JUN siRNA regulates matrix metalloproteinase-2 expression, microvascular endothelial growth and retinal neovascularisation

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Summary

Transcription factors link changes in the extracellular environment with alterations in gene expression. As such, these molecules serve as attractive targets for intervention in pathological settings. Since JUN has been linked with microvascular disease in humans, we hypothesised that small interfering RNA (siRNA) targeting this immediate-early gene may be useful agents that suppress endothelial growth and neovascularisation. Here we show that Jun siRNA inhibits Jun mRNA and protein expression in murine microvascular endothelial cells, blocks cell proliferation and suppresses migration in a scratch-wound assay. It also inhibits three-dimensional tubular formation on basement membrane extracts and reduces angiogenesis in mice bearing Matrigel plugs as subcutaneous implants. Single intravitreal administration of Jun siRNA reduces neovascularisation in a murine model of proliferative retinopathy, and suppresses endothelial JUN and matrix metalloproteinase-2 (MMP-2) immunoreactivity in retinal vessels, data supported by its repression of MMP-2 expression and gelatinolytic activity in vitro. Co-administration of TGFβ with the siRNA reverses this neovascular inhibitory effect, which is in turn abrogated by cis-9-octadecenyl-N-hydroxylamide, consistent with the involvement of a metalloproteinase such as MMP-2. Thus, JUN siRNA can serve as a specific inhibitor of aberrant endothelial and neovascular growth.

Key words: JUN, siRNA, Gene targeting, Endothelial cells, Retinal neovascularisation

Introduction

The angiogenic process involves a complex series of cellular events including microvascular sprouting, endothelial proliferation, migration and tubule formation. Although it is known that some of these events are controlled at the level of transcription by nuclear regulatory factors such as Ets-1 (Iwasaka et al., 1996), hypoxia-inducible factor-1 (Boudreau et al., 1997), FOS (Marconcini et al., 1999) and early growth response-1 (Fahmy et al., 2003), more work is needed to provide greater understanding of the transcription factors involved. This information would help facilitate the design of factor-specific, anti-angiogenic strategies.

Recent studies by our group using gene-specific catalytic DNA (DNAzymes) demonstrated a role for the basic region leucine zipper protein and immediate-early gene product, JUN in tumour angiogenesis (Zhang et al., 2004). JUN, a member of the AP-1 superfamily, is activated by multiple angiogenic stimuli including vascular endothelial growth factor (Zhang et al., 2004), (basic) fibroblast growth factor-2 (Viard et al., 1993) and serum (Zhang et al., 2004). JUN has been linked with neovascular disorders in humans. For example, JUN is expressed in the proliferative membranes of patients with proliferative diabetic retinopathy but not in patients with quiescent retinopathy (Ren et al., 2000). JUN might also be one of the factors influenced by endostatin in its inhibition of angiogenesis (Abdollahi et al., 2004).

Small interfering RNAs (siRNAs) provide a new approach to gain insight into the function(s) of the targeted gene in biological systems. siRNAs are RNA duplexes of 21-23 nucleotides (nt) in length that facilitate mRNA destruction in a RISC (RNA-induced silencing complex)-dependent manner (Elbashir et al., 2001; Hamilton and Baulcombe, 1999). In the present study, we evaluated the capacity of novel siRNA molecules targeting Jun mRNA to serve as inhibitors of JUN and JUN-dependent gene expression, microvascular endothelial cell proliferation, migration, tubule formation and retinal neovascularisation.

