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Inhibition of Angiogenesis using RNAi technology

Yunsang Lee
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Yunsang Lee entitled "Inhibition of Angiogenesis using RNAi technology." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Barry T. Rouse, Major Professor

We have read this dissertation and recommend its acceptance:

Robert N. Moore, Albert T. Ichiki, Steve J. Kennel, Mark Sangster

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Inhibition of Angiogenesis using RNAi technology

A

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For the Doctor of Philosophy Degree

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Yunsang Lee

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ABSTRACT

Ocular infection of Herpes Simplex Virus-1 (HSV-1) causes Herpetic Stromal Keratitis (HSK), which is a leading cause of infectious blindness. Although complex interactions of molecular and cellular events involve in the development of HSK, it has been known that angiogenesis is a key step for the HSK pathogenesis. Treatment of neutralizing antibody against vascular endothelial growth factor (VEGF) to inhibit VEGF activity reduced angiogenesis and HSK severity caused by infection of HSV-1 in mouse eyes. In addition, inactivation of cytokine which induces VEGF production and angiogenesis also reduced angiogenesis and HSK severity. Therefore, it has been proposed that VEGF or molecules which induce VEGF production or angiogenesis can be good target molecules for treating HSK. In this study, we investigated whether targeting VEGF or IL-1 receptor type I (IL-1RI) using RNAi technology could reduce angiogenesis in mouse eyes.

A general introduction and overview of RNAi were provided in Part I. Results in Part II demonstrated that intrastromal injection of VEGF short hairpin RNA (shRNA) could reduce VEGF production and angiogenesis caused by CpG motif in mouse eyes. Implantation of pellets containing bio-active CpG motifs following intrastromal injection with a plasmid expressing shRNA against VEGF reduced angiogenesis and VEGF production. Results in Part III showed that intrastromal injection of IL-1RI shRNA could reduce angiogenesis caused by IL-1α through reduction of VEGF production. The results in this dissertation indicate that targeting VEGF or IL-1RI using RNAi technology can reduce angiogenesis in mouse eyes. Additionally, these results imply that an eye is a

suitable organ to apply RNAi technology. Thus, this technology may help to understand corneal biology as well as to treat corneal diseases in the near future.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

bFGF.....basic fibroblast growth factor

CCR5.....CC chemokine 5

dsRNA.....double stranded RNA

EGFP.....Enhanced green fluorescence protein

HBV.....Hepatocyte B virus

HCV.....Hepatocyte C virus

HIV-1.....Human immunodeficiency virus 1

HPV.....Human papiloma virus

HSK.....Herpetic stromal keratitis

HSV-1.....Herpes simplex virus 1

IL-1.....Interleukin 1

IL-1RI.....Interleukin 1 receptor type 1

NS5B.....Non structural protein 5B

RISC.....RNA induced silencing complex

RNAi.....RNA interference

RSV.....Respiratory syncytial virus

shRNA.....short hairpin RNA

siRNA..... small interfering RNA

TNF-α..... Transforming necrosis factor α

VEGF.....Vascular endothelial growth factor

Part I

Background and overview

Discovery of RNA interference (RNAi)

In 1990 Richard Jorgensen attempted to make transgenic petunias with a deep purple color by introducing a pigment-producing gene under powerful promoter. Many of the flowers, however, showed variegated or even white pigmentation (1). This bizarre phenomenon, which was named co-suppression because the expression of both the transgene and the homologous endogenous gene were suppressed, was mysterious until 1998 when Andrew Fire and Crag Mello discovered RNAi, the sequence-specific gene silencing mediated by double stranded RNA (dsRNA) (2). They showed that the presence of dsRNA inhibited the expression of the gene which was homologous to the dsRNA in *C. elegans*. Multicopy transgenes in plant can produce low levels of dsRNA. From this discovery, now we know that dsRNA is the molecule responsible of the previously unsolved phenomenon, co-suppression, which is also named post transcriptional gene silencing (PTGS) in plants. From further work it was established that co-suppression in plant and RNAi in *C. elegans* share a common mechanism and that the RNAi phenomenon occurs in many other organism including *Drosophila* and mammals.

Mechanisms of RNAi

The results from several *in vivo* and *in vitro* experiments have elucidated the mechanism of RNAi. Baulcomb and Hamilton discovered the first key step. In plants undergoing co-suppression, they identified RNAs of about 25nt nucleotides in length that matched the sequence of the gene being silenced and which were absent in non-silenced plants (3). Further work done by Zamore *et al.*(4) using *Drosophila* embryo lysates and

an *in vitro* system from S2 cells showed that dsRNA added to *Drosophila* embryo lysates was processed to length of 21-23nt and homologous endogenous mRNA was cleaved. These results revealed that in the first step, referred to as the initiation step, long dsRNA was digested into 21-23nt small interfering RNAs (siRNAs) (5,6,7) Biochemical experiments showed that siRNA have a 2-3nt 3' overhang, 5' phosphate and a 3' hydroxyl group (8). This structure is the characteristic cleavage pattern for the RNase III family of ribonucleases. Evidence has shown that cloned enzyme, Dicer, a member of the RNase III family, cleaves dsRNA in an ATP-dependent manner to 19-21bp siRNA. These results suggested that the enzyme responsible for cleavage of long dsRNA to make siRNA in the initiation step was Dicer.

In the effector step, siRNAs bind to an RNA-induced silencing complex (RISC). The siRNA is unwound, the sense strand is removed and the antisense strand remains to lead RISC to its target homologous mRNA. Finally, the RISC promotes cleavage of the mRNA (5, 6, 7, 9).

In plants and *C. elegans,* an amplification step has been proposed (5-7). Amplification could occur through using the antisense strand of siRNA as a primer by RNA-dependent RNA polymerase (RdRp). Therefore, more dsRNAs could be made through this amplification step, which provieds plant cells and *C. elegans* with a more effective RNAi. Mammalian cells, however, do not have an amplification step because they lack of RdRp. Fig. 1 shows a model for the mechanism of RNAi.

Another mechanism of RNAi is transcriptional gene silencing (TGS). This TGS accomplished via DNA methylation or heterochromatin formation by histone methylation. This mechanism has been studied mainly in plants (10, 11). When dsRNA degradation

mediated PTGS occurs in plants, the genomic DNA regions homologous to dsRNA are often found methylated at almost all the sensitive cytosine residues. This process is generally referred to as RNA-dependent DNA methylation, which renders the corresponding part of the genome, especially the promoter region transcriptionally silent. The initiator of RNA-dependent DNA methylation could be either the transgene-derived dsRNA or siRNA (10, 11). Depending on the sequence information of the dsRNA, RNA-dependent DNA methylation was found to occur in the open reading frame or at the promoter region of the genome (12, 13). If methylation occurs only in the open reading frame, TGS is not affected. However, RNA-dependent DNA methylation at the promoter sequences induces TGS and is stable and heritable (14).

RNA dependent DNA methylation has been reported mainly in plants and yeast. However, very recently, Morris et al., (15) showed that human cells also have siRNA-induced transcriptional gene silencing. Using siRNA which targets the promoter region, they showed transcriptional silencing is associated with DNA methylation. Therefore, it seems that TGS is an active pathway in many organisms. However, more investigation is necessary in order to understand RNA dependant DNA methylation in human cells.

Some evidence implies that heterochromatin formation could be the other cause of TGS by siRNA (16-18). In *C. elegans*, which does not have DNA methylation, some mutations of genes involved in RNAi machinery, *mut7* and *rde2*, derepress transgenes which are repressed by polycomb proteins, which maintain the chromatin heterochromatin status. In addition, it has been found that polycomb proteins MES3, MES4, and MES6 are required for RNAi under some experimental condition (16, 17).

