Available online at www.sciencedirect.com
SCIENCE DIRECT
Experimental Eye Research 81 (2005) 32–37
EXPERIMENTAL EYE RESEARCH
www.elsevier.com/locate/yexer

SiRNA strategy against overexpression of extracellular matrix in diabetic retinopathy

Toshiyuki Oshitari[a], David Brown[b], Sayon Roy[a,*]

[a]Departments of Medicine and Ophthalmology, Boston University School of Medicine, 715 Albany Street, Boston, MA 02118, USA
[b]Ambion, Inc., 2130 Woodward Street, Austin, TX 78744-1837, USA

Received 30 September 2004; accepted in revised form 10 January 2005
Available online 22 March 2005

Abstract

Increased synthesis of extracellular matrix (ECM) contributes to the development of vascular BM thickening, a prominent abnormality in diabetic retinopathy. RNA interference (RNAi) approach was used in this study to examine the effect of small interfering RNAs (siRNAs) for their ability to inhibit ECM-specific gene overexpression under high glucose condition in rat microvascular endothelial cells (RMECs). Four fibronectin (FN)-siRNAs, three collagen IV (Coll IV)-siRNAs, and four laminin (LM)-siRNAs, a total of 11 siRNAs were screened. RMECs were transfected with 10, 30, or 100 nM of each siRNAs in the presence of 8 μM lipofectin and subjected to analysis 72 hr after transfection. In long-term studies siRNA-transfected cells were examined after 12 days. Two FN siRNAs, two Coll IV siRNAs, and two LM siRNAs significantly reduced the respective target expressions. Findings from this study indicate that high glucose-induced abnormal expression of BM components may contribute to increased vascular permeability. SiRNA may be a useful tool in preventing excess vascular permeability, a characteristic feature of early diabetic retinopathy.

Keywords: RNA interference; small interfering RNA; extracellular matrix components; microvascular endothelial cells; monolayer cell permeability; high glucose; transfection; Western blot analysis

1. Introduction

Vascular basement membrane (BM) thickening is a prominent characteristic lesion of diabetic retinopathy (Osterby, 1990; Stitt et al., 1994). Several studies have shown that synthesis of ECM components such as fibronectin (FN), laminin (LM) or collagen IV (Coll IV) is upregulated by high glucose or diabetes and that these changes are associated with the development of BM thickening (Roy et al., 1990, 1994, 1996, 2003; Sato et al., 2001).

RNA interference (RNAi) is a double-strand RNA (dsRNA)-mediated gene silencing process against specific gene expression observed in many eukaryotic cells. Recent report suggests that small interfering RNA (siRNA) provides a powerful way to regulate specific gene expression in mammalian cells (Elbashir et al., 2001a). Adenovirus-induced siRNA targeting vascular endothelial growth factor (VEGF) significantly reduced the VEGF expression in mice retinas and inhibited choroidal neovascularization (Reich et al., 2003).

Increased vascular permeability is observed in the early stages of diabetic retinopathy, which can result in macula edema and cause visual disturbances. However, the precise mechanism by which vascular permeability increases in early diabetic retinopathy remains unclear. Recently we have reported that monolayer cell permeability is increased in rat microvascular endothelial cells (RMECs) grown in high glucose medium and that reduction of FN, LM, and Coll IV overexpression can significantly improve monolayer cell permeability in vitro (Chadda et al., 2003). We hypothesize that BM thickening is associated with increased vascular permeability, and that siRNA-mediated reduction

* Corresponding author. Dr Sayon Roy, Department of Ophthalmology, Boston University School of Medicine, 715 Albany Street, Boston, MA02118, USA.
E-mail addresses: tarii@aol.com (T. Oshitari), dbrown@ambion.com (D. Brown), sayon@bu.edu (S. Roy).

0014-4835/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.exer.2005.01.006

of FN, LM and Coll IV expression may inhibit vascular permeability.

In this study we have identified siRNAs that are effective against specific ECM component overexpression under high glucose condition in RMECs. We also examined long-term efficacy of the siRNAs and determined the effect of the siRNAs mentioned above in high glucose-induced increased monolayer cell permeability by measuring transelectrical resistance (TER).

2. Materials and methods

2.1. Cell culture

Rat microvascular endothelial cells ERMECs positive for von Willebrand factor were used in this study (Marcum and Rosenberg, 1985). Cells were grown in M199 supplemented with 10% FBS, antibiotic and antimycotics. At subconfluency, cells were transfected with siRNAs designed to reduce ECM expression by these cells. Seventy-two hours post-transfection cells were harvested and protein isolated for Western Blot analysis. Long-term studies were performed 12-days post-siRNA transfection and involved cell passaging.