Results

Jun siRNA inhibits JUN and MMP-2 protein expression, and proliferation of murine microvascular endothelial cells

Synthetic siRNA targeting nts 2465-2485 of murine Jun mRNA (GenBank Acc. No. J04115) was introduced into growth-quiescent bEND-3 cells using the commercial transfection agent, RNAiFect, before incubation in medium containing fetal bovine serum (10%) for 2 hours. Murine, rather than human, microvascular endothelial cells were used for in vitro experiments because the siRNA would be evaluated in murine models. Serum induced JUN protein expression (Fig. 1A), which was inhibited by Jun siRNA but not by its scrambled counterpart, Jun siRNAscr at 0.1 and 0.4 μM, relative to levels of β-actin (Fig. 1A).
To complement these data, and demonstrate the functional relevance of siRNA inhibition of JUN protein, we used luciferase activity as a surrogate measure of JUN-AP-1 DNA-binding activity. Two tandem JUN-AP-1 DNA binding sites were subcloned into the SmaI site of pGL3-prom 5’ to the SV40 promoter upstream of firefly luciferase cDNA. Inducible JUN-AP-1-dependent reporter gene expression in transfected bEND-3 cells was inhibited by the Jun siRNA in a sequence-dependent manner (Fig. 1B). RT-PCR analysis also revealed that Jun mRNA expression was suppressed by the siRNA (Fig. 1C). An siRNA molecule (Egr-1 siRNAscr) of irrelevant sequence but identical size as Jun siRNA, like Jun siRNAscr, failed to influence inducible Jun mRNA (Fig. 1C) or JUN-AP-1-dependent reporter activity (Fig. 1B). Jun siRNA also suppressed TGFβ-inducible mRNA expression of matrix metalloproteinase-2 (MMP-2), a JUN-dependent gelatinase (Zhang et al., 2004) implicated in extracellular proteolysis during angiogenesis (Pepper, 2001) at 0.4 μM (Fig. 1D). The siRNA inhibited TGFβ-inducible gelatinolytic activity in the culture supernatant corresponding to 72 kDa, the size of pro-MMP-2 (Fig. 1E).

We next examined the effect of siRNA on bEND-3 proliferation by quantifying cell numbers 2 days following serum stimulation of growth-quiescent cells. bEND-3 proliferation was strongly induced by serum (Fig. 2) and inhibited by the Jun siRNA, whereas siRNAscr did not inhibit at any concentration tested (Fig. 2). Cell counts in the scrambled siRNA cohort were not different from those in the mock-transfected group (Fig. 2). A second siRNA targeting Jun (siRNA2) also inhibited endothelial cell proliferation, whereas its own scrambled counterpart (siRNA2scr) failed to affect cell growth (Fig. 2).

**Jun siRNA inhibits bEND-3 regrowth after injury**

The capacity of Jun siRNA to block bEND-3 cell regrowth after wounding was next assessed by quantifying cell numbers in the denuded zone. In this system, cells regrow into the denuded zone virtually to completion within 2 days in the absence of siRNA but in the presence of vehicle (Fig. 3A,B). This reparative process, which involves both migration and proliferation, was suppressed by the Jun siRNA. siRNA inhibition was sequence specific because cell counts in the scrambled siRNA cohort did not differ from those in the mock-transfected group (Fig. 3A,B).

**Jun siRNA blocks bEND-3 tubular formation on Matrigel in vitro**

Seeding an endothelial cell suspension onto Matrigel (basement membrane extract) facilitates the relatively rapid formation of a three-dimensional tubular capillary-like network. The Jun siRNA inhibited tube formation within 6 hours (Fig. 4), whereas network formation in the presence of Jun siRNAscr was indistinguishable from the vehicle control (Fig. 4).

**Jun siRNA inhibits angiogenesis in murine Matrigel implants**

Subcutaneous Matrigel implantation in C57BL/6 mice triggers an angiogenic response from the host vasculature and the eventual colonisation of the plugs within 14 days (Fahmy et al., 2003). Jun siRNA inhibited angiogenesis in these plugs by ~50% (Fig. 5). By contrast, Jun siRNAscr possessed no inhibitory effect on new blood vessel formation within the plug (Fig. 5) indicating again, sequence-specific inhibition by Jun siRNA.

