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

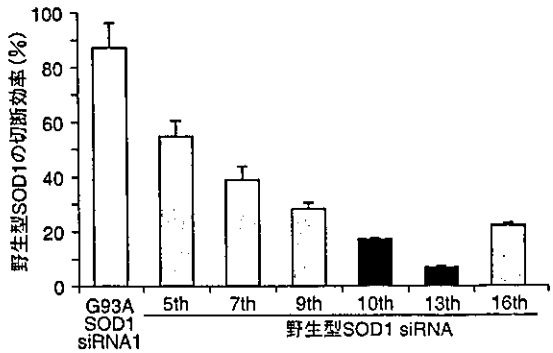

図2.1.1 siRNAへの標的遺伝子とのミスマッチ変異挿入位置によるsiRNA効果への影響

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

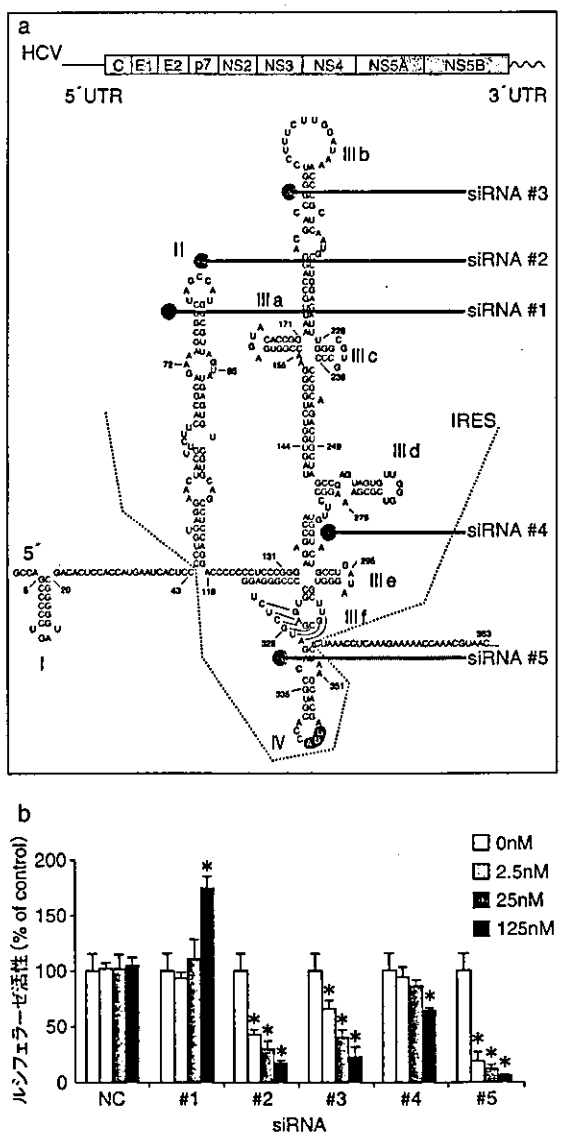

図2.1.2 HCV遺伝子とsiRNA（文献7より改変転載）

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

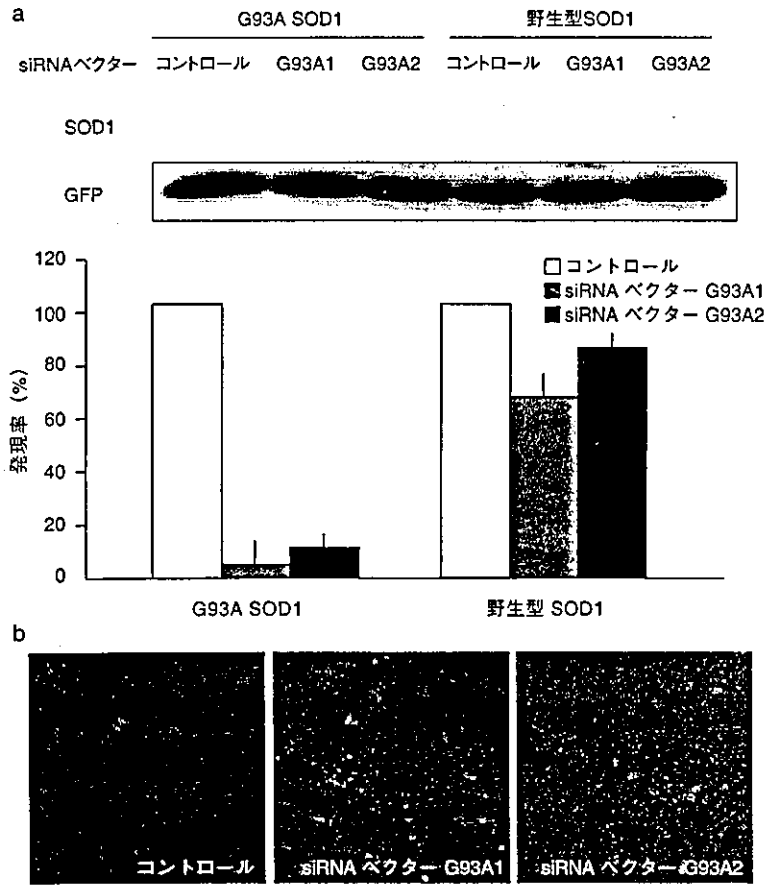

図 2.1.3 変異 SOD1 に特異的に作用する siRNA（文献 13 より改変転載、口絵 7 参照）

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

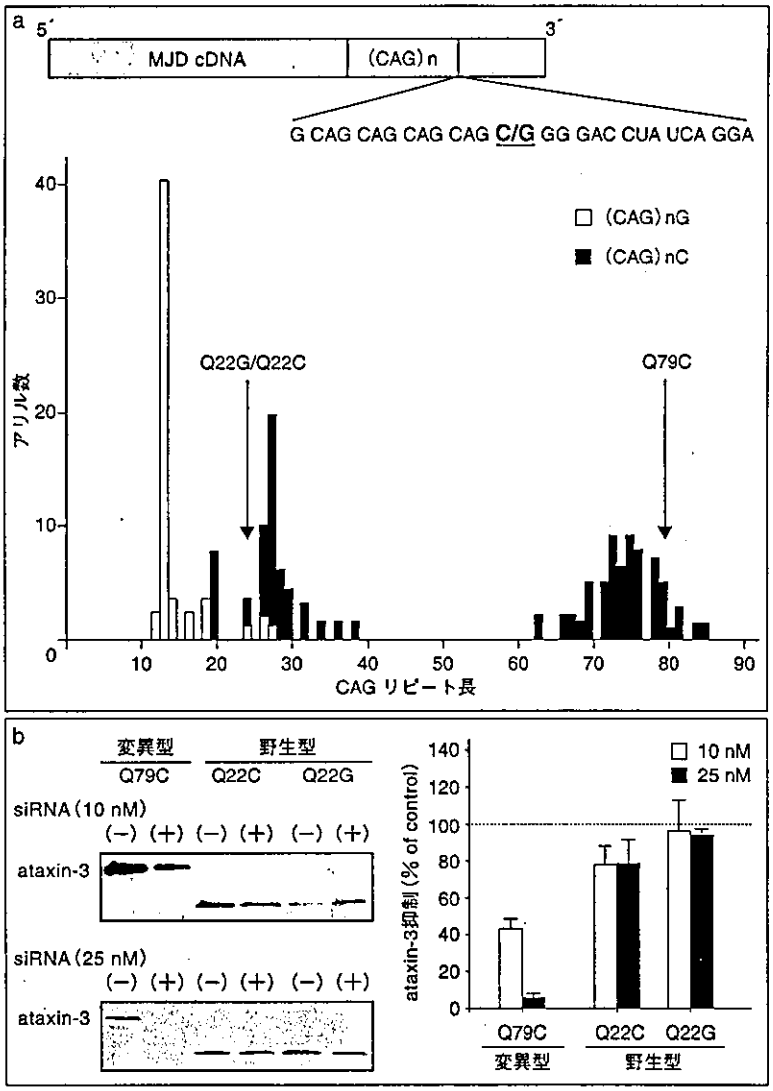

図2.1.4 MJD RNAに対する配列変異アリル特異的な1次配列非依存的なsiRNAの切断（文献16より改変転載）

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SNPs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。

文献

1) Brummelkamp TR, Bernards R, Agami R: A system for stable expression of short interfering RNAs in mammalian cells. Science 29: 550-553, 2002
2) Holen T, Amarzguioui M, Wiiger MT, et al: Positional effects of short interfering RNAs targeting the human coagulation trigger Tissue Factor. Nucleic Acids Res 30: 1757-1766, 2002
3) Amarzguiou M, Holen T, Babaie E, et al: Tolerance for mutations and chemical modifications in a siRNA, Nucleic Acids Res 31: 589-595, 2003
4) Jackson AL, Bartz SR, Schelter J, et al: Expression profiling reveals off-target gene regulation by RNAi. Nature Biotechnol 21: 635-637, 2003
5) Bridge AJ, Pebernard S, Ducraux A, et al: Induction of an interferon response by RNAi vectors in mammalian cells. Nature Genet 34: 263-264 2003
6) Sledz CA, Holko M, de Veer MJ, et al: Activation of the interferon system by short – interfering RNAs. Nature Cell Biol 5: 834-839, 2003
7) Yokota T, Sakamoto N, Enomoto N, et al: Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. EMBO Rep 4: 602-608, 2003
8) Song E, Lee SK, Dykxhoorn DM, et al: Sustained small interfering RNA-mediated human immunodeficiency virus type 1 inhibition in primary macrophages. J Virol 77: 7174-7181, 2003
9) Garrus JE, von Schwedler UK, PornillosOW, et al: Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. Cell 107: 55-65, 2001
10) Surabhi RM, Gaynor RB: RNA interference directed against viral and cellular targets inhibits human immunodeficiency Virus Type 1 replication. J Virol 76: 12963-12973, 2002
11) Arteaga HJ, Hinkula J, van Dijk-Hard I, et al: Choosing CCR5 or Rev siRNA in HIV-1. Nat Biotechnol 21: 230-231, 2003
12) Sorensen DR, Leirdal M, Sioud M: Gene silencing by systemic delivery of synthetic siRNAs in adult mice. J Mol Biol 327: 761-766, 2003
13) Yokota T, Miyagishi M, Hino T, et al: siRNA-based inhibition of superoxide dismutase expression; potential use in familial Amyotrophic lateral sclerosis. Biochem Biophys Res Commun 314: 283-291, 2004
14) Gonzalez-Alegre P, Miller VM, Davidson BL, et al: Toward therapy for DYT1 dystonia: allele-specific silencing of mutant Torsin A. Ann Neurol 53: 781-787, 2003
15) Miller VM, Xia H, Marrs GL, et al: Allele-specific silencing of dominant disease genes. Proc Natl Acad Sci USA 100: 7195-7200, 2003
16) Li Y, Yokota T, Taira K, et al: Sequence dependent and independent discrimination siRNA-based Inhibition of Mutant ataxin 3 Gene Expression by siRNA. Ann Neurol 56: 124-129, 2004
17) McCaffrey AP, Meuse L, Pham TT, et al: RNA interferece in adult mice. Nature 418: 38-39, 2002
18) Song E, Lee S-K, W.ang J, et al: RNA interference targeting Fas protects mice from fulminant hepatitis. Nature Med 9: 347-351, 2003
19) Xia H, Mao Q, Paulson HL, et al: siRNA-mediated gene silencing in vitro and in vivo. Nat Biotechnol 20: 1006-1010, 2002
20) Qin XF, An DS, Chen IS, et al: Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. Proc Natl Acad Sci USA 100: 183-188, 2003
21) Barton GM, Medzhitov R: Retroviral delivery of small interfering RNA into primary cells. Proc. Natl Acad Sci USA 99: 14943-14945, 2002
22) Hommel JD, Sears RM, Georgescu D, et al: Local gene knockdown in the brain using viral-mediated RNA interference. Nature Med 9: 1539 -1544, 2003

