nt). (C) Effect of Dz394, Rz405, and siRNA396 on wild-type SOD1 proteins (with no tag) expressed in 293T cells as detected by Western blotting with anti-SOD1 antibody. Expression level of target protein was adjusted by level of co-transfected GFP expression. (D) Western blot analysis of the effect of siRNA 396 on endogenous wild-type SOD1 in 293T cells. Values are means and SEM.

DNA-vector based expressing siRNA

The 0.5 and 2.0 μg of DNA-vector based expressing siRNAs were co-transfected with 0.25 μg of wild-type or mutant SOD1-containing plasmids in 293T and Neuro2a cells. Both expressing siRNA vectors for G93A, siRNA V. G93A.1 and 2, significantly suppressed expression of G93ASOD1 protein, and the suppression ratios were comparable to those by siRNA G93A.1 and 2 of oligonucleotide with very little effect on wild-type SOD1 expression (Fig. 5A). There was not much difference in efficiency between 0.5 and 2.5 μg of expressing siRNAs (data not shown). The expressing siRNA vector for wild-type SOD1, siRNA V. 396, could inhibit markedly the expressions of wild-type, G93A, and A4V SOD1 proteins in similar degrees to siRNA 396 of oligonucleotide (Fig. 5B). The siRNA V. 396 suppressed the endogenous human SOD1 in 293T cells (Fig. 5C) and mouse SOD1 in N2a cells (data not shown).

Cell-toxicity assay

The suppressive effect of siRNA on mutant-SOD1-induced cell toxicity was tested using Neuro2a cells. The survival rates of mutant transformants were significantly decreased compared with wild-type transformant and vector controls in the presence of lactacystin, a proteasome inhibitor. siRNA G93A.2 increased the survival rate of G93A, but not that of A4V transformant. In contrast, siRNA396 increased the survival rates of both G93A and A4V transformants (Fig. 6). Similar effects were observed with transfection of siRNA V.G93A.2 and 396 (data not shown).