**Jun siRNA inhibits JUN protein expression and retinal neovascularisation in mice following hyperoxia-induced proliferative retinopathy**

We reasoned that because Jun siRNA blocks matrix angiogenesis in mice (Fig. 5), the molecule may be useful in a pathophysiologically relevant angiogenic setting such as ocular angiogenesis. Exposure of neonatal mice to hyperoxic conditions followed by normoxia results in retinal neovascularisation (Smith et al., 1994) (Fig. 6A). Single intravitreal administration of the JUN siRNA inhibited retinal neovascularisation compared with mice treated with siRNAscr (Fig. 6A). Blood vessel counts in mice treated with siRNAscr did not differ from those treated with vehicle alone (Fig. 6A) indicating that siRNA inhibition was sequence specific and not merely due to a mass effect. Histological examination further indicated that the siRNA did not produce adverse morphological effects in the retina, nor did terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) immunostaining indicate apoptosis (data not shown).

Recent studies have demonstrated that retinal angiogenesis is compromised in MMP-2-deficient mice (Ohno-Matsui et al., 2003). Since JUN regulates MMP-2 expression (Fig. 1D) (Zhang et al., 2004), we evaluated the effect of the siRNA on JUN and MMP-2 expression in retinal blood vessels by immunohistochemistry. JUN and MMP-2 are poorly expressed in vessels of normal mice but dramatically induced by hyperoxia or normoxia (Fig. 6B,C). The siRNA suppressed inducible JUN (Fig. 6B) and MMP-2 (Fig. 6C) expression in murine microvascular endothelial cells. (A) Growth-quiescent bEND-3 cells transfected with 0.1 μM (thin bar) or 0.4 μM (thick bar) of Jun siRNA, siRNAscr or mock-transfected and incubated in medium containing serum for 2 hours before western blot analysis (for Jun or β-actin) of whole cell extracts. (B) Growth-quiescent bEND-3 cells transfected with 15 μg of pGL3-prom bearing two JUN/AP-1 binding sites and 0.1 μM (thin bar) or 0.4 μM (thick bar) of Jun siRNA, siRNAscr, Egr-1 siRNAscr or mock-transfected were incubated in medium containing serum for 24 hours before determination of luciferase activity in the cell lysates. (C) RT-PCR analysis for Jun using extracts of growth-quiescent bEND-3 cells transfected with 0.1 μM (thin bar) or 0.4 μM (thick bar) of Jun siRNA, siRNAscr, Egr-1 siRNAscr or mock-transfected and incubated in medium containing serum for 24 hours. (D) RT-PCR analysis for Jun using extracts of growth-quiescent bEND-3 cells transfected with 0.1 μM (thin bar) or 0.4 μM (thick bar) of Jun siRNA, siRNAscr, Egr-1 siRNAscr or mock-transfected and incubated in serum-free medium containing 10 ng/ml TGFβ for 24 hours. (E) Gelatin zymography demonstrating MMP-2 bioactivity in supernatants of transfected bEND-3 cells. The cells were transfected with 0.1 μM (thin bar) or 0.4 μM (thick bar) of Jun siRNA or siRNAscr and incubated in serum-free medium containing 10 ng/ml TGFβ for 24 hours. Data are representative of at least two independent experiments performed in duplicate or triplicate and are mean values ± s.e.m. *P<0.05 compared with no siRNA control by Student’s t-test.
Fig. 1. See previous page for legend.
MMP-2 (Fig. 6C) expression in these vessels. By contrast, the siRNA had no effect on levels of the zinc-finger transcription factor Sp1 whose levels were unaltered by hyperoxia or hypoxia (Fig. 6D). We hypothesised that local co-administration of recombinant TGFβ2 with the JUN siRNA would override the suppressive effect of the siRNA alone, because TGFβ2 can rapidly stimulate JUN expression (Davidson et al., 1993; Tang et al., 1998). Retinal vascularity in the siRNA group was increased by TGFβ2 (Fig. 6A). Rescue by the growth factor was blocked by the presence of 9-octadecenoyl-N-hydroxylamide (Fig. 6A). These findings demonstrate that TGFβ2 can override siRNA inhibition of retinal vascularity in a metalloproteinase-dependent manner (Fig. 6A).