For examining the effect of siRNA against high glucose-induced FN, Coll IV, and LM overexpression, RMECs were cultured in normal (5 mM) or high (30 mM) glucose medium for 7 days followed by siRNA transfection, Western Blot analysis, and TER measurement performed 3 days post-transfection.

To examine cell monolayer permeability, TER was measured using a fixed pair of electrodes connected to a voltohmmeter (World Precision Instruments, Inc., Sarasota, FL) designed specifically to perform routine TER measurement in tissue culture. Briefly, cells were plated on inserts of the upper chamber in 24-well plates, grown according to experimental conditions, and 3 days after siRNA transfection TER was measured.

2.2. SiRNAs

We screened several 21-nucleotide, double-stranded siRNAs for their ability to inhibit ECM-specific gene expression in RMECs. SiRNAs were selected based on Tuschl rules (AA + 19 adjacent nucleotides) (Elbashir et al., 2001a). Many of the siRNAs targeted sites encompassed the translation initiation site of either fibronectin (FN), collagen IV (Coll IV), or laminin (LM) EFN, Coll IV, or LM transcript. We tested four FN siRNAs, three Coll IV siRNAs, and four LM siRNAs, a total of 11 siRNAs (Table. 1) that were prepared using the siRNA Construction Kit (Ambion, Austin, TX). Non-specific siRNA, niR16 was used as control in this study.

To determine the optimal concentration of siRNA efficacy, RMECs were grown to subconfluency and transfected with 10, 30, or 100 nM of each of the siRNAs in the presence of 8 μM lipofectin and subjected to analysis 72 hr after transfection. SiRNA-transfected cells were compared to non-transfected cells or cells transfected with a non-specific siRNA as control for specificity of the RNAi strategy. The efficacies of the siRNAs were determined by Western blot analysis. Chemically synthesized siRNAs matching the sequences of the most effective FN, Coll IV, and LM siRNAs were prepared and tested for their long-term efficacy. SiRNAs that showed long-term efficacy were further tested against high glucose-induced extracellular matrix gene overexpression.

Table 1
Sequences of siRNAs tested in this study

SiRNAs	Sequence	Accession number	Map location
FN2557	5′-aagtacatcgtcaacgtctat-3′	X15906	2557–2577
FN1371	5′-aacttcaaattatgaacaaga-3′	X15906	1371–1391
FN379	5′-aagcattatcagataaatcag-3′	X15906	379–399
FN5110	5′-ccatcccagatgcaggt-gacg-3′	X15906	5110–5130
Coll IV-*n*	5′-gcgccaccatggggccc-3′	U85606	3432–3449
Coll IV-1	5′-aacttgtcaactctagaaaga-3′	U85606	2461–2481
Coll IV-2	5′-aaagatacattcaaagtcccc-3′	U85606	2477–2497
LM-*n*	5′-cacggcgggatgacgg-3′	L46862	1098–1114
LM-1	5′-aagttgcgtatgtcatcatta-3′	XM_237536.2	743–763
LM-2	5′-aatacacagtgtcttacgaca-3′	XM_237536.2	2153–2173
LM-3	5′-aatggaaagaacctc-caatag-3′	XM_237536.2	5298–5318
niR16	5′-aauauuggcguuaagauu-cua-3′	N/A	N/A

Four FN siRNAs, three Coll IV siRNAs, four LM siRNAs, non-specific siRNA (niR16) were examined.

2.3. Western blot analysis

RMECs were isolated and incubated in 25 mM Tris, pH 7.4, containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 0.1% Triton X-100. Western blots were performed with 50 μg protein per lane and transferred onto nitrocellulose membrane according to Towbin's procedure (Towbin et al., 1979). After blocking with 5% fat-free milk in tribromosalicylanilide, the membrane was incubated with the blocking solution containing either rabbit anti-FN, anti-Coll IV or anti-LM antibody (1:500, 1:500, 1:1000, respectively) overnight. After washing, the membrane was incubated with anti-rabbit antibody conjugated with horseradish peroxidase (1:15 000) for 1 hr. Immuno-Star Chemiluminescent Protein Detection System was used to detect protein levels of FN, Coll IV and LM. Densitometric analysis of the luminescent signal was performed at non-saturating exposures with a laser scanning densitometer and NIH Image software.