Hall et al., (18) has shown that DNA repeats, which are responsible for dsRNA, are important for heterochromatin formation using a yeast system. They inserted a 3.6kb centromere H repeat in a euchromatin position. The insertion of the repeats induced silencing of a linked reporter gene and methylation of histone H3-K9. From these results, it was suggested that dsRNA could induce heterochromatin formation. Fig. 2 shows the basic mechanisms of TGS induced by dsRNA.

Biological function of RNAi

Many plant viruses encode suppressors of PTGS that are essential for pathogenesis. Therefore, it seems that RNAi has an important role in pathogen resistance (19, 20). RNAi also control endogenous transposons. In *C. elegans*, some RNAi-deficient strains showed increased mobility of endogenous transposons (21, 22), which was inactivated by heterochromatin formation. Therefore RNAi may stabilize the genome by preventing transposition.

Natural RNAi not only prevents the transposable elements from disrupting the integrity of genomes but also is responsible for organism development. Mutations of *ego1* and *dicer*, involved in RNAi pathway in *C. elegans* cause developmental defects (23, 24). Thus, genetic evidence implies that a natural role of the RNAi machinery is the control of development of the organism. In many organisms, micro-RNAs have been identified (25). Unlike siRNA micro-RNA is not perfect dsRNA. Micro-RNAs have hair-pin structure and do not trigger mRNA degradation. Instead, micro-RNAs hybridize to mRNAs and prevent translation. It seems that the micro-RNA regulates endogenous gene expression negatively. In *C.elegans*, let-7 and lin-4 mi-RNAs have been cloned. Lin-4

micro-RNA inhibits endogenous *lin14* and *lin28* gene expression. The mutation of many micro-RNAs result in a developmental defect and the expression of micro-RNA is temporal. Therefore, it appears that micro-RNAs regulate development-related genes transiently.

Gene silencing by siRNA in mammalian cells

Since RNAi was discovered in *C. elegance* the RNAi technology mediated by the introduction of long dsRNA has been used to investigate gene function in many organisms including *Arabidopsis* and *Drosophila*. However this long dsRNA can not be used in mammals because the introduction of dsRNA longer than 30nt induces an interferon response (26). Interferon induces 2'-5' oligoadenylate synthase which activate RNaseL leading to non-specific RNA degradation (27). In addition, long dsRNA activate the protein kinase PKR, which phosporylates and inactivate the translation initiation factor eIF2a leading to inhibition of translation. Because dsRNA smaller than 30nt do not activate interferon responses and long dsRNA are cleaved to form 21-23nt siRNA, Tuschl and colleagues introduced chemically synthesized siRNA into mammalian cells to test whether siRNA could induce gene silencing without an interferon response (26). Introduction of synthetic siRNAs induced gene silencing effectively in a sequence-specific manner. This finding has led to the widespread use of the RNAi technology to determine unknown gene function and to degrade mRNA which causes disease in mammalian cells

Types of dsRNA inducing RNAi

Long dsRNA: Long dsRNA in cells can give rise to siRNAs through digestion of the dsRNA by Dicer. The siRNAs bind RISC and target homologous mRNA to induce effective gene silencing. Because various siRNAs which target the same mRNA are made from long dsRNA, defining an optimal sequence for siRNA is not necessary. Therefore, long dsRNA is widely used in plants, *C. elegans*, and *Drosophila*. However, long dsRNA can not be used in mammalian cells because of the interferon response.

Synthetic siRNA: Because of its convenience, chemically synthesized siRNA is widely used to silence genes. However, the synthetic siRNAs have some limitations. First, the silencing effect is transient. Although the duration of the silencing effect depends on the cell types, generally the silencing effect lasts only 4-5days in mammalian cells because of the lack of an amplification step. Therefore, if long term inhibition of gene expression in mammalian cells is necessary, synthetic siRNAs can not be used. Second, synthetic siRNA is not renewable. Third, some cell lines are not transfected efficiently, so synthetic siRNA can be used only with cell lines having a high transfection efficiency.

Short hairpin RNA (shRNA): To overcome the limitations of synthetic siRNA, plasmid or viral vectors expressing shRNA have been developed (28, 29, 30). These systems use the polIII promoter that produces short RNA species such as transfer RNA and 5s RNA which are not translated into protein. Two polIII promoters have been used predominantly, the U6 promoter and H1 promoter. Many researchers have shown that expression of 19-22nt shRNAs from U6 or H1 promoter is effective. It has been shown that the size of the loop is not the main parameter for efficient gene silencing since some several different sized loops have been efficient (31).

Brummolkamp et al., (32) designed a plasmid vector expressing shRNA against E-cadherin and p53 driven by the H1 promoter and coupled to a selective marker. Cell lines transfected stably with the plasmid showed that specific silencing for more than 2 month. Therefore, vectors expressing shRNA can be used for long-term gene silencing. In addition, these vector systems can be used in cell lines having low transfection efficiency because transfected cells can be selected by use of a reporter gene or selection marker.

Micro-RNA: The first micro-RNA was identified by Ambros and colleagues. They isolated a *lin4* mutant of *C. elegans* which was arrested at the first larval stage (33). Later on, the *let7* mutation was isolated in the same system, which was responsible for development through the fourth larval stage (34). Both *lin4* and *let7* encode 22nt RNAs and were called short temporal RNA because they control the development of *C. elegans* temporally. The mature lin4 RNA inhibited the mRNA expression of *lin14* and *lin28* genes and controls the fate of cells during the first three larval stages. Recent studies have revealed that the short temporal RNAs are members of micro-RNA.

In *D. melanogaster*, *C. elegans*, plants and humans, more than 600 micro-RNAs have been identified (25, 35, 36). An analysis of micro-RNA expression in cell lines and tissues suggests cell or tissue specific expression. For example, micro-RNA1 is specifically expressed in human heart and stage specifically in mouse embryogenesis (36). The regulated expression patterns of these micro-RNAs are suggestive of their functions in developmental control.

Since micro-RNAs are derived from their precursor dsRNAs and are similar in size to siRNAs, the generation of siRNA and micro-RNAs is similar. In fact, both siRNA and micro-RNAs are processed by Dicer activities in animals as well as in plants (37- 39).

Human recombinant Dicer can process pre-*let7* RNA to mature *let7* efficiently *in vitro* (40).

Delivery of siRNA

Transfection reagent: The most widely used method to deliver synthetic siRNA or plasmid expressing shRNA into cells is transfection using a lipid-based transfection reagent. This transfection reagent gives high transfection efficiency in some cell lines including HeLa cell (41)

Electroporation: Some researchers have used electroporation to deliver synthetic siRNA or plasmid producing shRNA to cells. However, many cells die after electroporation (42)

Viral vector: Although plasmid vector mediated shRNAs mediate effective silencing, it has the critical disadvantage of low transfection efficiency in many cells. Stable transfection of the plasmid vector could be one solution. However, this process requires a long time. To overcome this problem, several researchers developed new viral vector systems (43-47).

1) Retrovirus vector

Retrovirus vectors integrate transgenes into host genome very efficiently, which makes it possible for shRNAs to be constitutively expressed and silence the target mRNA for long times. Because of this property, several retroviral vectors expressing shRNA have been produced. For example, Paddison and Hannon (43) inserted U6 expression cassette containing template for shRNA against p53 into a MMLV-based vector and showed that infection of the vector silenced p53 stably.

2) Lentivirus vector

Lentivirus vectors have two advantages over retrovirus vectors. First, lentivirus can infect both dividing and non-dividing cells while retrovirus infect only dividing cells (44). Second, retrovirus undergoes proviral silencing during development (45). However, lentivirus vectors are resistant to this silencing. Several groups have used the lentivirus vector to deliver shRNA to primary cells, which are not easily transfected. Stewart et al. (31) transduced dendritic cells with a lentivirus vector to target endogenously expressed GFP, and showed significant reduction of the target gene expression. Mouse and human primary T cells also have been transduced with a lentivirus targeting CD25, or CCR5, HIV-1 co-receptor expression. Mouse primary T cells transduced with a lentivirus targeting CD25 showed significantly reduced proliferation in the presence of IL-2 (46). Targeting CCR5 in human peripheral T cells with lentivirus-derived shRNA showed a 10-fold reduced CCR5 expression and reduced infection when the cells were infected with CCR5-tropic HIV-1 (47).