(横田隆徳、水澤英洋)

EMBO
reports

scientific report

Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs

Takanori Yokota[1,4+], Naoya Sakamoto[2*++], Nobuyuki Enomoto[2], Yoko Tanabe[2], Makoto Miyagishi[3,4], Shinya Maekawa[2], Li Yi[1], Masayuki Kurosaki[2], Kazunari Taira[3,4], Mamoru Watanabe[2] & Hidehiro Mizusawa[1]*

[1]Department of Neurology and Neurological Science and [2]Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Yushima, Bunkyo-ku, Tokyo, Japan, [3]Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan, and [4]Gene Function Research Laboratory, National Institute of Advanced Industrial Science and Technology, Higashi, Tsukuba Science City, Japan

Small interfering RNAs (siRNAs) efficiently inhibit gene expression by RNA interference. Here, we report efficient inhibition, by both synthetic and vector-derived siRNAs, of hepatitis C virus (HCV) replication, as well as viral protein synthesis, using an HCV replicon system. The siRNAs were designed to target the 5′ untranslated region (5′ UTR) of the HCV genome, which has an internal ribosomal entry site for the translation of the entire viral polyprotein. Moreover, the 5′ UTR is the most conserved region in the HCV genome, making it an ideal target for siRNAs. Importantly, we have identified an effective site in the 5′ UTR at which ~80% suppression of HCV replication was achieved with concentrations of siRNA as low as 2.5 nM. Furthermore, DNA-based vectors expressing siRNA against HCV were also effective, which might allow the efficient delivery of RNAi into hepatocytes *in vivo* using viral vectors. Our results support the feasibility of using siRNA-based gene therapy to inhibit HCV replication, which may prove to be valuable in the treatment of hepatitis C.

EMBO reports 4, 602–608 (2003)
doi:10.1038/sj.embor.embor840

[1]Department of Neurology and Neurological Science and [2]Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
[3]Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0013, Japan
[4]Gene Function Research Laboratory, National Institute of Advanced Industrial Science and Technology (AIST), Central 4, 1-1-1 Higashi, Tsukuba Science City 305-8562, Japan
*These authors contributed equally to this work
+Corresponding author. Tel: +81 3 5803 5234; Fax +81 3 5803 0169;
E-mail: tak-yokota.nuro@tmd.ac.jp
++Corresponding author. Tel: +81 3 5803 5877; Fax +81 3 5803 0268;
E-mail: nsakamoto.gast@tmd.ac.jp

Received 27 November 2002; revised 3 April 2003; accepted 3 April 2003
Published online 9 May 2003

INTRODUCTION

Hepatitis C virus (HCV) is one of the main causes of liver-related morbidity and mortality. The virus establishes a persistent infection in the liver, leading to the development of chronic hepatitis, liver cirrhosis and hepatocellular carcinomas (Alter, 1997). A satisfactory treatment for HCV infection has yet to be developed because investigations of HCV have been hampered by the lack of a stable cell-culture system and of a small-animal model. An HCV replicon that has been reported recently is a selectable sub-genomic HCV RNA, which replicates efficiently and continuously in human hepatoma Huh7 cells (Lohmann *et al.*, 1999). The development of the replicon system has allowed various molecular studies of HCV replication, host–cell interactions and antiviral strategies.

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing that is initiated by double-stranded RNA (dsRNA). RNAi is a multi-step process that involves the generation of small interfering RNA (siRNA), 21–23 nucleotides long, that results in degradation of RNA that is complementary to the siRNA (Sharp, 2001). In mammalian cells, however, this provokes a strong cytotoxic response, leading to the non-specific degradation of RNA transcripts and a general shutdown of host-cell protein translation (Baglioni & Nilsen, 1983; Williams, 1997). This problem has been overcome recently by using a synthetic siRNA that is long enough to mediate gene-specific suppression, but is short enough to evade the adverse effects of long dsRNAs (Elbashir *et al.*, 2001a). RNAi has become a powerful tool for the analysis of gene function and has potential therapeutic applications. Recently, suppression of human immunodeficiency virus (HIV) and poliovirus replication by siRNA has been reported (Gitlin *et al.*, 2002; Jacque *et al.*, 2002). The successful use of siRNA in mammalian cells encouraged us to develop an siRNA expression vector (Miyagishi & Taira, 2002) and to apply RNAi to the exploration of anti-HCV strategies using the HCV replicon system as the target.

©2003 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

The HCV genome is a positive-stranded RNA that contains a single, long open reading frame that encodes structural and non-structural proteins. Translation of the viral genome is mediated by an internal ribosomal entry site (IRES), which is located in the untranslated region at the 5′ terminus (the 5′ UTR; Tsukiyama-Kohara *et al.*, 1992). The HCV genome varies considerably between HCV strains. However, the 5′ UTR and the upstream portion of the core region are the most conserved parts of the genome, with a nucleotide identity of 99.6% (Choo *et al.*, 1991; Okamoto *et al.*, 1991). Because sequence mismatches between the siRNA and the target affect the efficiency of RNAi, the 5′ UTR would seem to be an ideal target for siRNA.

Here, we engineered siRNAs and DNA-based siRNA-expressing vectors to target HCV RNA, and evaluated the effects on viral replication using an HCV replicon system. We report that viral replication was inhibited successfully both by vector-derived siRNA and by an extremely low concentration of a synthetic siRNA that targets the conserved 5′ UTR of the HCV genome.

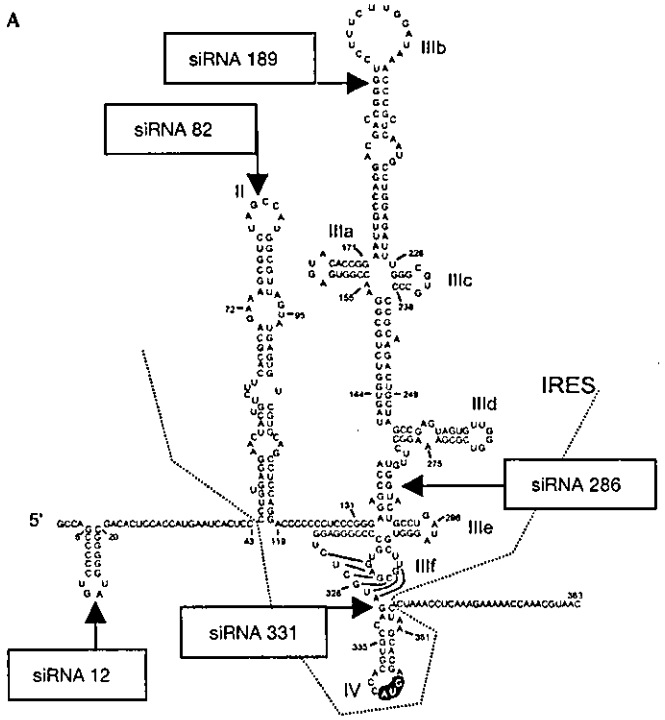

Fig. 1 | Design of small interfering RNAs and target constructs. **(A)** Predicted secondary structure of the 5′ internal ribosomal entry site (IRES) in the 5′ untranslated region (UTR) of the hepatitis C virus (HCV) genome (nucleotides 1–341) and target sites of small interfering RNAs (siRNAs; Brown *et al.*, 1992). **(B)** Structures of the HCV IRES reporter and replicon plasmids. BGH pA, bovine growth hormone polyadenylation site; CMV P/E, cytomegalovirus early promoter/enhancer; ΔC, truncated HCV core region (nucleotides 342–377); EMCV, encephalomyocarditis virus; *FLuc*, firefly luciferase gene; *NPT*, neomycin phosphotransferase gene; *NS3*, *NS4*, *NS5A* and *NS5B*, genes that encode HCV non-structural proteins; pT7, T7 promoter; Rep-Feo, replicon that expresses a chimeric protein consisting of NPT and Fluc.

RESULTS

siRNAs directed against the HCV 5′ untranslated region

siRNAs were designed to target the 5′ UTR of HCV RNA (Table 1). The target sequences were directed to a single-stranded region, according to the secondary structure of the 5′ UTR predicted by Brown *et al.* (1992; Fig. 1A). Sequences of the form $(AA/CA/GA)N_{19}$ (where N indicates any nucleotide) and with a GC content of less than 70% were selected from this region (Elbashir *et al.*, 2001b). A guanine located after the 5′-AA/CA/GA is required for efficient RNA polymerase initiation when the siRNA is expressed in a DNA-based vector. The selected 19-nucleotide RNAs, followed by TT, were synthesized chemically.