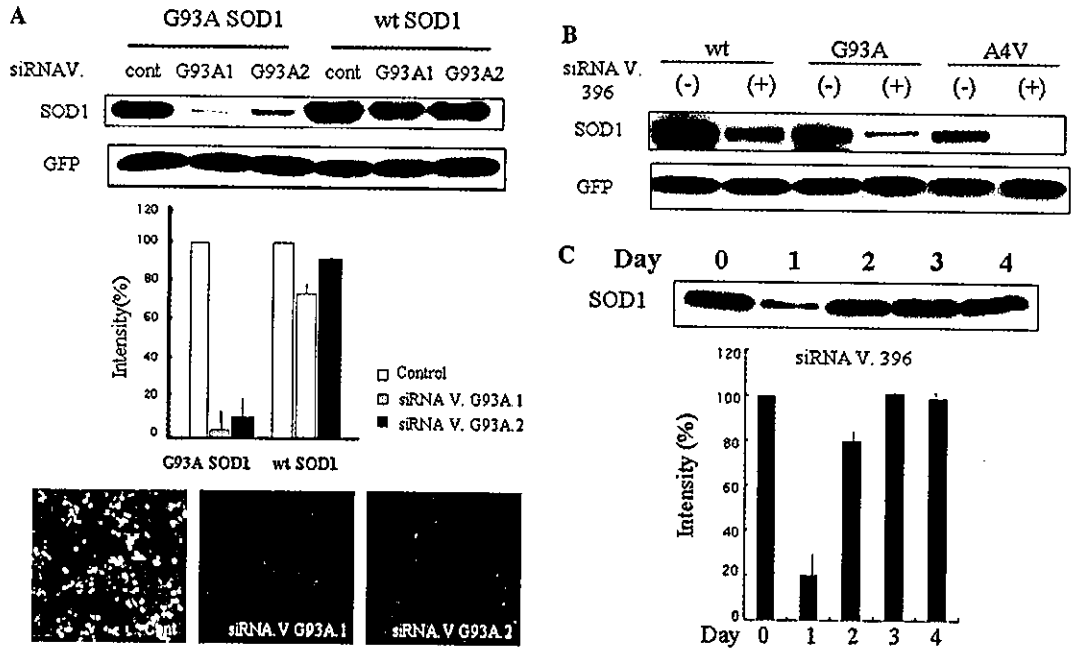

Fig. 5. Effect of DNA-vector based expressing siRNAs (siRNA V.). (A) Effect of expressing siRNA vectors for G93A SOD1 (siRNA V. G93A.1 and 2) on G93A and wild-type SOD1 protein (with no tag) expressed in Neuro2a cells as detected by Western blotting with anti-SOD1 antibody. Expression level of target protein was adjusted by level of co-transfected GFP expression (upper panel). Fluorescence of GFP-fused SODs was also suppressed by siRNA V. G93A.1 and 2 (lower panel). Expression level of target protein was adjusted by level of co-transfected DsRed fluorescence (data not shown). Data are at 48 h after transfections. Values in the figure are means and SEM. (B) Effect of expressing siRNA vectors for wild-type SOD1 (siRNA V.394) on wild-type, G93A, and A4V SOD1 protein (with no tag) expressed in 293T cells as detected by Western blotting with anti-SOD1 antibody. Expression level of target protein was adjusted by level of co-transfected GFP expression. (C) Western blot analysis of effect of siRNA V.396 on endogenous wild-type SOD1 expression in 293T cells. Values are means and SEM.

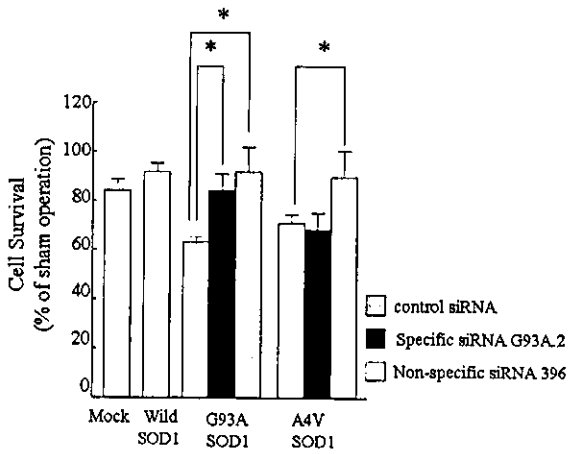

Fig. 6. siRNAs rescue the mutant SOD1-induced toxicity in mammalian cells. The relative viability of the wild-type, mutant, and mock transfectants treated with lactacystin (20 μM) compared with each without treatment of lactacystin. Lactacystin enhances the mutant SOD1-related toxicity in Neuro2a cells. siRNA G93A.2 increased the viability of G93A SOD1-transformant to the similar level of mock-transformant, but did not improve that of A4V SOD1-transformant. siRNA 396 increased the viabilities of both G93A and A4V SOD1-transformants. Values are means and SEM.

Discussion

In autosomal dominant disease, such as familial ALS, the most effective therapeutic approach requires the reduction of the aberrant mutant protein leaving wild-type protein intact. Possible candidate methods include ribozyme, DNA enzyme, and siRNA to cleave mutant RNA selectively. Antisense RNA or DNA has been used to reduce the expression of target protein, but these do not discriminate between the wild-type and mutant mRNA of SOD1, most of which has a point mutation [15]. siRNA G93A.1 and 2, which were designed to target the G93A mutant specifically, suppressed the expression of approximately 90% of G93A SOD1 protein, a suppression rate that is similar to that described in the original report on siRNA [5]. In contrast, they had much less effect on the expression of wild-type SOD1 and especially siRNA G93A.2 had close to no effect on wild-type SOD1 expression. While nucleotides in the 3′- and 5′- terminal region (except for the 3′ overhang of the siRNA sequence) do not contribute to the specificity of target recognition, nucleotides in the center of the siRNA sequence are important specificity determinants [5,16]. That the mutation site in siRNA G93A.2 is located more in the center of the sequence than that in siRNA G93A.1 might explain the lesser effect of G93A.2 on wild-type SOD1 compared to G93A.1.

Ribozymes and DNA enzymes also can discriminate even a single nucleotide mismatch. In our experiments the ribozyme and DNA enzyme, designed for SOD1 mutants at codon 93, were absolutely specific for the mutant