3) Adenovirus

Adenovirus has also been used to deliver shRNA in vivo (48). The advantages of adenovirus vector are 1) high titers, 2) infection of dividing and non-dividing cells, and 3) infection of various species and cell types. However, adenovirus vectors induce immune responses and do not allow long-lasting expression of shRNA. Therefore, this delivery system is not suitable for therapeutic purposes but can be used for short-term inhibition for research purposes.

4) Adeno associated virus (AAV)

Some researchers have used AAV vector to express shRNA in cells because it has advantages (49) of infecting various cell types and both dividing and non-dividing cells. In addition, it maintains long-term expression and has low immunogenicity. AAV integrates at a preferred site, in human chromosome 19, but generally genomic insertion is rare. Therefore, insertional mutation is not a big problem. However, the disadvantages of AAV vector are that insert sizes are small and some patients may have pre-existing antibodies against AAV.

Applications of RNAi

RNAi and functional genomics

RNAi has evolved into a powerful tool for identification of unknown gene function. Specifically, the power of *C.elegans* and *Drosophila* genetics provides the opportunity to use siRNA or shRNA libraries to identify genes rapidly which are responsible for the specific phenotype. For example, Lee et al. (50) systemically inactivated 5690 genes in *C. elegans* using siRNA to identify those involved in its life span. They found that mutation of the leucyl-tRNA synthetase gene increased the life span. Recently several groups have reported the method to construct siRNA or shRNA libraries (51, 52, 53, 54). Large-scale screening using siRNA or shRNA libraries will make it easy to determine specific genes which are responsible for distinct phenotypes.

Disease therapy

After the demonstration that siRNAs can silence genes in mammalian cells without an interferon response, many studies have focused on disease treatment using RNAi technology. The most widely studied disease models are viral diseases, cancers, and

genetic diseases. Because of the potency and specificity of siRNA, it is very attractive for therapeutic purpose. However, there are important issues to be addressed before siRNA becomes useful for therapy. First, there is no effective method for delivery of the siRNA to the specific sites *in vivo*. Although some studies show efficient delivery *in vivo* (Table 2), delivering the RNAi to specific cells still limits its use in therapeutic applications in humans. Second, potential toxicity of siRNA are uncertain in humans. Despite these problems, many *in vitro* and some *in vivo* studies show potential for treatment of diseases

Targeting viral RNA with siRNA

siRNA has been shown to inhibit the production of a retrovirus, a negative-stranded RNA virus, and a positive-stranded RNA virus. Cells transfected with siRNA corresponding to the viral genome induced an obvious reduction in virus production, indicating that viral RNA may be targeted by RNA silencing machinery.

HIV-1

Treating HIV-infected patients with combinations of antiviral drugs is reasonably effective, but there are some problems concerning drug toxicity and the emergence of drug-resistant HIV-1 strains. Since RNAi was demonstrated to be active in mammalian cells and HIV-1 use RNA intermediates in their replication cycles, many researchers have tried to use RNAi technology to inhibit HIV-1 replication as an alternative strategy (Fig. 3). Recently, several studies have shown that siRNAs can inhibit HIV-1 replication and virus production *in vitro* (47, 55-63). These studies demonstrate that the targeting of early or late viral transcripts, such as gag, pol, env, nef, rev, vif, or tat, may inhibit protein

expression and viral replication. Although HIV-1 production and replication may be inhibited by RNAi, the question remains whether RNAi targets the incoming genomic RNA, the newly synthesized transcripts, or both. If the incoming genomic viral RNA can be targeted by RNAi, it will be helpful in preventing the cells from forming a provirus. Evidence has suggested that RNAi does indeed target the incoming genomic RNA for destruction (56, 58, 60). In these studies, the amount of the integrated provirus was found to be reduced when cells were pre-treated with siRNAs. However, other studies showed little or no reduction in integrated proviral DNA (55, 59, 62). It remains to be proved whether incoming genomic DNA can be targeted effectively. However, it is clear that de novo synthesized viral transcripts are degraded efficiently by siRNA. Tat is necessary for efficient viral transcription after the establishment of a provirus. Therefore, targeting tat mRNA reduces not only tat expression, but also the expression of other viral proteins. Targeting the viral RNA directly presents a potential problem for clinical application because of the high viral mutation rate. Mutational variants may escape being targeted. Therefore, some researchers have used siRNA against cellular transcripts to inhibit the infection of HIV-1. siRNA against CD4, which is the receptor for HIV-1, decreased HIV-1 infection. The treatment of siRNA against CCR5 and CXCR4 also showed a 48% and 68% reduction in CCR5 and CXCR4 expression, and inhibited the CCR5 tropic and CXCR4 tropic HIV-1 infections respectively (48,58, 64). Another host factor that is important for HIV-1 replication is the transcription factor NF-κB. Binding NF-κB to motifs in the LTR promoter of the integrated provirus is required for viral transcription. A five-fold reduction in HIV-1 production was measured by silencing the p65 subunit of NF-κB with siRNA (62). Although targeting the host co-factor has shown an effective

inhibition of HIV-1 production, the cellular co-factors have important roles in cellular functions such as transcription (NF-κB) and immuno-regulation (CD4). Therefore, targeting a co-factor with RNAi has negative effects. However, CCR5 is an exception. Individuals who are defective for CCR5 show a resistance to infection from HIV-1 and are healthy. Therefore, CCR5 siRNA may be a good candidate for the therapeutic intervention of HIV-1 infections.

Other RNA Viruses

HCV is a major cause of chronic hepatitis and hepatocellular carcinoma. HCV belongs to Flaviviridae with a positive single stranded RNA genome. Because there is no cell culture system for HCV replication, RNAi studies have used replicon in Huh-7 cells as a model for HCV replication. These replicons support HCV RNA transcription and protein synthesis, but do not produce infectious viruses. Synthetic siRNAs against NS3 and NS5B, non-structural proteins, resulted in inhibition of HCV replication in vitro (65-68). The internal ribosomal entry site (IRES) that is required for translation, has also been targeted by synthetic siRNA or shRNA and shown strong inhibition of the replication. (67-69).

The influenza A virus is a member of Orthomyxoviridae and a major cause of infection of the human respiratory tract. The genome of influenza A virus is encoded by eight segmented negative single stranded RNAs. It has been shown that siRNAs against conserved regions of the influenza genome could inhibit virus production in cell culture and embryonated chicken eggs (70). Among 20 siRNAs tested, those against nucleocapsid (NP) and RNA polymerase (PA) reduced the accumulation of all eight viral

RNAs. The NP and PA proteins play an important role in viral transcription and replication, so these siRNAs may be more effective than others.

For other RNA viruses, polio virus and respiratory syncytial virus (RSV), inhibition of the viral replications and viral protein production was also demonstrated. The siRNA against poliovirus capsid and RNA polymerase reduced viral titer in human cells (71), and the P and F proteins of RSV were reduced by targeting their mRNA using siRNA (72).

DNA Viruses

In contrast to RNA viruses, DNA viruses are less likely to escape from siRNA because of their lower mutation frequency, but siRNAs against DNA viruses can only target the viral mRNAs, and not the viral genomes. Nonetheless, efficient inhibition of DNA viruses with siRNAs has been shown (73-76). For example, HBV is a member of the Hepadnaviridae and its genome is a 3.2kb ds circular DNA. During infection, four RNAs are transcribed that encode the coat protein (CP), polymerase (P), surface antigen (S) and the transcription activator (X). HBV production in Huh-7 cells could be reduced up to 20-fold by shRNA against the X mRNA (73). HBV is the first virus to be inhibited by RNAi in vivo in mammals. When HBV and shRNA against HBV are co-delivered by hydrodynamic injection, significant repression of HBV antigen expression in liver was shown (74).