Effect of siRNA oligonucleotides on pIRES–Fluc

First, we tested the HCV-directed siRNAs for their ability to suppress HCV IRES-mediated translation. An HCV IRES-reporter-gene vector, pIRES–Fluc, which expresses messenger RNA that consists of the HCV 5′ UTR and the upstream part of the core region (nucleotides 1–377), connected in-frame with the firefly luciferase (FLuc) gene (Fig. 1B), was used as the target. The pIRES–FLuc construct and the siRNA oligonucleotides were cotransfected into 293T cells. siRNAs 189 and 331 suppressed luciferase activity significantly in a dose-dependent manner in the range 2.5–125 nM

Table 1 | Sequences of small interfering RNAs used to target the 5′ untranslated region of hepatitis C virus RNA

Name	Sequence
siRNA 12	5′-gcccccgauuggggggcgacTT-3′
	3′-TTcgggggcuaacccccgcua-5′
siRNA 82	5′-gcgucuagccauggcguuaTT-3′
	3′-TTcgcagaucgguaccgcaau-5′
siRNA 189	5′-ggacgaccgggguccuuucuTT-3′
	3′-TTccugcuggcccaggaaaga-5′
siRNA 286	5′-ggccuuugguguacugccugTT-3′
	3′-TTccggaacaccaugacggac-5′
siRNA 331	5′-ggucucguagaccgugcacTT-3′
	3′-TTccagagcaucuggcacgug-5′
Control	5′-gcagcagcagcagcgggacTT-3′
siRNA	3′-TTcgucgucgucgcccug-5′
331-shuffle	5′-ucggggcacugcuagauccTT-3′
	3′-TTagccccgugacgaucuagg-5′
331-mutant	5′-ggucucguaugccgugcacTT-3′
	3′-TTccagagcauacggcacgug-5′

siRNA, small interfering RNA.
Uppercase letters indicate deoxyribonucleotides.

©2003 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

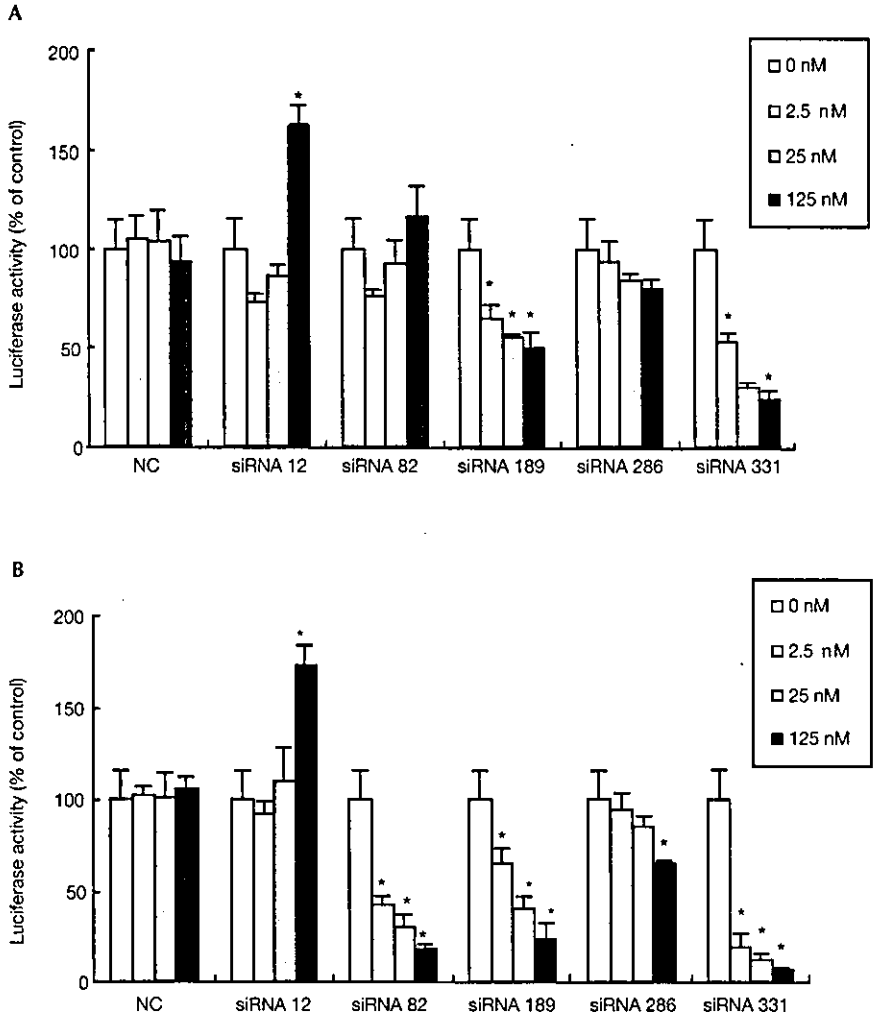

Fig. 2 | Effects of small interfering RNA oligonucleotides on the internal ribosomal entry site reporter and the hepatitis C virus replicon. (A) 293T cells were transfected with pIRES–FLuc, pRL-RSV and small interfering RNAs (siRNAs) at the concentrations indicated, or with a control siRNA (NC). The cells were harvested 48 h after transfection, and luciferase activities were measured. (B) Huh7 Rep-Feo cells were transfected with siRNA oligonucleotides or control siRNAs (NC). The internal luciferase activities were measured 48 h after transfection. Values are shown as percentages of the siRNA negative control, as the mean ± s.d. Asterisks indicate $p < 0.05$. FLuc, firefly luciferase; IRES, internal ribosomal entry site; pRL–RSV, *Renilla* luciferase expression plasmid; Rep-Feo, replicon that expresses a chimeric protein, consisting of NPT and Fluc.

(Fig. 2A). siRNA 331, which is directed against a region just upstream of the translation start codon, was the most effective, and decreased luciferase activity by 74% compared with the control. By contrast, siRNA 12, which was directed against helix 1 of the 5′ UTR, increased luciferase expression when used at 125 nM.

Effect of siRNA on HCV Feo-replicon cells

To assess the effects of the siRNAs on the intracellular replication of HCV, an HCV replicon was used as the target. We constructed a replicon that expresses a chimeric protein consisting of neomycin phosphotransferase (NPT) and Fluc (Rep-Feo; Fig. 1B). The fusion protein, which we called Feo, enables the selection of cells that continously carry the replicon, and also enables the quantification of replication levels by measuring luciferase activity. Transfection of the siRNA into Huh7 Rep-Feo cells, which stably express the HCV Feo replicon, showed that siRNAs 82, 189, 286 and 331 inhibited replication of the HCV RNA significantly in a dose-dependent manner (Fig. 2B). siRNA 82, which was ineffective against pIRES–FLuc, suppressed the replicon. Suppression profiles for luciferase expression obtained from assays using Huh7–Rep-Feo cells closely matched those obtained from the cotransfection of siRNA and pIRES–FLuc. The most effective siRNA, siRNA 331, suppressed luciferase activity by 81% at a concentration as low as 2.5 nM, and the suppression rate increased to 94% at 125 nM. The siRNAs suppressed the expression of the HCV replicon more potently than they did that of pIRES–FLuc. The levels of suppression of the replicon as compared with those of pIRES–Fluc, respectively, using 125 nM of the siRNAs were: 82% compared with 17% for siRNA 82; 77% compared with 50% for siRNA 189; and 94% compared with 75% for siRNA 331.

©2003 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

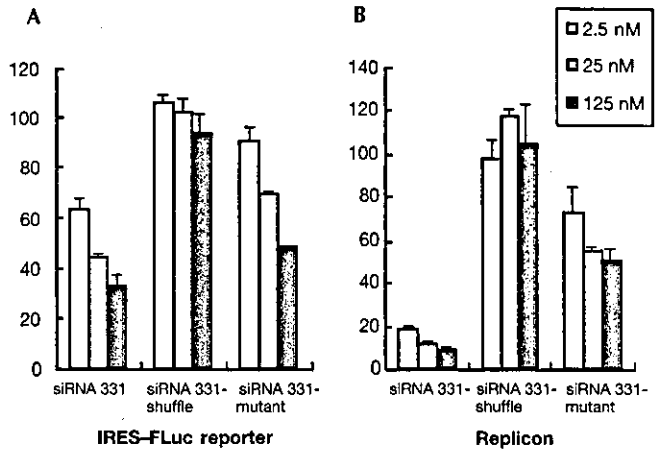

Fig. 3 | Reversal of suppression by negative-control small interfering RNAs. Two small interfering RNA (siRNA) controls were used to analyse further the effects of siRNA 331. The controls used were an siRNA with a shuffled sequence of siRNA 331 (331-shuffle) and an siRNA with mutations at the tenth and eleventh nucleotides from the 5′ end of the siRNA 331 sequence (331-mutant). siRNAs 331, 331-shuffle and 331-mutant were cotransfected with pIRES-Fluc into 293 cells (A) or were transfected into Huh7 Rep-Feo cells (B). Luciferase assays were performed 48 h after transfection. pIRES–FLuc, a reporter-gene vector that expresses messenger RNA consisting of the hepatitis C virus 5′ untranslated region and the upstream part of the core region, connected in-frame with firefly luciferase.