**Discussion**

This study demonstrates that siRNA targeting the transcription factor JUN inhibits serum-inducible microvascular endothelial cell proliferation and blocks cellular regrowth in an in vitro model of wounding. The siRNA suppressed three-dimensional tubular formation on basement membrane extracts in a time-dependent manner, and blocked angiogenesis in mice implanted with subcutaneous Matrigel plugs. Moreover, a single intravitreal administration of the siRNA suppressed retinal neovascularisation in mice subjected to the hyperoxia-induced retinopathy of prematurity model, which has been used to evaluate the anti-angiogenic properties of several compounds including VEGF antisense oligonucleotides (Robinson et al., 1996), Macugen/EYE-001 (Eyetech, 2002), combretastatin-A4 (Griggs et al., 2002) and soluble EphA2 receptor (Chen et al., 2006). TGFβ2, co-administered with the siRNA, increased retinal vascularity, which was abrogated by cis-9-octadecenoyl-N-hydroxylamide, consistent with the involvement of a metalloproteinase such as MMP-2 (Aye et al., 2004; Emonard et al., 1999; Liao et al., 2003). The scrambled counterpart of the JUN siRNA had no effect in any of these systems indicating the sequence specificity of the siRNA. siRNA targeting VEGF and the VEGF receptors have previously been used to inhibit angiogenesis in animal models, and are presently under evaluation in clinical trials in patients with age-related macular degeneration (Kim et al., 2004; Reich et al., 2003; Shen et al., 2006; Tolentino et al., 2004).

JUN, like JUNB and JUND, is a member of the JUN sub-family of dimeric basic region leucine zipper proteins, and is known to regulate cell proliferation, differentiation, and apoptosis. siRNA targeting JUN expression has been shown to inhibit angiogenesis in various animal models. The findings of this study suggest that siRNA targeting JUN could be a potential therapeutic strategy for the treatment of angiogenesis-related diseases such as age-related macular degeneration and diabetic retinopathy.
JUN regulates MMP-2, EC growth and neovascularisation

(bZIP) proteins in AP-1. The AP-1 family regulates a plethora of cellular processes, including proliferation, differentiation, apoptosis and survival (Shaulian and Karin, 2002). Gene transactivation by JUN is mediated by physical interactions with promoter recognition elements (5'-TGAG/CTCA-3'), which are also known as phorbol 12-O-tetradecanoate-13-acetate (TPA) response elements (Angel et al., 1987) and/or interactions with other factors. JUN proteins are able to form dimers with bZIP proteins, such as for example, members of the CREB-ATF family of proteins and the oncogenic transcription factor Maf. JUN also dimersifies with the p65 subunit of NF-κB, Sp1, CBP/p300 (CRE-binding-protein binding protein), SMAD-3 and -4, and retinoblastoma protein (Rb), among others (Chinenov and Kerppola, 2001). JUN does not necessarily have to bind DNA to influence gene transcription, as has been observed in the human 12(S)-lipoxygenase promoter where Sp1 serves as an anchor protein (Chang and Chen, 2005). The spectrum of genes influenced by JUN, directly and indirectly, is therefore impressive, and includes those regulating the cell cycle, growth, the extracellular matrix, membrane and cytoskeleton (Vogt, 2001).

The JUN siRNA inhibited gelatinolytic activity corresponding to 72 kDa, the size of pro-MMP-2. Our previous studies using DNAzymes have demonstrated that MMP-2 is a JUN-dependent gene (Zhang et al., 2004). JUN proteins have been found to regulate MMP-2 transcription (Bergman et al., 2003). Our inability to detect a smaller migrating species in vitro corresponding to the active form of MMP-2 (59-65 kDa) may reflect the limit of detection of zymography. It is also possible that although these cells produce pro-MMP-2, they may express insufficient levels or lack essential co-factor(s)/enzymes that activate MMP-2 in culture. These co-factor(s) are, nevertheless likely to be present during retinal neovascularisation which is inhibited by cis-9-octadecenoyl-N-hydroxylamide (Fig. 6A) and unlike the in vitro setting, involves a variety of cell types. Indeed, studies have demonstrated that MMP-2, MMP-9 and MT1-MMP are all upregulated in the retina of mice undergoing a neovascular response (Majka et al., 2001). Previous studies have also demonstrated the appearance of a single band on a zymogram corresponding to the 72 kDa form of MMP-2 (Steinbrenner et al., 2003). A limitation of this study is that of the plethora of genes JUN controls, the influence of the siRNA on the altered expression of just one metalloproteinase was investigated. Thus, JUN siRNA can inhibit endothelial cell growth and neovascularisation and this may involve suppression of MMP-2 production.