Recently, Bhuyan et al. have demonstrated that siRNA against Herpes Simplex Virus -1 (HSV-1) can target viral mRNA (76). They target glycoprotein E, which mediates cell-

to cell spread and immune evasion, and the transfection of the siRNA results in the phenotype of a gE-deletion mutant virus *in vitro*.

Another DNA virus Human papillomavirus (HPV) is an important therapeutic target because the constitutive expression of viral proteins E6 and E7 is required for carcinogenic growth. Jiang and Milner have shown that targeting HPV E6 and E7 with siRNA induces selective degradation of E6 and E7 mRNA in cells (77). Reduction of E6 with siRNA induces cell growth suppression and inhibition of E7 production causes apoptosis (77).

RNAi as an Antiviral Therapy

These initial studies of the effect of RNAi on viral replication in mammalian cells generated much hope for the use of RNAi as a novel antiviral therapy. However, there are some obstacles to hurdle in order to use RNAi technology as a useful therapeutic strategy. First, inhibition of viral replication using siRNA is largely based on transient transfection of synthetic siRNAs. These synthetic siRNAs induce only temporal inhibition. An approach for the long-term inhibition of viral replication has to be developed. shRNA transcribed from plasmid or viral vector might be one solution, although shRNAs have not yet been extensively employed.

A second problem is delivery of siRNA. So far no efficient delivery system has been developed, although some in vivo experiments show positive signs. At this point the viral vector seems to be the most reasonable approach. However, until an improved delivery system is developed, RNAi technology might be used as a genetic tool for the viral study.

Finally, as mentioned previously, viruses, especially, RNA viruses, are likely to evade siRNA by mutations of the target sequences. Treatment of multiple siRNAs or targeting a conserved sequence might be answers, but they have not yet been evaluated. An alternative approach is targeting host mRNA instead of viral RNA. As mentioned, CCR5 is a good candidate for inhibition of HIV-1 infection. There is another example showing prevention of virus-induced disease by silencing of host mRNA. HBV and HCV infection trigger Fas –mediated apoptosis of hepatocytes. Song et al. showed that hydrodynamic injection of siRNA against fas could block fulminant hepatitis induced by a fas-specific antibody in mice (78). Hydrodynamically-injected siRNA against fas delivered to the liver and showed that 82% of the treated mice survived for 10 days while control mice died within 3 days (78). Hydrodynamic tail vein injection delivered siRNA or shRNA mainly to the liver. Thus, this delivery method might be ideal to silence gene expression in hepatocytes in mice. However, it has not been shown that this injection is effective in humans.

Although many reports showing inhibition of viral replication with siRNA have been published, therapeutical approach of siRNA is still limited by many problems concerning the delivery, toxicity and viral escape. However, RNAi technology will elucidate the viral gene function, and help to understand the interactions between viruses and their hosts.

Cancer

Tumor cells can result from uncontrolled cell growth mediated by oncogenic genes or by resistance to cell death (apoptosis) by dysfunction of pro-apoptotic and/or anti-apoptotic molecules. Therefore, RNAi technology could be applied to inhibit oncogene or

anti-apoptotic gene expression for cancer therapy. Cioca et al., (79) demonstrated targeting oncogene c-raf and anti-apoptotic gene, bcl-2 in a myeloid leukemia cell line decreased the protein expression level and induced apoptosis in the cell line. Other targets for cancer therapy include pro angiogenic molecules because tumor cells require blood supplies. Co-treatment of experimental tumors with chemotherapy and siRNA against vascular endothelial growth factor (VEGF) showed promise for control of tumors (80).

Dominant genetic diseases

Another field in which siRNAs are being tested for therapeutic purposes is dominant genetic diseases. Genetically dominant diseases result from mutation of one copy of the gene even though the other copy of the gene is normal. Some *in vitro* studies have shown that dominant genetic diseases might be treated by degradation of only the mutant transcript targeted with siRNA (81, 82). For example, Only mutant transcripts of the *sod* gene in amyotropic lateral sclerosis, *tau* gene in frontotemporal dementia, and *app* gene in familial Alzheimer's disease were degraded *in vitro* (81-83). Therefore, dominant genetic diseases caused by one or a few common mutations are good candidates for clinical application of RNAi technology

Why siRNA?

siRNA and antisense oligonucleotides share some features. They are nucleotides, require delivery systems and induce post-transcriptional gene silencing. However, there are also important differences between them. siRNAs are more potent and efficient for the inhibition of target gene expression than antisense oligonucleotide (84,85). Although there is a report claiming that siRNA and antisense oligonucletide have similar potency

(86), many researchers believe siRNA is more potent and effective because siRNA is naturally occurring as a form of microRNA and uses cellular machinery, while antisense oligonucleotide does not exist naturally. Other advantage of siRNAs over antisense oligonucleotide is that siRNA is highly effective without any chemical modifications, while antisense oligonucleotide is not stable without chemical modification (87). This lack of requirement of chemical modification of siRNA reduces toxicity of siRNA compared to antisense oligonucleotides (88). In addition, the duration of gene silencing of endogenous targets with siRNA is longer than that with chemically modified antisense oligonucleotides (88). Therefore, longer inhibition, low toxicity and strong potency make siRNAs better molecules to effect gene silencing. Another RNA molecule, ribozyme, has been tried to inhibit gene expression, but its potency remains in question and there is no sign of success as a potential therapeutics. Therefore, it appears that siRNA is a better molecule to inhibit gene expression *in vivo* as well as *in vitro*.

RNAi *in vivo*

Although there are many articles claiming successful gene silencing with siRNA in mammalian cell cultures, only limited reports in which siRNA is used *in vivo* have been published because of lack of suitable delivery systems. The first paper where siRNA was used in vivo was published in 2002. In the paper, Kay and colleagues co-injected NS5B-luciferase fusion gene with NS5B siRNA and then showed reduced luciferase expression using bioluminescence imaging (89). Although they targeted exogenous gene by co-injection with corresponding siRNA, this was the first result demonstrating that siRNA can induce RNAi *in vivo*. Several months later, Xia et al. demonstrated inhibition of

endogenous gene expression with siRNA (90). They inhibited β-glucuronidase production in the liver using adenovirus expressing shRNA. Many *in vivo* experiments targeted liver cells because a delivery system is available. Tail vain injection with high pressure, which is called hydrodynamic injection, delivers nucleic acid mainly to liver cell. Fas, Caspase8, HCV, and HBV are targeted at the liver (Table 2). The brain is also targeted by a few researchers. They delivered siRNA or shRNA into the brain by direct local injection. AGRP, tyrosine hydrocyclase, and ataxin-1 are targeted in the brain (91-93). Solid tumor cells are also easily accessible, so mutated p53, vascular endothelial growth factor (VEGF), and endothelial growth factor receptor (EGFR) are targeted in order to reduce tumor mass *in vivo* (80, 94, 95). Transforming Growth Factor-β (TGF-β), CRX, and NRN, and VEGF are reduced in eyes by local injection (96-98). We summarized *in vivo* experiments using siRNA or shRNA in Table 2. As shown in the table, many *in vivo* experiments using systemic injection targeted the liver or other particular tissues, such as the brain, eyes, and tumors are targeted because local injection of siRNA or shRNA was possible. Because of the limited delivery system, application of RNAi technology into mice has not reached its potential yet. However, RNAi does have great potential if the development and improvement of delivery systems for RNAi technology is achieved.