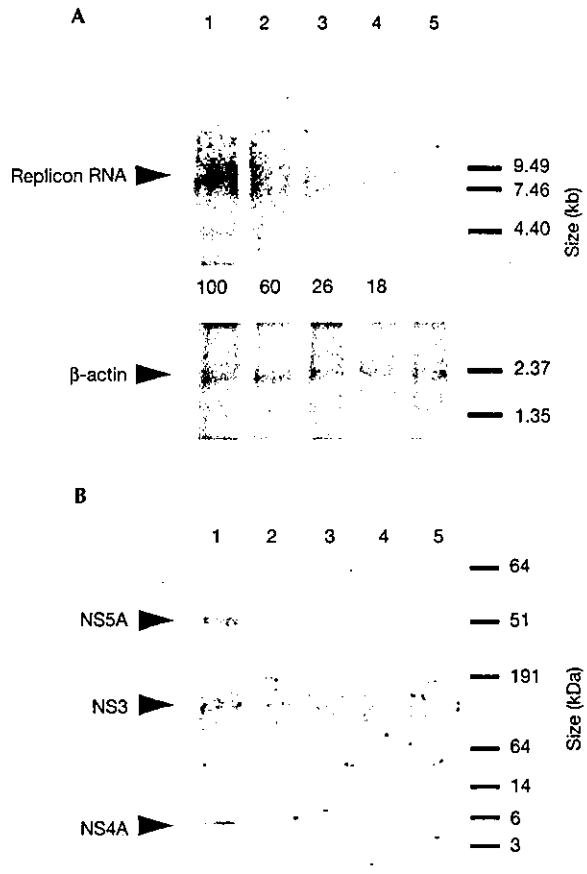

Fig. 4 | Suppression of replicon RNA and replicon-derived hepatitis C virus non-structural-protein synthesis by small interfering RNA 331. (A,B) Human hepatoma Huh7 Rep-Feo cells (which stably express the hepatitis C virus (HCV) Feo replicon) were mock-transfected (lane 1) or were transfected with 2.5 nM (lane 2), 25 nM (lane 3) or 125 nM (lane 4) of small interfering RNA (siRNA) 331. Lane 5, untransfected Huh7 cells. The cells were harvested 48 h after transfection. (A) Northern blotting of the HCV replicon and β-actin RNA. Numbers below the replicon fluorogram show the results of densitometric analysis shown as a percentage of the mock-transfected control. (B) Western blotting using the monoclonal anti-NS5A, anti-NS3 and anti-NS4A antibodies. Densitometry readings (as percentages of the mock-transfected control) for lanes 1–4 were 100, 30, 10 and 10, respectively, for NS5A, 100, 25, 56 and 43 for NS3, and 100, 4, 10, 0 for NS4A.

The control siRNA, which was unrelated to the HCV RNA sequence, did not have any inhibitory effects on the activity of the IRES–FLuc reporter or on the replication of the HCV replicon. For further analysis of the suppression at the most effective site (that targeted by siRNA 331), two additional control siRNAs were tested; these were an siRNA in which the sequence of siRNA 331 was shuffled (331-shuffle), and a mutant version of siRNA 331, which had mismatches at the tenth and eleventh nucleotides from the 5′ end (331-mutant; Table 1). The siRNAs were cotransfected with pIRES–Fluc into 293T cells or were transfected into Huh7 Rep-Feo cells (Fig. 3). The 331-shuffle siRNA had no effect on the activity of IRES–FLuc or on the replication of the HCV replicon, but unexpectedly, the 331-mutant siRNA had a partial inhibitory effect. Because the reduction of the replicon RNA level by 331-mutant siRNA was not obvious by northern blotting (see supplementary information online), the 331-mutant snRNA might function, at least in part, as a translational repressor by acting as a small temporal RNA (Doench *et al.*, 2003). The MTS (see Methods section) assays of the cells transfected with the siRNAs showed no significant effects on cell growth and viability (data not shown). These data showed that the decrease in luciferase activity was due to specific suppressive effects of the siRNAs on HCV replication, and not due to cell death induced by the siRNAs.

Northern and western blotting analyses

In northern blotting analyses (Fig. 4A), levels of the Feo-replicon RNA, which was detectable in mock-transfected control cells, were reduced substantially after transfection of siRNA 331 at 2.5 nM, 25 nM and 125 nM. Densitometric analysis of the replicon RNA showed its intracellular levels in Huh7 Rep-Feo cells correlated well with the luciferase activities. Similarly, in western blotting analyses (Fig. 4B), levels of the HCV non-structural proteins NS3, NS4A and NS5A, which are translated from the HCV replicon, were decreased by corresponding amounts in response to treatment with the siRNA 331.

DNA-vector-based siRNAs suppressed HCV replication

On the basis of the results obtained with the siRNA oligonucleotides, we constructed DNA-based vectors that expressed the siRNA 331 sequence. This was done by modifying previously reported methods, and using two different vectors, the tandem type (Miyagishi & Taira, 2002) and the stem–loop type (Brummelkamp *et al.*, 2002). The tandem-type vector contained 19-nucleotide

©2003 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

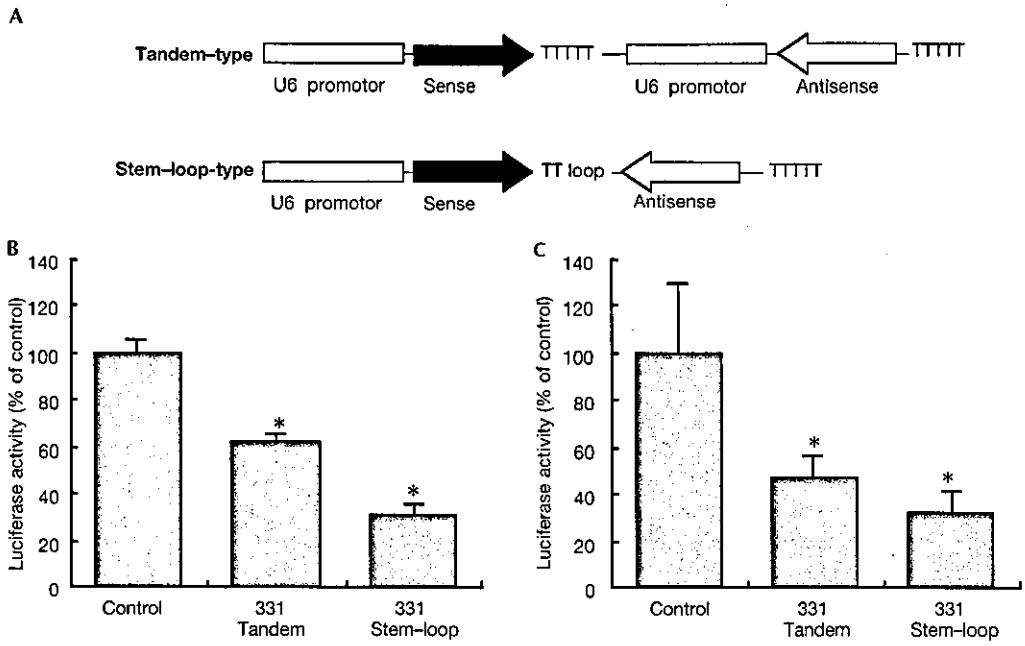

Fig. 5 | Effects of expressing small interfering RNA 331 from a DNA-based vector on hepatitis C virus internal-ribosomal-entry-site-mediated translation and replication. The structures of small interfering RNA (siRNA) expression vectors are shown in (A). Tandem- or stem–loop-type siRNA 331 expression vectors were co-transfected with pIRES-Fluc and pRL–RSV (a *Renilla*-luciferase expression plasmid) into 293T cells (panel (B), or were transfected into Huh7 Rep-Feo cells (C). Luciferase activities were measured 48 h after transfection. Values are shown as percentages of the siRNA-negative control as the mean ± s.d. Asterisks indicate $p<0.05$. pIRES–FLuc, a reporter-gene vector that expresses messenger RNA consisting of the hepatitis C virus 5′ untranslated region and the upstream part of the core region, connected in-frame with firefly luciferase.

sense and antisense siRNA sequences with a 3′ overhang of 4 uridines. Each of the sequences was placed under the control of the U6 promoter. The stem–loop-type vector expressed siRNA hairpins under control of the U6 promoter, which contained the 3′ end of the sense strand and the 5′ end of the antisense strand, connected by a nine-nucleotide loop sequence (Fig. 5A). The siRNA-331-expressing vectors were cotransfected with pIRES–FLuc into 293T cells. Both the tandem and stem–loop siRNA-expressing vectors suppressed luciferase expression, but the stem–loop type was more efficient than the tandem type (Fig. 5B). In addition, both the tandem-type and stem–loop-type vectors significantly suppressed HCV RNA replication in the replicon cells (Fig. 5C).

DISCUSSION

RNAi is an ancient defence mechanism of plant and invertebrate cells and silences foreign gene expression, such as that from viruses or transposons. Since the recent success of highly specific RNAi in mammalian cells using an siRNA (Elbashir *et al.*, 2001a), the technique has been applied to pathogenic human viruses such as HIV and poliovirus, which are suitable targets. Recently, a fragment of the HCV NS5B RNA polymerase gene, which was transiently cotransfected with siRNA into mouse liver by hydrodynamic injection, was reported to be cleaved after treatment with siRNA (McCaffrey *et al.*, 2002). However, this experiment only showed suppression of protein expression from a plasmid that was transfected exogenously, and it has not been shown that siRNA can block HCV replication itself. Here, taking advantage of a newly developed HCV replicon system, we showed that siRNA targeted the HCV 5′ UTR efficiently and cleaved the target specifically. More importantly, it was shown that the cleavage of HCV RNA not only suppressed viral protein synthesis, but also blocked the replication of sub-genomic viral RNA. Although, during the review of this manuscript, other studies have demonstrated the effectiveness of siRNA against HCV replication (Randall *et al.*, 2003; Seo *et al.*, 2003), we present here the effectiveness of vector-derived siRNAs and synthetic siRNAs and describe the most effective site for the inhibition of HCV replication. As shown in Fig. 2B, more than 80% suppression was obtained using an siRNA concentration of only 2.5 nM.

Viruses, particularly RNA viruses such as HCV, are notoriously prone to errors during their replication, and continuously produce mutated viral proteins to escape immune-system defence mechanisms (Carmichael, 2002). These mutations may also escape attack by siRNAs. The protein-coding sequence of the HCV genome that was targeted in the study by McCaffrey *et al.* (2002) varies considerably among different HCV genotypes, and even among strains of the same genotype (Okamoto *et al.*, 1991). In addition, given the high error rate of the non-proofreading HCV RNA-dependent RNA polymerase, so-called 'siRNA escape mutants', which have silent mutations in the protein-coding sequence, could emerge quickly. By contrast, the 5′ UTR, which was selected as the target in the present study, is almost identical among the known strains of HCV. Moreover, structural constraints on the 5′ UTR, in terms of its ability to direct internal ribosome entry and translation of viral proteins, would not permit escape mutations. Therefore, the 5′ UTR of the HCV genome appears to be an ideal target for siRNA in clinical applications.