Materials and Methods

Cell culture and siRNA transfection

Murine microvascular endothelial cells (bEND-3) (gift from the Inflammatory Research Unit, University of New South Wales, Australia) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) with 2 mM glutamine, 5 U/ml penicillin-strepto-
mycin and 10% fetal bovine serum (FBS) at 37°C, 5% CO₂. Jun siRNA was synthesised with the sequence, 5'-r(CAGCUUCCGCUUUGUAA)dTT-3'. The scrambled counterpart, Jun siRNAscr, had the sequence 5'-r(GAUUACUAGCCGUAG)dTT-3'. Sequence of Jun siRNA2 was 5'-r(GCGCAUGAGGAACCGCAUU)dTT-3' and its scrambled counterpart, Jun siRNA2scr, which also served as a negative control as indicated, had the sequence 5'-r(GCCGAACUAUGCGCUAG)dTT-3'. Egr-1 siRNAscr, which was used to deliver the siRNA according to the manufacturer's instructions 6 hours after the change of culture medium to serum-free conditions to initiate growth arrest at subconfluence. The 'No siRNA' group was mock transfected with vehicle alone. Eighteen hours after transfection, the cells were transfected again and the medium was replaced with medium containing 10% serum.

### Western blot analysis

Serum-starved bEND-3 cells transfected twice with siRNA, siRNAscr or mock transfected with vehicle alone were incubated in 10% FBS for the indicated time. The cells were washed in phosphate-buffered saline (PBS), and then harvested into RIPA buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1% Triton X-100, 5 mM EDTA, 1% aprotinin, 2 mM phenylmethyl sulphonlfyl fluoride (PMSF), followed by two freeze-thaw cycles and centrifugation at 8000 g in a microfuge (Sigma, USA). The supernatant was collected and stored at -80°C until Western blot analysis was performed.

### Fig. 6

siRNA targeting Jun inhibits neovascularisation in mice following hyperoxia-induced proliferative retinopathy. Postnatal day 6 (P6) C57BL/6 mice were exposed to hyperoxia (75% oxygen) for 4 days. P10 mice were returned to normoxia, and a bolus intravitreal injection of 20 µg of either Jun siRNA or siRNAscr was administered. Where indicated the siRNA was co-administered with 0.08 µg TGFβ with or without 20 µM cis-9-octadecenoyl-N-hydroxylamide (cis-9-o-N-h). Mice were left at room oxygen for a further 7 days before P17 pup eyes were enucleated and fixed in 10% formalin in PBS. (A) Serial cross-sections of the eyes were stained with Haematoxylin and Eosin and blood vessels in the retina were quantified by light microscopy under 400× magnification and expressed as the mean ± s.e.m. Representative images at 100× magnification (upper panels). Jun siRNA inhibits hyperoxia-induced neovascularisation (lower panel). *P<0.05 by Student’s t-test between groups as indicated. Immunoreactivity for (B) JUN, (C) MMP-2 or (D) Sp1 in retinal microvessels of mice treated intravitreally (20 µg) by single administration of Jun siRNA or siRNAscr or vehicle (no siRNA). Western blot analysis in C and D was performed with bEND-3 lysates as described in the Materials and Methods. Arrows in B-D indicate specific immunostaining (magnification, 1000×).
Germany) for 10 minutes at 4°C to remove cell debris. 2 µg of protein was resolved on 12% polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) nylon membranes (Immobilon™, Millipore, Bedford, MA). The membranes were then probed with rabbit polyclonal antibodies to JUN (1:1000 (0.2 µg/ml), Santa Cruz Biotechnology, CA), MMP-2 (1:1000 (0.2 µg/ml), Santa Cruz Biotechnology, CA) and Sp1 (1:1000 (0.2 µg/ml), Santa Cruz Biotech) in TBPS [0.05% Tween 20 in PBS (v/v)] and incubated with horseradish-peroxidase-conjugated swine-anti rabbit IgG (1:1000, Dako, Denmark). The protein bands were visualised using the Western Lightning Chemiluminescence kit (PerkinElmer Life Sciences, Boston, MA).