Specific Aims and Rationale

Pathogenesis of herpetic stromal keratitis (HSK) involves many molecules and a complex mechanism (99). However, it has been shown that angiogenesis is a critical step in the in development of HSK after herpes simplex virus-1 (HSV-1) infection (100).

Treatment of neutralizing Ab against VEGF reduced angiogenesis and HSK severity caused by HSV-1 infection (100). In addition, it has been demonstrated that inhibition of IL-1 signaling, which is induced by HSV-1 infection, reduced VEGF production, angiogenesis, and HSK severity (101). Therefore, it has been proposed that inhibition of angiogenesis is a valuable approach to control HSK.

Recently, it has been shown that introduction of siRNA or shRNA into mammalian cells inhibits target gene expression, which is called RNAi. Although the RNAi technology has been successful *in vitro*, only limited in vivo experiments were published because of delivery problems. Although most in vivo experiments target the liver, RNAi technology was also successfully used in mouse eyes (96-98).

Therefore, in this study, we investigated the potential value of RNAi technology for inhibition of angiogenesis-related gene expression in mouse eyes using intrastromal injection. Treatment of plasmid expressing shRNA in vitro was performed to determine whether shRNA can inhibit exogenous and endogenous target genes. More importantly, it was also demonstrated that intrastromal injection of plasmid DNA expressing shRNA against VEGF or IL-1 receptor type I can reduce angiogenesis induced by CpG motif and IL-1α treatment respectively. These studies demonstrating application of RNAi in mouse eyes is a valuable approach in the study of corneal biology and in the treatment of corneal diseases.

LIST OF REFERENCES

List of references

1. Jorgensen, R. Altered gene expression in plants due to trans interaction between homologous genes. Trends Biotechnol. 8, 340-344 (1990).
2. Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806-811 (1998).
3. Hamilton, A.J. and Baulcombe, D.C. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286, 950-952 (1999).
4. Zamore, P.D., Tuschl, T., Sharp, P.A.and Bartel D.P. RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 101, 25-33 (2000).
5. Hammond, S.M., Caudy, A.A. and Hannon, G,J. Post-transcriptional gene silencing by Double-stranded RNA. Nature Rev Gen 2, 110-119 (2001).
6. Sharp P.A. RNA interference 2001. Genes Dev 15, 485-490 (2001).
7. Hutvagner, G. and Zamore, P.D. RNAi: nature abhors a double-sstrand. Curr Opin Genetics & Development 12, 225-232 (2002).
8. Elbashir, S.M., Lendeckel, W., and Tuschl, T. RNA interference is mediated by 21-and 22- nucleotide RNAs. Genes Dev. 15, 188-200 (2001).
9. Nykanen, A., Haley, B., and Zamore, P.D. ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell 107, 309-321 (2001).

10. Jones, L., Ratcliff, F., and Baulcombe, D.C. RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. Curr. Biol. 11, 747-757 (2001).

11. Vaistij, F.E., Jones, L, and Baulcombe, D.C. Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. Plant Cell 14, 857-867 (2002).

12. Aufsatz, W., Mette, M.F., Van der Winden, J., Matzke, A.J., and Matzke, M. RNA-directed DNA methylation in Arabidopsis. Proc. Natl. Acad. Sci. USA 99, 16499-16506 (2002).

13. Matzke, M., Matzke, A.J., and Kooter, J.M. RNA: guiding gene silencing. Science 293, 1080-1083 (2001).

14. Hannon, G.J. RNA interference. Nature 418, 244-251 (2001).

15. Morris, K.V., Chan, S. W-L., Jacobsen, S.E. and Looney, D.J. Samll interfering RNA-induced transcriptional gene silencing in human cells. Science 305, 1289-1292 (2004).

16. Dudley, N.R., Labbe, J.C., and Goldstein, B. With RNA interference to identify genes required for RNA interference. Proc. Natl. Acad. Sci. USA 99, 4191-4196 (2002).

17. Korf, I., Fan, Y., and Strome, S. The Polycome group in *Caenorhabditis* elegans and maternal control of gerline development. Development 125, 2468-2478 (1998).

18. Hall, I.M., Shankaramarayana, G.D., Noma, K.-I., Ayoub, N., Choen, A., and Grewal, S.L. Establishment and maintenance of a heterochromatin domain. Science 297, 2232-2237 (2002).

19. Mourrain, P., Ceclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J-B., Jouette, D., Lacombe, A-M., Nikic, S., Picault, N., Remoue, K., Sanial, M., Vo, T-A. and Vaucheret, H. *Arabidopsis SGS2* and *SGS3* genes are required for posttranscriptional gene silencing and natral virus resistance. Cell 101, 533-542 (2000).

20. Voinnet, O., Lederer, C. and Baulcombe, D.C. A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana*. Cell 103, 157-167 (2000).

21. Tabara, H. eta l. The rde-1 gene, RNA interference, and transposon silencing in *C.elegans*. Cell 99, 123-132 (1999).

22. Ketting, R.F., Haverkamp, T.H., van Luenen, H.G. and Plasterk, R.H. *mut-7* of *C. elegans*, required for transposon silencing and RNA interference, in a homolog of Werner syndrome helicase and RNaseD. Cell 99, 133-141 (1999).

23. Knight, S. W., and Bass, B.L. A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in C. elegans Science 293, 2269-2271 (2001).

24. Smardon, A., Spoerke, J.M., Stacey, S.C., Klein, M.E., Mackin, N., and Maine, E.M. EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans* Curr. Biol. 10, 169-178 (2000).

25. He, L. and Hannon, G.J. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet. 5, 522-31 (2004).

26. Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Klaus, W. and Tuschl, T. Duplexed of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494-498 (2001).

27. Minks, M.A., West D.K., Benvin S. and Baglioni, C. Structureal requirements of double-stranded RNA for the activation of 2'-5'-oligo (A) polymerase and protein kinase of interferon-treated HeLa cells. J. Biol. Chem. 254, 10180-10183 (1979).

28. Paddison, P.J., Caudy, A.A., and Hannon, G.J. Stable suppression of gene expression by RNAi in mammalian cells. Proc. Naatl. Acad. Sci. USA 99, 1443-1448 (2002)

29. Sui, G., Soohoo, C., Affar, E_B., Gay, F., Shi, Y., Forrester, W.C., and Shi, Y. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc. Natl. Acad. Sci. USA 99, 5515-5520 (2002)

30. Stewart, S.A. Dykxhoom, D.M., Palliser, D., Mizuno, H. Yu, E.Y., An, D.S., Sabatini, D.M., Chen, I.S., Hahn, W.C., Sharp, P.A., Weinberg, R.A and Novina, C.D. Lentivirus-delivered stable gene silencing by RNAi in primary cells. RNA 9, 493-501 (2003).

31. Dykxhoorn, D.M., Novina, C.D. and Sharp, P.A. Killing the messenger: short RNAs that silence gene expression. Nat Rev Mol Cell Biol. 4, 457-467 (2003).

32. Brummelkamp, T.R., Bernards, R. and Agami, R. A system for stable expression of short interfering RNA in mammalian cells. Science 296, 550-553 (2002)

33. Lee, R.C., Feinbaum, R.L., and Abvros, V. The C. elegans geterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. Cell 75, 843-854 (1993)

34. Beinhart, B.J. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditiss elegans. Nature, 403, 901-906 (2000).

35. Lau, N.C., Lim, L.P., Weinstein, E.G., and Bartel, D.P. An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans* Science 294, 858-862 (2001).

36. Lee, R.C., and Ambros, V. An extensive class of small RNAs in *Caenorhabditis elegans*. Science 294, 862-864 (2001).

37. Grishok, A., Pasquinnelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D.L., Fire, A., Ruvkun, G., Mello, C.C. Genes and mechanisms related to RNA interference regulate expressioin of the small temporal RNAs that control C.elegans developmental timing Cell, 106, 23-24 (2001).