Not all 5′-UTR-directed siRNAs were equally effective; among the siRNAs tested, siRNA 331, which is directed against a region upstream

©2003 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

of the start codon, was the most efficient, whereas siRNA 82, which is directed against helix II, had almost no effect on viral genome expression. These results may be due in part to the highly folded structure of the 5′ UTR, which may leave few single-stranded gaps that siRNAs can access. We reported previously that the target region of siRNA 331 is also an efficient target site for a catalytic RNA, a hammerhead ribozyme, for the suppression of HCV protein expression (Sakamoto *et al.*, 1996). Our results suggest that the secondary structure of the HCV RNA genome influences the efficiency of siRNAs at least in part.

Our results showed that the siRNAs suppressed the expression of an HCV replicon more potently than they did the IRES reporter vector (Fig. 2). This stronger suppressive effect of siRNA on the HCV replicon might be due to several effects on its autonomous replication mechanism. The blockage of the IRES-mediated synthesis of the non-structural proteins, which are essential for viral RNA synthesis, and the cleavage of elements in the 5′ UTR that are necessary to prime complementary RNA strand synthesis, may result in further suppression of viral replication. Thus, our siRNAs not only reduced viral protein synthesis, but also abolished intracellular replication of the viral genomic RNA, raising the possibility that RNAi could achieve the elimination of viruses from persistently infected host cells.

Cleavage of the HCV IRES by siRNAs may lead to complicated effects on protein translation. Treatment with a high concentration of siRNA 12, which was directed against helix 1 of the 5′ UTR, increased HCV protein expression and viral replication (see also supplementary information online). It has been reported that the most 5′ part of the 5′ UTR may negatively regulate the IRES function (Honda *et al.*, 1996). Moreover, deletion of the nucleotides that make up helix 1 leads to an increase in IRES-mediated translation (Rijnbrand *et al.*, 1995; Wang *et al.*, 1993). We speculate that the cleavage of helix I by siRNA 12 led to an enhancement of IRES-mediated translation through the inactivation of *cis*- or *trans*-acting negative regulatory elements of the IRES. Our results demonstrate clearly, for the first time, that careful selection of target sequences for siRNAs is mandatory, not only to achieve maximum efficiency (as with siRNA 331), but also to avoid adverse effects (as with siRNA 12) in therapeutic applications.

At present, several potential HCV therapies are under development. These include inhibitors of the NS3 protease (Sulkowski, 2003) and the NS5B RNA polymerase (Dhanak *et al.*, 2002), and recombinant vaccines (Choo *et al.*, 1994). Delivery methods for siRNAs to cells *in vivo* that are efficient and safe enough to suppress HCV replication in all infected cells have not been established. Chemically modified synthetic siRNA might easily be made and delivered into cells on their own; it was reported recently that serum (ribonuclease)-resistant modified siRNA can be delivered into cells without a cationic lipid carrier (Capodici *et al.*, 2002). Importantly, we demonstrated for the first time that DNA-based siRNA expression vectors are effective against HCV, and might allow the efficient delivery of RNAi to hepatocytes *in vivo* using viral vectors. As an alternative approach, we are at present analysing siRNA-331-encoding DNA constructs within adenovirus and adeno-associated virus vectors, as reported in Xia *et al.* (2002). Our preliminary data indicate that an adenovirus vector expressing siRNA 331 efficiently suppresses HCV replication *in vitro*.

In conclusion, the efficiency of our siRNAs and siRNA-expressing vectors in inhibiting HCV replication in cells suggests that this RNA-targeting approach might provide an effective therapeutic option for HCV infection, especially at the optimal site (the siRNA 331 target-site) within the conserved 5′ UTR.

METHODS

Preparation of siRNAs and DNA-based vectors expressing siRNA. Sense and antisense strands of siRNA oligonucleotides were synthesized, and were then annealed at 95 °C for 1 min, followed by slow cooling in PBS, pH 6.8, containing 2 mM $MgCl_2$. To construct siRNA-expressing vectors, inserts were made by PCR. These contained the human U6 promoter in the case of the tandem-type vector, and the loop sequence (5′-TTCAAGAGA-3′) flanked by sense and antisense siRNA sequences in the case of the stem–loop-type vector. These were inserted immediately downstream of the U6 promoter in pUC19. siRNA and siRNA-expressing vectors for an unrelated target, the Machado–Joseph Disease gene, were used as negative controls (Table 1).

HCV replicon expressing chimeric reporter genes. An HCV replicon plasmid, pHCVIbneo-delS, was derived from an infectious HCV clone, HC-N, genotype Ib (Guo *et al.*, 2001). The pHCVIbneo-delS plasmid was reconstructed by substituting the *NPT* gene with a fusion of *FLuc* and *NPT* (pRep–Feo; Fig 1B).

Cell culture and transfection. The human hepatoma cell line Huh7 and the human embryonic kidney cell line 293T were maintained in DMEM (Sigma), supplemented with 10% FCS, at 37 °C with 5% CO_2. Transfections of the siRNA oligonucleotides and the plasmids were performed in 24-well plates using Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's instructions. Thirty nanogrammes of the pIRES–FLuc construct and 2.5–125 nM of siRNA or 0.5 μg of siRNA-expressing vectors were transiently transfected with 10 ng of the *Renilla* luciferase expression plasmid (pRL–RSV; Promega). For transfection into Huh7 Rep-Feo cells, 2.5–125 nM of siRNA or 0.5 μg of siRNA-expressing vectors were transfected with 10 ng of pRL–RSV. In each transfection, FLuc activity was adjusted using the *Renilla* luciferase activity to normalize the transfection efficiency.

Luciferase assays. Luciferase activities were quantified using a luminometer (Lumat LB9501; Promega) using the Bright-Glo Luciferase Assay System (Promega). Assays were performed in triplicate, and the results were expressed as means ±s.d. as percentages of the controls.

MTS (dimethylthiazol carboxymethoxyphenyl sulphophenyl tetrazolium) assays. To evaluate the cytotoxic effects of the siRNAs, MTT assays were performed 48 h after siRNA transfections using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega).

Northern hybridization. Total cellular RNA was extracted from cells using ISOGEN (Wako). The RNA was separated by denaturing agarose–formaldehyde-gel electrophoresis and transferred to a Hybond-N+ nylon membrane (Amersham-Pharmacia Biotech.). The upper part of the membrane, which contained the HCV replicon RNA, was hybridized with a digoxigenin-labelled probe that was specific for the full-length replicon sequence, and the lower part of the membrane was hybridized with a probe specific for β-actin. The signals were detected in a chemiluminescence reaction using a Digoxigenin Luminescent Detection Kit (Roche Molecular Biochemicals) and were visualized using a Fluoro-Imager (Roche).

Western blotting. 10 μg of total cell lysate was separated using NuPAGE 4.12% Bis–Tris gels (Invitrogen) and blotted onto an Immobilon polyvinylidene difluoride membrane (Roche). The membrane was incubated with monoclonal anti-NS5A (BioDesign), anti-NS3 and anti-NS4A antibodies (Virogen), and detection was carried out in a chemiluminescence reaction (BM Chemiluminescence Blotting Substrate; Roche).

©2003 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

Statistical analyses. Statistical analyses were performed using the student's *t*-test; *p* values of less than 0.05 were considered as statistically significant.

Supplementary information is available at *EMBO reports* online (www.emboreports.org).

ACKNOWLEDGEMENTS

We thank C. Seeger for providing the replicon plasmid pHCV1bneo-delS. This study was supported by grants from the Ohuchi Atsuko, the Japan Society for the Promotion of Science (grant numbers 12557053, 14657129 and 14370175) and from the Ministry of Economy, Trade and Industry of Japan.

REFERENCES

Alter, M.J. (1997) Epidemiology of hepatitis C. *Hepatology*, **26**, 62S–65S.

Baglioni, C. & Nilsen, T.W. (1983) Mechanisms of antiviral action of interferon. *Interferon*, **5**, 23–42.

Brown, E.A., Zhang, H., Ping, L.H. & Lemon, S.M. (1992) Secondary structure of the 5′ nontranslated regions of hepatitis C virus and pestivirus genomic RNAs. *Nucleic Acids Res.*, **20**, 5041–5045.

Brummelkamp, T.R., Bernards, R. & Agami, R. (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science*, **296**, 550–553.

Capodici, J., Kariko, K. & Weissman, D. (2002) Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. *J. Immunol.*, **169**, 5196–5201.

Carmichael, G.G. (2002) Silencing viruses with RNA. *Nature*, **418**, 379–380.

Choo, Q.L. *et al.* (1994) Vaccination of chimpanzees against infection by the hepatitis C virus. *Proc. Natl Acad. Sci. USA*, **91**, 1294–1298.

Choo, Q.L. *et al.* (1991) Genetic organization and diversity of the hepatitis C virus. *Proc. Natl Acad. Sci. USA*, **88**, 2451–2455.

Dhanak, D. *et al.* (2002) Identification and biological characterization of heterocyclic inhibitors of the hepatitis C virus RNA-dependent RNA polymerase. *J. Biol. Chem.*, **277**, 38322–38327.

Doench, J.G., Petersen, C.P. & Sharp, P.A. (2003) siRNAs can function as miRNAs. *Genes Dev.*, **17**, 438–442.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. & Tuschl, T. (2001a) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*, **411**, 494–498.

Elbashir, S.M., Martinez, J., Patkaniowska, A., Lendeckel, W. & Tuschl, T. (2001b) Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J.*, **20**, 6877–6888.

Gitlin, L., Karelsky, S. & Andino, R. (2002) Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature*, **418**, 430–434.

Guo, J.T., Bichko, V.V. & Seeger, C. (2001) Effect of α-interferon on the hepatitis C virus replicon. *J. Virol.*, **75**, 8516–8523.

Honda, M., Ping, L.-H., Rijnbrand, C.A., Amphlett, E., Clarke, B., Rowlands, D.J. & Lemon, S.M. (1996) Structural requirement for initiation of translation by internal ribosome entry within genome-length hepatitis C virus RNA. *Virology*, **222**, 31–42.

Jacque, J.M., Triques, K. & Stevenson, M. (2002) Modulation of HIV-1 replication by RNA interference. *Nature*, **418**, 435–438.

Lohmann, V., Koerner, F., Koch, J.-O., Herian, U., Theilmann, L. & Bartenschlager, R. (1999) Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science*, **285**, 110–113.