RT-PCR

Growth-quiescent bEND-3 cells in 100 mm Petri dishes were transfected with siRNA or siRNAscr and incubated in medium containing 10% serum for the times indicated. Total RNA was prepared using Trizol reagent (Invitrogen). cDNA was generated in 20 µl reactions containing 4 µg total RNA, 200 U Superscript II reverse transcriptase (Invitrogen), 500 µM of each dNTP and 0.5 µg oligo (dT)15 (Invitrogen). PCR analysis was performed in 20 µl reactions containing reverse-transcribed cDNA template, 1 U DNA polymerase (Promega, Annandale, Sydney, Australia), 100 µM of each dNTP, 30 mM MgCl2 (Invitrogen) and 0.1 µM of forward and reverse primer (for JUN: 25 cycles at 95°C 25 seconds, 61°C 30 seconds and 72°C 45 seconds; for MMP-2: 22 cycles of 95°C for 30 seconds, 57°C for 30 seconds and 72°C for 40 seconds). The volume of cDNA template required to achieve equivalent levels of GAPDH (452 bp product) between samples was to amplify JUN cDNA (514 bp amplification), MMP-2 (445 bp amplification) or GAPDH (452 bp amplification). Primers were as follows: JUN (forward primer, 5’-GGAGAACGCTTCTCAGCAGAT-3’; reverse primer, 5’-GAAGTTGCTGAGGTTGGCGT-3’); MMP-2 (forward primer, 5’-GGGACAAGAACCAGATCACATAC-3’; reverse primer, 5’-CTTCACTCCTAGGTTGCGTG-3’); GAPDH (forward primer, 5’-ACCAAGTCCATGACCATAC-3’; reverse primer, 5’-TCCACACCCTGTTGCTGTA-3’).

JUN AP-1-dependent reporter gene expression

The pGL3-promoter (Promega) was digested with Smal and double-stranded oligonucleotides (Oligo JUN) (Zhang et al., 2004) bearing JUN-AP-1 binding sites (GGTCTGATGAGCAGGCGAATCAGTAGACTTGCGGAAA) were cloned upstream of the SV40 promoter driving Firefly luciferase. Transient transfections were performed at 70% confluence in 100 mm Petri dishes using FuGENE6 with 15 µl reporter plasmid construct and either 0.1 or 0.4 µM siRNA. Luciferase activity was determined 24 hours after transfection using the Luciferase assay system (Promega).

Gelatin zymography

Culture supernatants were collected from bEND-3 cells transfected with siRNA as indicated or mock transfected with vehicle alone and resolved (equal amounts of protein per lane) at 4°C on a 10% polyacrylamide gel (with a 4% polyacrylamide stacking gel) containing 1 mg/ml bovine type B gelatin (Sigma). The gel was soaked in 2.5% Triton X-100 (Sigma) for 1 hour then incubated in substrate buffer (50 mM Tris-HCl, 10 mM CaCl2, 0.02% NaN3) overnight at 37°C. The gel was stained in 0.2% Coomassie Blue R-250 (Bio-Rad) in water, methanol and glacial acetic acid at volume ratio (5:4:1) for 1 hour, then destained in H2O:methanol:glacial acetic acid (5:4:1) to reveal clear bands corresponding to protein bands with gelatinolytic activity.

Cell proliferation assay

bEND-3 cells seeded (3 x 10^5/well) in 96-well plate titers were generated as reported previously (Kachhian et al., 2002) with rabbit polyclonal MMP-2 and Spl antibodies purchased from Santa Cruz Biotechnology, CA.

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