38. Hutvagner, G., McLachlan, J., Pasquinelli, A.E., Balint, E., Tuschl, T., Zamore, P.D. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science 293, 834-838 (2001).

39. Ketting, R.F., Fischer, S.E., Bernstein, E., Sijen, T., Hannon, G.J., and Plasterk, R.H.A. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev. 15, 2654-2659 (2001).

40. Provost, P., Dishart, D., Doucet, J., Frendewey, D., Samuelsson, B., and Radmark, O. Ribonuclease activity and RNA binding of recombinant human Dicer. EMBO J. 21, 5864-5874 (2002).

41. McManus, M.T., Sharp, P.A. Gene silencing in mammals by small interfering RNAs. Nat Rev Genet 3, 737-747 (2002).

42. McManus, M.T., Haines, B.B., Dillon, C.P., Whitehurst, C.E., van Parijs L, Chen, J. and Sharp PA. Small interfering RNA-mediated gene silencing in T lymphocytes. J Immunol.169, 5754-5760 (2002).

43. Paddison, P.J. and Hannon, G.J. RNA interference: the new somatic cell genetics? Cancer Cell 2 17-23 (2002)

44. Naldini, L., U. Blomer, P. Gallay, D. Ory, R. Mulligan, F. H. Gage, I. M. Verma, and D. Trono. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272, 263-267 (1996)

45. Svoboda, J., Heinar,, J., Geryk, J., Elleder, D., and Vernerova, Z. Retroviruses in foreign species and the problem of provirus silencing. Gene 261, 181-188 (2000).

46. Rubinson, D.A. et al. A lentivirus-based system to silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nature Genet. 33, 401-406 (2003).

47. Qin, X.F., An, D.S., Chen, I.S. and Baltimore, D. 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).

48. Bain, J.R., Schisler, J.C., Takeuchi, K., Newgard, C.B., Becker, T.C. An adenovirus vector for efficient RNA interference-mediated suppression of target

genes in insulinoma cells and pancreatic is of langerhans. Diabetes 53, 2190-2194 (2004).

49. Tomar, R.S., Matta, H., and Chaudhary, P.M. Use of adeno-associated viral vector for delivery of small interfering RNA Oncogene 22, 5712-5715 (2003)
50. Lee S.S., Lee, R.Y., Fraser A.G., Kamath R.S., Ahringer J., and Ruvkun, G. A systemic RNAi screen identifies a critical role for mitochondria in C.elegans longevity. Nat. Genet 33, 40-48 (2002)
51. Zheng, L., Liu, J., Batalov, S., Zhou, D., Orth, A., Ding, S. and Schultz, P.G. An approach to genomewide screens of expressed small interfering RNAs in mammalian cells Proc. Natl. Acad Sci. USA 101, 135-140 (2004)
52. Luo, B., Heard, A.D. and Lodish, H.F. Small interfering RNA production by enzymatic engineering of DNA (SPEED). Proc. Natl. Acad. Sci. USA 10, 5494-5499 (2004)
53. Shirane, D. Sugao, K., Namiki, S., Tanabe, M., Iino, M., Hirose, K. Enzymatic production of RNAi libraries form cDNAs. Nature Genet. 36, 190-196 (2004)
54. Sen, G., Wehrman, T.S., Myers, J.W., and Blau, H.M. Restriction exzyme-generated siRNA (REGS) vectors and libraries. Nature Genet. 36, 183-189 (2004).
55. Coburn, G., and Cullen, B.R. Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. J. Viol. 76, 9225-9231 (2002).
56. Capodici, J., Kariko, K., and Weissman, D. Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. J. Immunol. 169, 5196-5201 (2002).

57. Lee, N.S., Dohjima, T., Bauer, G., Li, H., Li, M.J., Ehsani, A., Salvaterra, P., Rossi, J. Expresion of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. Nature Biotechnol. 20, 500-505 (2002).

58. Novina, C.D., Murray, M.F., Dykxhoom, D.M., Bereeford, P.J., Riess J., Lee, S.K., Collman, R.G., Lieberman, J., Shankar, P. and Sharp, P.A. siRNA-directed inhibition of HIV-1 infection. Nature Med. 8, 681-686 (2002).

59. Hu, W.Y., Myers, C.P., Kilzer, J.M., Pfaff, S.L., Bushman, F.D. 2002. Inhibition of retroviral pathogenesis by RNA interference. Curr Biol. 12, 1301-1311.

60. Jacque, J.M., Triques, K., Steverson, M. 2002. Modulation of HIV-1 replication by RNA interference. Nature 418, 435-438.

61. Park, W.S., Hayafune, M., Miyano-Kurosaki, N. and Takaku, H Specific HIV-1 env gene silencing by small interfering RNAs in human peripheral blood mononuclear cells. Gene Ther. 10, 2046-2050 (2003)

62. Surabhi, R.M., Gaynor, R.B. RNA interference directed against viral and cellular targets inhibits human immunodeficiency virus type 1 replication. J. Viol. 76, 12963-12973 (2002).

63. Yamamoto, T., Omoto, S., Mizuguchi, M. Mizukami, H., Okuyama, H., Okada, N., Saksena, N.K., Brisibe E.A., Otake, K., Fuji, Y.R. Double-stranded nef RNA interferes with human immunodeficiency virus type 1 replication. Microbiol Immunol. 46, 809-817 (2002).

64. Martinez, M.A., Gutierrez, A., Armand-Ugon, M., Blanco, J., Parera, M., Gomez, J., Clotet, B., Este, J.A. Suppression of chemokine receptor expression by RNA

interference allows for inhibition of HIV-1 replication. AIDS 16, 2385-2390 (2002).

65. Kapadia, S.B., Brideau-Andersen, A., Chisari F.V. Interference of hepatitis C virus RNA replication by short interfering RNAs Proc. Natl. Acad. Sci USA 100 2014-2018. (2003)

66. Randall, G., Grakoui, A., Rice, C.M. Clearance of replication hepatitis C virus replicaon RNAs in cell culture by small interfering RNAs. Proc. Natl. Acad. Sci USA 100, 235-240.(2003)

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

68. Wilson, J.A., Jayasena, S., Khvorova, A., Sabatinos, S., Rodrigue-Gervais, I.J., Arya, S., Sarangi, F., Harris-Brandts, M., Beaulieu, S. and Richardson C.D. RNA interference blocks gene expression and RNA synthesis from hepatitis C replicons propagated in human liver cells Proc. Natl. Acad Sci. USA 100, 2783-2788 (2003)

69. Yokota T., Sakamotot, N., Enomoto, N., Tanabe, Y., Miyagishi, M., Maekawa, S., Yi, L., Kurosai, M., Taira, K., Watanabe, M., Mizusawa, H. Inhibition of intracellular hepatitis C virus replication by intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. EMBO Rep 4, 1-7 (2003)

70. Ge, Q., McManus, M.T., Nguyen, T., Shen, C.H., Sharp, P.A., Eisen, H.N., Chen, J. RNA interference of influenza virus production by directly targeting mRNA for

degradation and indirectly inhibiting all viral RNA transcription. Proc. Natl. Acad. Sci USA 100, 2718-2723 (2003)

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

72. Bitko, V., and Bank, S. Phenotypic silencing of cytoplasmic genes using sequence-specific double-stranded short interfering RNA and its application in the reverse genetics of wild type negative-strand RNA viruses BMC Microbiol. 1, 34 (2001)

73. Shlomai, A., Shaul, Y. Inhibition of hepatitis B virus expression and replication by RNA interference. Hepatology 37, 764-770 (2003)

74. Hamasaki, K., Nakao, K., Matsumoto, K., Ichikawa, T., Ishikawa, H., Eguchi, K., Short interfering RNA-directed inhibition of hepatitis B virus replication. FEBB Lett 543, 51-54.(2003)

75. McCaffrey, A.P., Nakai, H., Pandey, K., Huang, Z., Salazar, F.H., Xu, H., Wieland, S.F., Marion, P.L., Kay, M.A. Inhibiton of hepatitis B virus in mice by RNA interference. Nature Biotechnol. 6, 636-644 (2003)

76. Bhuyan PK, Kariko K, Capodici J, Lubinski J, Hook LM, Friedman HM, Weissman D. Short interfering RNA-mediated inhibition of herpes simplex virus type 1 gene expression and function during infection of human keratinocytes. J Virol. 78, 10276-81 (2004).