McCaffrey, A.P., Meuse, L., Pham, T.T., Conklin, D.S., Hannon, G.J. & Kay, M.A. (2002) RNA interference in adult mice. *Nature*, **418**, 38–39.

Miyagishi, M. & Taira, K. (2002) U6 promotor-driven siRNAs with four uridine 3′ overhangs efficiently suppress targeted gene expression in mammalian cells. *Nature Biotechnol.*, **19**, 497–500.

Okamoto, H., Okada, S., Sugiyama, Y., Kurai, K., Iizuka, H., Machida, A., Miyakawa, Y. & Mayumi, M. (1991) Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: comparison with reported isolates for conserved and divergent regions. *J. Gen. Virol.*, **72**, 2697–2704.

Randall, G., Grakoui, A. & Rice, C.M. (2003) Clearance of replicating hepatitis C virus replicon RNAs in cell culture by small interfering RNAs. *Proc. Natl Acad. Sci. USA*, **100**, 235–240.

Rijnbrand, R., Bredenbeek, P., van der Straaten, T., Whetter, L., Inchauspe, G., Lemon, S. & Spaan, W. (1995) Almost the entire 5′ non-translated region of hepatitis C virus is required for cap-independent translation. *FEBS Lett.*, **365**, 115–119.

Sakamoto, N., Wu, C.H. & Wu, G.Y. (1996) Intracellular cleavage of hepatitis C virus RNA and inhibition of viral protein translation by hammerhead ribozymes. *J. Clin. Invest.*, **98**, 2720–2728.

Seo, M.Y., Abrignani, S., Houghton, M. & Han, J.H. (2003) Letter to the editor. Small interfering RNA-mediated inhibition of hepatitis C virus replication in the human hepatoma cell line Huh-7. *J. Virol.*, **77**, 810–812.

Sharp, P.A. (2001) RNA interference. *Genes Dev.*, **15**, 485–490.

Sulkowski, M. (2003) A promising new anti-HCV protease inhibitor. *Hopkins HIV Rep.*, **15**, 7.

Tsukiyama-Kohara, K., Iizuka, N., Kohara, M. & Nomoto, A. (1992) Internal ribosome entry site within hepatitis C virus RNA. *J. Virol.*, **66**, 1476–1483.

Wang, C., Sarnow, P. & Siddiqui, A. (1993) Translation of human hepatitis C virus RNA in cultured cells is mediated by an internal ribosome-binding mechanism. *J. Virol.*, **67**, 3338–3344.

Williams, B.R. (1997) Role of the double-stranded RNA-activated protein kinase (PKR) in cell regulation. *Biochem. Soc. Trans.*, **25**, 509–513.

Xia, H., Mao, Q., Paulson, H.L. & Davidson, B.L. (2002) siRNA-mediated gene silencing *in vitro* and *in vivo*. *Nature Biotechnol.*, **20**, 1006–1010.

©2003 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 310 (2003) 1062–1066
BBRC
www.elsevier.com/locate/ybbrc

Introduction of short interfering RNA to silence endogenous E-selectin in vascular endothelium leads to successful inhibition of leukocyte adhesion

Yasunobu Nishiwaki,[a,c,1] Takanori Yokota,[b,1] Megumi Hiraoka,[a] Makoto Miyagishi,[d] Kazunari Taira,[d] Mitsuaki Isobe,[c] Hidehiro Mizusawa,[b] and Masayuki Yoshida[a,*]

[a] *Department of Medical Biochemistry, Tokyo Medical and Dental University Tokyo, Japan*
[b] *Department of Neurology and Neurological Science, Tokyo Medical and Dental University Tokyo, Japan*
[c] *Department of Cardiology, Tokyo Medical and Dental University Tokyo, Japan*
[d] *Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo, Japan*

Received 28 August 2003

Abstract

Short interfering RNAs (siRNAs) are powerful sequence-specific reagents that suppress gene expression in mammalian cells. We report for the first time that gene silencing of endothelial E-selectin by siRNAs leads to successful inhibition of leukocyte–endothelial interaction under flow. siRNAs designed to target human E-selectin were tranfected into human umbilical vein endothelial cells (HUVEC). Western blotting analysis revealed that transfection of these siRNAs, but not the scrambled control siRNA (100 nM each), attenuated E-selectin expression in HUVEC activated with TNF-α (10 ng/ml, 4 h) without affecting expression of ICAM-1. Moreover, a leukocyte adhesion assay under flow (shear stress = 1.0 dyne/cm^2) demonstrated that HUVEC transfected with a siRNA against E-selectin (siE-01) supported significantly less HL60 adhesion as compared to those transfected with the control siRNA (scE-01) after activation ($p < 0.03$). This technique provides a powerful strategy to dissect a specific function of a given molecule in leukocyte–endothelial interaction.
© 2003 Elsevier Inc. All rights reserved.

Keywords: Endothelial cells; Adhesion molecules; Molecular biology; Inflammation

Introduction of double-stranded RNAs (dsRNAs) has led to the development of a powerful sequence-specific tool to suppress gene expression during development in plants, invertebrates, and vertebrates [1]. In mammalian cell cultures, dsRNA-mediated interference of gene expression has also been accomplished by transfection of synthetic RNA oligonucleotides [2] or plasmids [3], with the requirement that the active fragments to be composed of 21 or 22 base pairs (short interfering RNA, siRNA) to ensure their specificity [4]. Application of siRNAs to abrogate endogenous gene expression in mammalian tissues is now regarded as a potentially powerful technique to define the function of a given molecule, as well as a possible method for gene therapy [5]. In the present study, we demonstrated that oligonucleotide-mediated delivery of siRNAs into vascular endothelium specifically reduced endogenous expression of E-selectin upon cytokine stimulation and subsequently inhibited leukocyte adhesion to the endothelium under flow. Knowledge of the ability of siRNAs to silence a specific gene in vascular endothelium will extend their application to novel gene therapies in cardiovascular medicine.

Materials and methods

Molecular biology procedures. siRNAs were designed to target the coding sequence of human E-selectin cDNA. The target sequences were directed to the single-strand region according to the predicted secondary RNA structure and sequences of the form $(AA/CA)N_{19}$ with

* Corresponding author. Fax: +81-3-5800-3380.
E-mail address: masavasc@tmd.ac.jp (M. Yoshida).
[1] These two autohors equally contributed to the work.

0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.09.125

GC contents of less than 70% were selected from this region [6]. Nineteen nucleotide RNAs followed by TT/TG were selected, then chemically synthesized, and gel-purified. For generation of double-stranded siRNAs, both strands were mixed, then heated to 95 °C for 2 min, and 70 °C for 1 min after which they were allowed to cool to room temperature. The final concentration of the siRNAs was 20 μM in 2 mM of magnesium chloride in 1 mM phosphate-buffered saline, pH 7.4. Sequences corresponding to the siRNAs were as follows: siRNA-01, nucleotide numbers 163–183 for the E-selectin coding region (GenBank Accesion No. NM000450); siRNA-02, 266–286; siRNA-03, 301–322; siRNA-04, 1243–1263; and siRNA-05, 1364–1384. Western blotting was performed using standard protocols with ECL reagents (Amersham–Pharmacia), with purified mouse antibodies against E-selectin (7A9 [7]) and ICAM-1 (BBIG-I1, Wako Chemical, Japan). Based on the results of the siRNA oligonucleotides, we constructed DNA-based vectors that expressed the sequence of siRNA-01 by our original method [3]. The vector expressed siRNA hairpins under control of the U6 promoter, which contained the 3′ end of the 21 nt sense-strand and the 5′ end of the 21 nt antisense-strand connected by a 21 nt loop sequence (UAGAAUUACAUCAAGGGAGAU). These sequences were inserted immediately downstream of the U6 promoter in a pUC19 (Takara).

Cell culture and transfection. HEK293 cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen). Human umbilical vein endothelial cells (HUVEC) were established from umbilical cords as previously described [8]. Typically, cells were seeded at 5×10^4 in each C-6 well and then transfected the next day with 2 μl Lipofectin 2000 (Invitrogen) combined with 3 μl (HEK293 cells) or 5 μl (HUVEC) with 20 μM of the siRNA in 1 ml Optimem (final concentration of siRNA was 60 nM for HEK293 cells and 100 nM for HUVEC). In some experiments, 1 μg E-selectin cDNA (pAdRSV4-E-sel [7]) or a

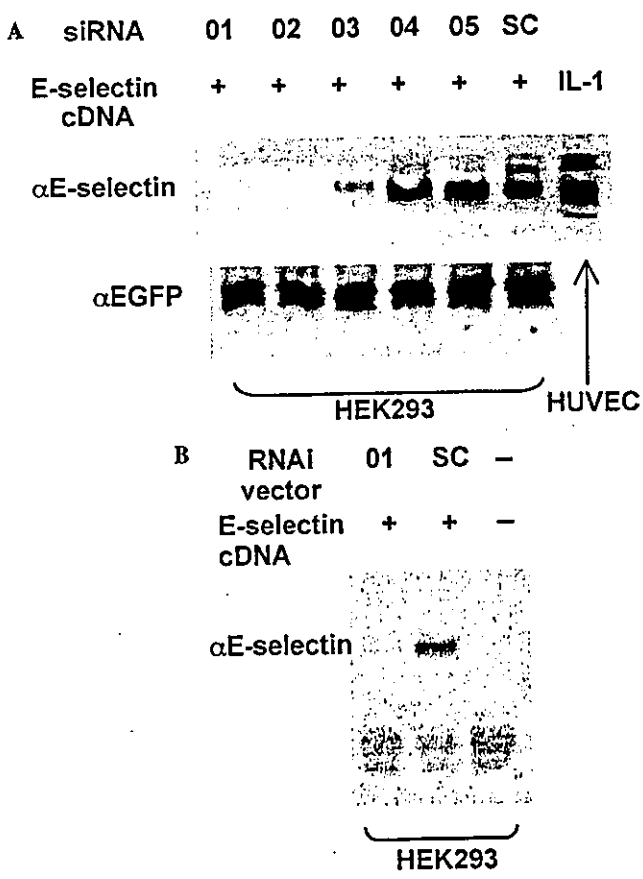

Fig. 1. (A) siRNA silencing of exogenous E-selectin gene expression in HEK293 cells. siRNA to human E-selectin (01 ~ 05) and a scrambled control siRNA 01 sequence (SC), each at 60 nM, were co-transfected into HEK293 cells with E-selectin cDNA (1 μg) and EGFP cDNA (1 μg) as described in Materials and methods. The cell lysates were subjected to Western blotting analysis using anti-E-selectin mAb (7A9). The lysate from HUVEC stimulated with IL-1β (IL-1) was used as positive control. Blots are representative of 4 similar experiments. (B) Efficacy of siRNA-vector against exogenous E-selectin gene expression in HEK293 cells. A U6 driven siRNA-vector to human E-selectin (01, 100 ng) and the control (SC, 100 ng) were co-transfected into HEK293 cells with E-selectin cDNA (1 μg) as described in Materials and methods. Western blotting analysis was carried out using an anti-E-selectin mAb (7A9). Blots are representative of 3 similar experiments.