77. Jiang, M. and Milner, J. Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treated with siRNA, a primer of RNA interference. Oncogene 21, 6041-6048 (2002).

78. Song, E., Lee, S.K., Wang, J., Ince, N., Ouyang, N., Min, J., Chen, J., Shankar, P., Lieberman, J. RNA interference targeting Fas protects mice form fulminant hepatitis Nature Med 9 347-351 (2003)

79. Cioca, D.P., Aoki, Y. and Kiyosawa, K. RNA interference is a functional pathway with therapeutic potential in human myeloid leukemia cell lines. Cancer Gene Ther. 10, 125-133

80. Filleur, S., Courtin, A., Ait-Si-Ali, S., Guglielmi, J., Merle, C., Herel-Bellan, A., Clezardin, P. and Florence, C. siRNA-mediated inhibition of vascular endothelial growth factor severely limits tumor resistance to antiangiogenic thrombospondin-1 and slows tumor vascularization and growth. Cancer Res 63, 3919-3922 (2003).

81. Miller, V.M., Gouvion, C.M., Davidson, B.L., and Paulson, H.L. Targeting Alzheimer's disease genes with RNA interference: an efficient strategy for silencing mutant alleles. Nucleic Acids Res. 32. 661-668 (2004).

82. Miller, V.M., Xia, H., Marrs, G.L., Gouvion, C.M., Lee, G., Davidson, B.L.and Paulson, H.L. Allele-specific silencing of dominant disease genes Proc. Natl. Acad. Sci. USA 100 7195-7200 (2003).

83. Ding H, Schwarz, D.S. Keene A., Affar, el B., Fenton, L., Xia, X., Shi, Y., Zamore, P.D. and Xu, Z. Selective silencing by RNAi of a dominant allele that causes amyotrophic lateral sclerosis. Aging Cell 2, 209-217 (2003).

84. Zamore, P.D., Tuschl, T., Sharp, P.A., and Bartel, D.P. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 101, 25-33. (2000).

85. Caplen, N.J., Parish, S., Imani, F., Fire, A., and Morgan, R.A. Specific inhibition of gene expression by small dougle-stranded RNAs in invertebrate and vertebrate systems Proc. Natl. Acad. Sci. 98, 9742-9747 (2001).

86. Vickers, T.A., Koo, S., Bennett, C.F., Crooke, S.T., Dean, N.M., Baker, B.F. Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents. A comparative analysis. J. Biol. Chem. 278, 7108-7118 (2003).

87. Braasch, D.A., Jensen, S., Liu, Y., Kaur, K., Arar, K., White, M.A., Corey, D.R. RNA interference in mammalian cells by chemically-modified RNA. Biochemistry 42, 7967-7975. (2003).

88. Hough, S.R., Wiederholt, K.A., Burrier, A.C., Woolf, T.M., and Taylor, M.F. Why RNAi makes sense Nature biotech. 21, 731-732 (2003).

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

90. Xia, H., Mao, Q., Paulson, H.L., and Davidson, B.L. siRNA-mediated gene silencing in vitro and in vivo. Nat biotechnol 20, 1006-1010. (2002).

91. Makumura, H., Muzuno, T.M., Mastaitis, J.W., Agami, T., and Mobbs, C.V. Reducing hypothalamic AGRP by RNA interference increase metabolic rate and decreases body weight without influencing food intake. BMC Neurosci 3, 18 (2002).

92. Xia H, Mao Q, Eliason SL, Harper SQ, Martins IH, Orr HT, Paulson HL, Yang L, Kotin RM, Davidson BL. RNAi suppresses polyglutamine-induced

neurodegeneration in a model of spinocerebellar ataxia. Nat Med.10,816-20 (2004).

93. Hommel, J.D., Sears, R.M., Georgescu, D., Simmons, D.L., and DiLeone, R.J. Local gene knockdown in the brain using viral-mediated RNA interference. Nat Med. 9, 1539-1544 (2004).

94. Zhang, Y., Zang, Y.F., Bryant, J., Charles, A., Boado, R.J. and Pardridge, W.M. Intravenous RNA interference gene therapy targeting the human epidermal growth factor receptor prolongs survival in intracranial brain cancer. Clin. Cancer Res. 10, 3667-3677 (2004).

95. Hemann, M.T., Fridman, J.S., Zilfou, J.T., Hernando, E., Paddison, P.J., Cordon-Cardo, C., Hannon, G.J., and Lowe, S.W. An epi-allelic series of p53 hypomorphs created by stable RNAi produces distinct tumor phenotypes in vivo. Nat Genet 33, 396-400 (2003).

96. Nakamura, H., Siddiqui, S.S., Shen, X., Malik, A.B., Pulido, J.S., Kumar, N.M., Yue, Y.J.T. RNA interference targeting transforming growth factor-β type II receptor suppresses ocular inflammation and fibrosis. Mol Vis. 10, 703-710 (2004).

97. Matsuda, T. and Cepko, C. Eletroporation and RNA interference in the rodent retinal in vivo and in vitro. Proc. Natl. Acad. Sci. USA. 101, 16-22 (2004).

98. Riech, S.J., Fosnot, J., Kuroki, A., Tang, W., Yang, S., Maguire, A.M., Bennett, J., and Tolentino, M.J. Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. Mol Vis 9, 210-216 (2003).

99. Thomas, J.T. and Rouse, B.T. Immunopathogenesis of herpetic ocular disease.

Imm. Research. 16/4, 375-386 (1997).

100. Zheng, M., Deshpande, S., Lee, S., Ferrara, F., and Rouse, BT. Contribution of vascular endothelial growth factor in the neovascularization process during the pathogenesis of herpetic stromal keratitis. J Viol. 75, 9828-9835 (2001).

101. Biswas, PS., Banerjee, K., Kim, B., and Rouse, BT. Mice transgenic for IL-1 receptor antagonist protein are resistant to herpetic stromal keratitis: possible role for IL-1 in herpetic stromal keratitis pathogenesis. J. Immunol. 172, 3736-3744 (2004).

102. Zender, L., Hutker, S., Liedtke, C., Tillmann, H.L., Zender, S., Mundt, B., Waltemathe, M., Gosling, T., Flemming, P., Malek, N.P., Trautwein, C., Manns, M.P., Kuhnel, F., and Kubicka, S. Caspase 8 small interfering RNA prevents acute liver failure in mice. Proc. Natl. Acad. Sci. USA 100, 7797-7802 (2003)

103. Dorn, G., Patel, S., Wotherspoon, G., Hemmings-Miszczak, M., Barclay, J., Natt, F.J.C., Bevan, S., Fox, A., Ganjy, P., Wishart, W., and Hall, J. siRNA relieves chronic neuropathic pain Nucleic Acid Res 32, e49 (2004)

104. Kong, X.C., Barzaghi, P. and Ruegg, M.A. Inhibition of synapse assembly in mammalian muscle in vivo by RNA interference. EMBO Rep 5, 183-188 (2004)

105. Tompkins, S.M., Lo, C-Y., Tumpey, T.M., and Epstein, S. Protection against lethal influenza virus challenge by RNA interference in vivo. Proc. Natl. Acad. Sci. 101, 8682-8686. (2004)

106. Lewis, D.L., Hgstrom, J.E., Loomis, A.G., Wolff, J.A.and Herweijer, H. Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. Nat Genet 32, 107-108. (2002)

107. Sorensen, D.R., Leirdl, M. and Sioud, M. Gene silencing by systemic delivery of synthetic siRNAs in adult mice. J. Mol. Biol. 327, 761-766. (2003).