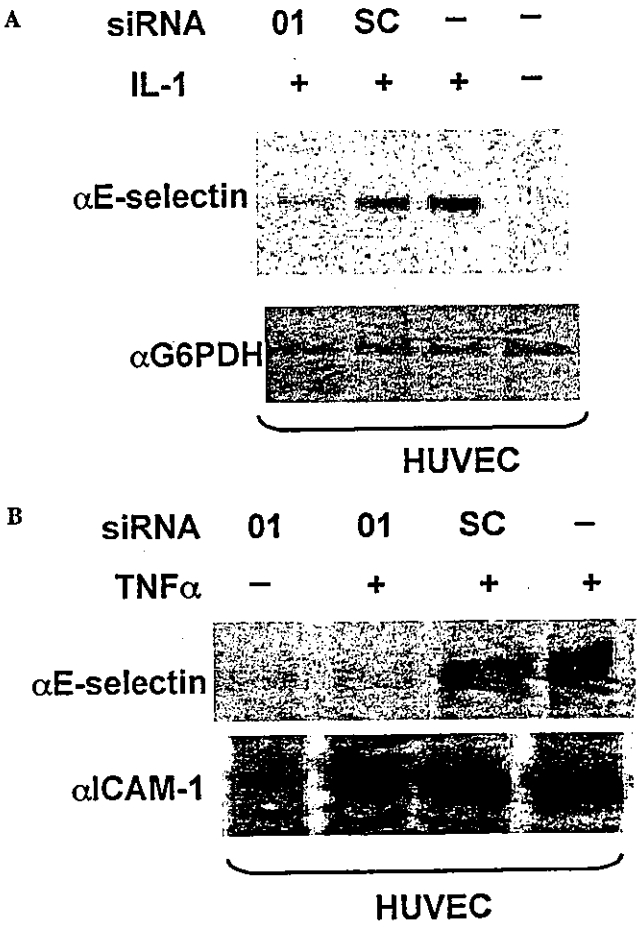

Fig. 2. (A) siRNA silencing of endogenous E-selectin gene expression in HUVEC. siRNA to human E-selectin (01) and a scrambled control of the siRNA 01 sequence (SC), each at 100 nM, were transfected into HUVEC as described in Materials and methods. Western blotting analysis was carried out using lysates prepared 24 h after transfection. The expression levels of G6PDH were also examined. Blots are representative of 3 similar experiments. (B) siRNA of E-selectin specifically inhibits gene expression of E-selectin but not ICAM-1 in activated HUVEC. siRNAs were transfected into HUVEC as described above, then 24 h after transfection, the HUVEC were stimulated with TNF-α (10 ng/ml) for 4 h, after which the cell lysates were prepared for Western blotting analysis to detect E-selectin and ICAM-1 expression.

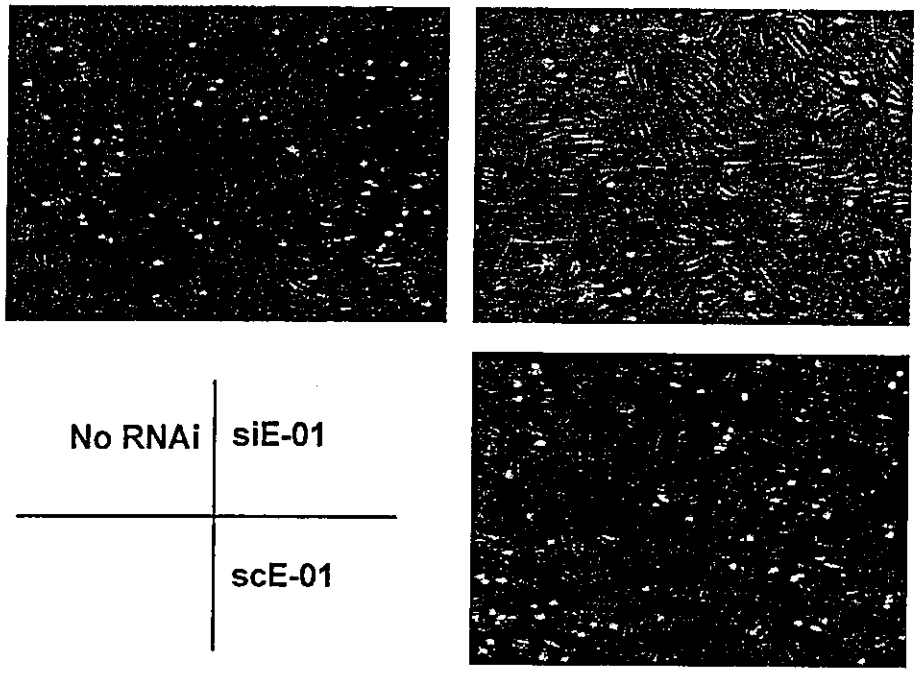

Fig. 3. siRNA of E-selectin specifically inhibits leukocyte adhesion to activated HUVEC under flow. siRNA to human E-selectin (siE-01) and a scrambled control of the siRNA 01 sequence (scE-01), each at 100 nM, were transfected into HUVEC and plated on a glass coverslip as described in Materials and methods. Twenty-four hours after transfection, the HUVEC were stimulated with TNF-α (10 ng/ml) for 4 h and an adhesion assay was carried out in the presence of laminar flow. Representative micrographs taken from video recording during the flow assay. HL-60 cells (white particles) were perfused over a TNF-activated HUVEC monolayer (gray background) that had been transfected with siE-01, scE-01, or lipofectin alone (no RNAi).

control plasmid containing enhanced green fluorescence protein (pEGFP-C2, Clontech) was co-transfected. Transfection mixtures were left on the cells for 16 h (HEK293 cells) or 4 h (HUVEC) and then replaced with regular media.

In vitro flow assay. The parallel-plate flow chamber used in the present study has been described previously in detail [9]. Briefly, HUVEC on coverslips were transfected with siRNA and then positioned in a flow chamber mounted on an inverted microscope. The monolayers were perfused for 5 min with perfusion medium [10] and HL60 cells (10^5 cells/ml) were then drawn through the chamber at controlled flow rate to generate a calculated wall shear stress of 1.0 dyne/cm^2 for 10 min. The entire period of perfusion was on videotape and then transferred to a PC for image analysis.

Statistical analyses. Results are presented as means ± SD. Data were analyzed using analysis of variance (ANOVA), with $p < 0.05$ considered significant.

Results and discussion

First, we analyzed the efficacy of double-stranded, siRNAs to silence E-selectin gene expression using HEK293 cells co-transfected with human E-selectin cDNA. Five different siRNAs against the coding sequence of human E-selectin cDNA were designed and Western blotting analysis was carried out using cell lysates that had been prepared 24 h after transfection of the siRNAs. As shown in Fig. 1A, HEK293 cells transfected with 3 of the siRNAs (siE-01, siE-02, and siE-03) were able to inhibit co-transfected E-selectin expression; however, the other 2 (siE-04 and siE-05) as well as nucleic-acid-scrambled double-stranded RNA (scE-01) failed to inhibit E-selectin expression. Moreover, the specificity of gene silencing was confirmed using simultaneous transfection of pEGFP-C2 (Fig. 1A). Next we examined the efficacy of plasmid-mediated RNAi with E-selectin. RNAi-vectors were co-transfected with the E-selectin cDNA plasmid into HEK293 cells. As shown in Fig. 1B, Western blotting analysis 48 h after transfection clearly demonstrated that co-transfection of U6-siE-01 but not U6-control significantly reduced exogenous expression of E-selectin.

Based on these experiments, we decided to use siE-01 to suppress endogenous E-selectin expression in HUVEC. siE-01 and scE-01 (100 nM each) were transfected into HUVEC using a cationic-liposome-mediated method. To exclude the possibility of a non-specifc toxicity of the siRNAs, DAPI staining of HUVEC was carried out. The level of apoptotic HUVEC ratio was around 0.9%, suggesting that our RNAi transfection did not cause significant cell damage (data not shown). Twenty-four hours after siRNA transfection, HUVEC were activated with interleukin (IL) 1β (10 U/ml, 4 h). As shown in Fig. 2A, the IL-1β-induced E-selectin expression was significantly inhibited by siE-01 but not by scE-01. Further, the expression level of G6PDH was not changed by

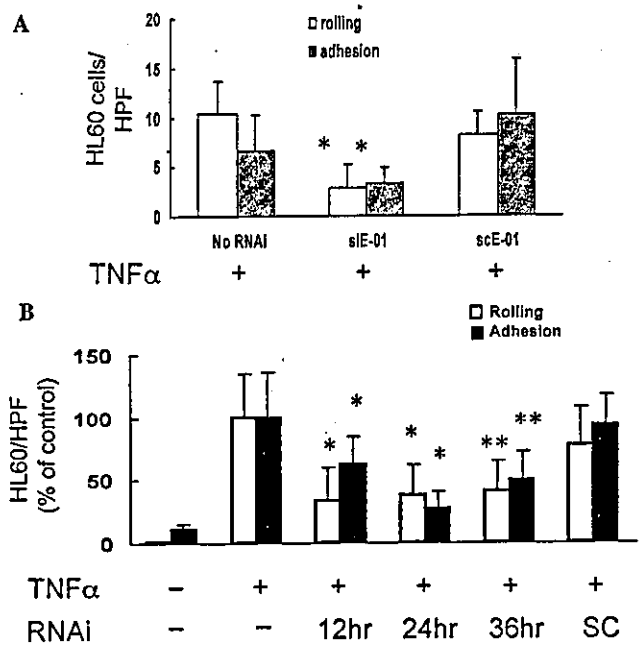

Fig. 4. Transfection of RNAi into HUVEC was performed as described in Fig. 3 and adhesion assay was carried out under flow (shear stress = 1.0 dyne/cm^2). (A) The number of adherent and rolling cells was quantitated from captured images using image analysis software and described as the number of interacting cells in each high power filled in the microscope area view (shear stress = 1.0 dyne/cm^2). *$p < 0.05$ vs. NoRNAi. (B) A quantitative adhesion assay under flow was carried out using HUVEC treated with siE-01 for various incubation times and compared to those treated with control RNA (SC) for 24 h (shear stress = 1.0 dyne/cm^2). *$p < 0.0005$ vs. TNF (+) RNAi (–), **$p < 0.005$ vs. TNF (+) RNAi (–). Data are representative of 4 similar experiments.

siRNA transfection. To validate the specificity of the inhibitory effects of siRNA, the expression level of ICAM-1 was also examined, however, it was not significantly altered after transfection of siE-01 (Fig. 2B). These results strongly suggested that introduction of siE-01 specifically silenced the expression of E-selectin.

Finally, leukocyte adhesion assay under flow was carried out using HUVEC transduced with siRNAs. As shown in Figs. 3 and 4A, the TNF-α-activated HUVEC monolayer supported a significantly greater adhesion of HL-60 cells under flow (shear stress = 1.0 dyne/cm^2) as compared to the non-activated HUVEC monolayer. Notably, the siE-01-transduced HUVEC monolayer failed to exhibit a comparable adhesion of HL-60 cells, whereas no inhibition of HL-60 adhesion was observed with the HUVEC monolayer transduced with sCE-01. We were able to inhibit E-selectin expression in activated HUVEC for up to 36 h after siE-01 transfection (Fig. 4B).

Our results may lead to the establishment of a novel experimental system to critically access the function of E-selectin in vascular endothelium using siRNA-mediated post transcriptional gene silencing. This is the first observation in the field of cardiovascular research of this novel technique, indeed, enabling us to manipulate a gene expressed in the vasculature. For the first time, we demonstrated that the lack of E-selectin gene expression significantly disrupted leukocyte–endothelial interaction in vascular endothelium and also showed that DNA-based siRNA expression vectors are effective against E-selectin in HEK293 cells. When considering applications for this siRNA approach to a potential gene therapy [11], an oligonucleotide-based system is advantageous in regard to biosafety. However, with gene therapy for atherosclerosis in vivo, a method to achieve long-term expression of siRNA in endothelium must be developed. For this purpose, we engineered similarly effective DNA-vector-based expressing siRNA, which might allow for long-term gene suppression in vivo with virus vectors, such as adeno-associate virus or renti-virus.

In conclusion, the efficacy of siRNA and our siRNA-expressing vector model to inhibit E-selectin-dependent leukocyte adhesion suggests that this RNA-targeting approach may provide a novel therapeutic option for inflammation and atherosclerotic disorders.

Acknowledgments

This study was supported in part by grants from the Ministry of Education, Science, Sports and Culture of Japan, and from Ministry of Health, Labour and Welfare of Japan. We gratefully acknowledge the expert assistance of Noriko Nitta in working with the cell cultures.

References

[1] G.J. Hannon, RNA interference, Nature 418 (2002) 244.
[2] S.M. Elbashir, W. Lendeckel, T. Tuschl, RNA interference is mediated by 21- and 22-nucleotide RNAs, Genes Dev. 15 (2001) 188.
[3] M. Miyagishi, K. Taira, U6 promoter-driven siRNAs with four uridine 3′ overhangs efficiently suppress targeted gene expression in mammalian cells, Nat. Biotechnol. 20 (2002) 497.
[4] L. Manche, S.R. Green, C. Schmedt, M.B. Mathews, Interactions between double-stranded RNA regulators and the protein kinase DAI, Mol. Cell. Biol. 12 (1992) 5238.
[5] D.P. Cioca, Y. Aoki, K. Kiyosawa, RNA interference is a functional pathway with therapeutic potential in human myeloid leukemia cell lines, Cancer Gene Ther. 10 (2003) 125.
[6] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, Nature 411 (2001) 494.
[7] M. Yoshida, B.E. Szente, J.M. Kiely, A. Rosenzweig, M.A. Gimbrone Jr., Phosphorylation of the cytoplasmic domain of E-selectin is regulated during leukocyte-endothelial adhesion, J. Immunol. 161 (1998) 933.
[8] M. Yoshida, W.F. Westlin, N. Wang, D.E. Ingber, A. Rosenzweig, N. Resnick, M.A. Gimbrone Jr., Leukocyte adhesion to vascular endothelium induces E-selectin linkage to the actin cytoskeleton, J. Cell Biol. 133 (1996) 445.
[9] A. Kawakami, A. Tanaka, K. Nakajima, K. Shimokado, M. Yoshida, Atorvastatin attenuates remnant lipoprotein-induced

monocyte adhesion to vascular endothelium under flow conditions, Circ. Res. 91 (2002) 263.
[10] M. Yoshida, T. Sawada, H. Ishii, R.E. Gerszten, A. Rosenzweig, M.A. Gimbrone Jr., Y. Yasukochi, F. Numano, HMG-CoA reductase inhibitor modulates monocyte–endothelial cell interaction under physiological flow conditions in vitro: involvement of RhoA GTPase-dependent mechanism, Arterioscler. Thromb. Vasc. Biol. 21 (2001) 1165.
[11] J.M. Jacque, K. Triques, M. Stevenson, Modulation of HIV-1 replication by RNA interference, Nature 418 (2002) 435.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 312 (2003) 1342–1348
BBRC
www.elsevier.com/locate/ybbrc

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

Takanori Yokota,[a,*] Kanako Sugawara,[a] Kaoru Ito,[a] Ryosuke Takahashi,[b] Hiroyoshi Ariga,[c,d] and Hidehiro Mizusawa[a]

[a] *Department of Neurology and Neurological Science, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan*
[b] *Laboratory for Motor System Neurodegeneration, RIKEN Brain Science Institute (BSI), Saitama 351-0198, Japan*
[c] *Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan*
[d] *CREST, Japan Science and Technology Corporation, Kawaguchi, Saitama 332-0012, Japan*

Received 2 October 2003

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.
© 2003 Elsevier Inc. All rights reserved.

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

* Corresponding author. Fax: +81-3-5803-0134.
E-mail address: tak-yokota.nuro@tmd.ac.jp (T. Yokota).

0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.11.056

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).

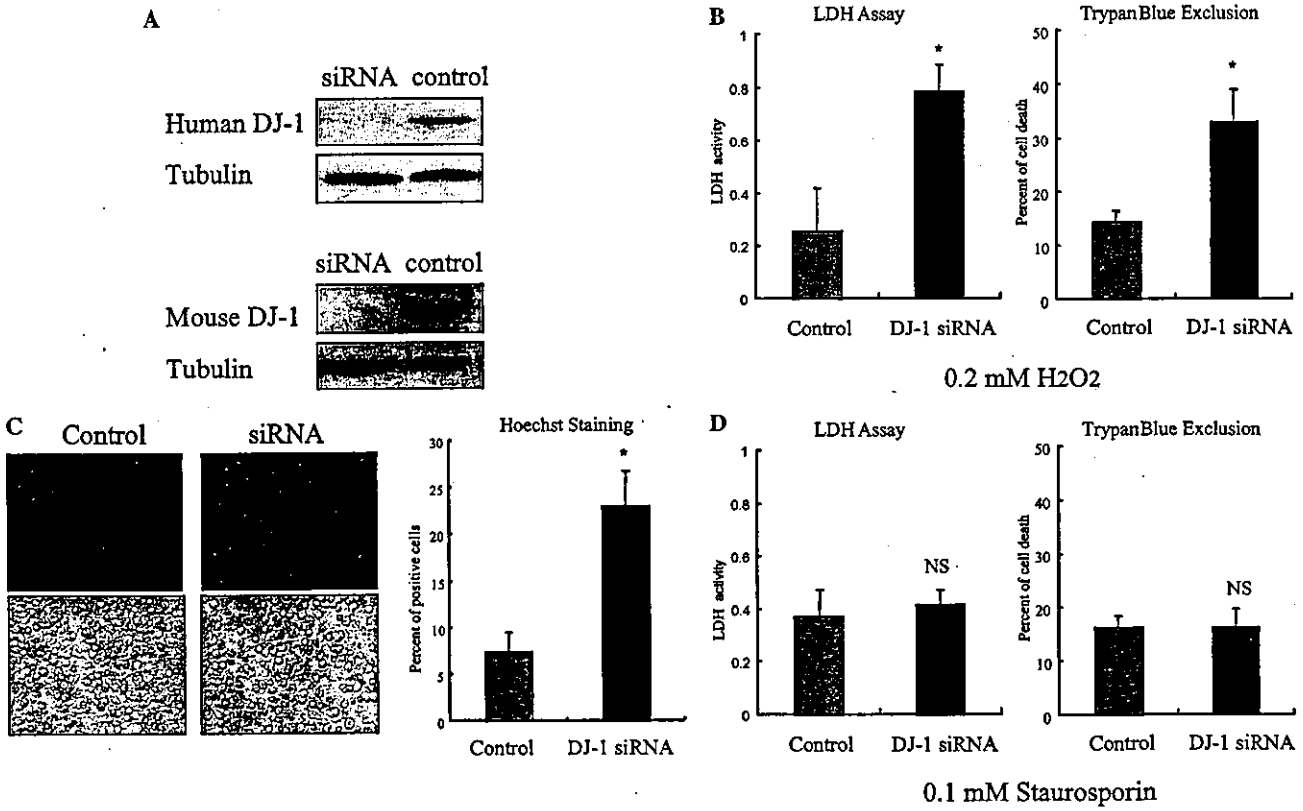

Fig. 1. Effect of down regulation of endogenous DJ-1 on cell death induced by oxidative stress with hydrogen peroxide (H_2O_2). (A) Down regulation of endogenous DJ-1 with siRNA-expressing vector on Western blotting after 48 h after transfection. One microgram of the siRNA-expressing vector for human 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.