108. Gildi, H., Ketzinel-Gilad, M., Rivkin, L., Felig, Y., Nussbaum, O. and Galun, E. Small interfering RNA inhibits hepatitis B virus replication in mice. Mol Ther. 8, 769-776. (2003).

109. McCaffrey, A.P., Nakai, H., Pndey, K., Huang, Z., Salzar, F.H., Xu, H., Wielnd, S.F., Marion, P.L. and Kay, M.A. Inhibition of hepatitis B virus in mice by RNA interference. Nat bitechnol. 21, 639-644. (2003).

110. Zhng, Y., Boado, R.J. and Ruegg, M.A. Inhibition of synapse assembly in mammalian muscle in vivo by RNA interference. EMBO Rep 5, 183-188 (2004)

APPENDIX

Fig. 1 A model for the mechanism of RNAi.

Dicer enzyme processes the introduced long dsRNA into short interfering RNA (siRNA). The siRNAs bind RISC and the complexes become activated by unwinding of the siRNA. Such complexes degrade target mRNA.

This figure is adapted from Nature 418, 244-251(2002)

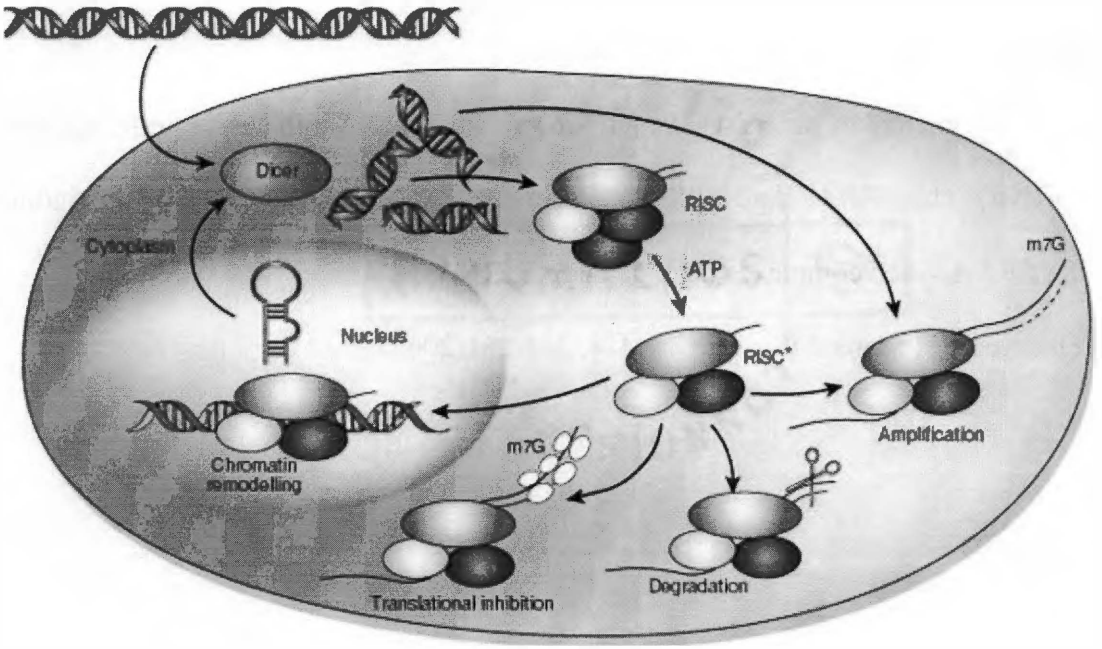
Dicer
Cytoplasm
RISC
ATP
m7G
Nucleus
RISC*
Amplification
Chromatin remodelling
m7G
Translational inhibition
Degradation

Fig. 2 A role of the RNAi process in heterochromatinization in nuclear DNA.

dsRNA could inhibit gene expression by methylation of target DNA or histone protein.

This mechanism is studied mainly in plant and *C. elegans*.

This figure is adapted from Nature 430, 161-164 (2004)

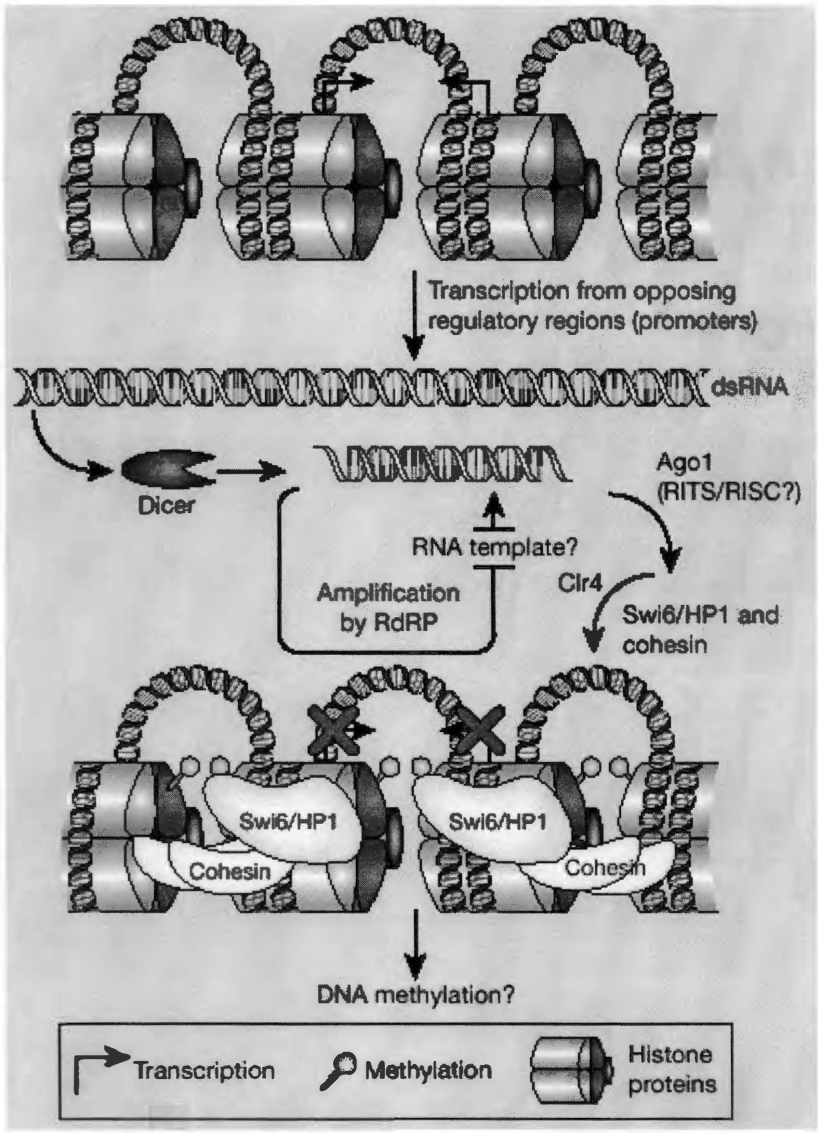
Transcription from opposing regulatory regions (promoters)
dsRNA
Dicer
Ago1 (RITS/RISC?)
RNA template?
Amplification by RdRP
Clr4
Swi6/HP1 and cohesin
Swi6/HP1
Cohesin
Swi6/HP1
Cohesin
DNA methylation?
Transcription
Methylation
Histone proteins

Fig. 3 RNA interference susceptible targets in the HIV-1 replication cycle.

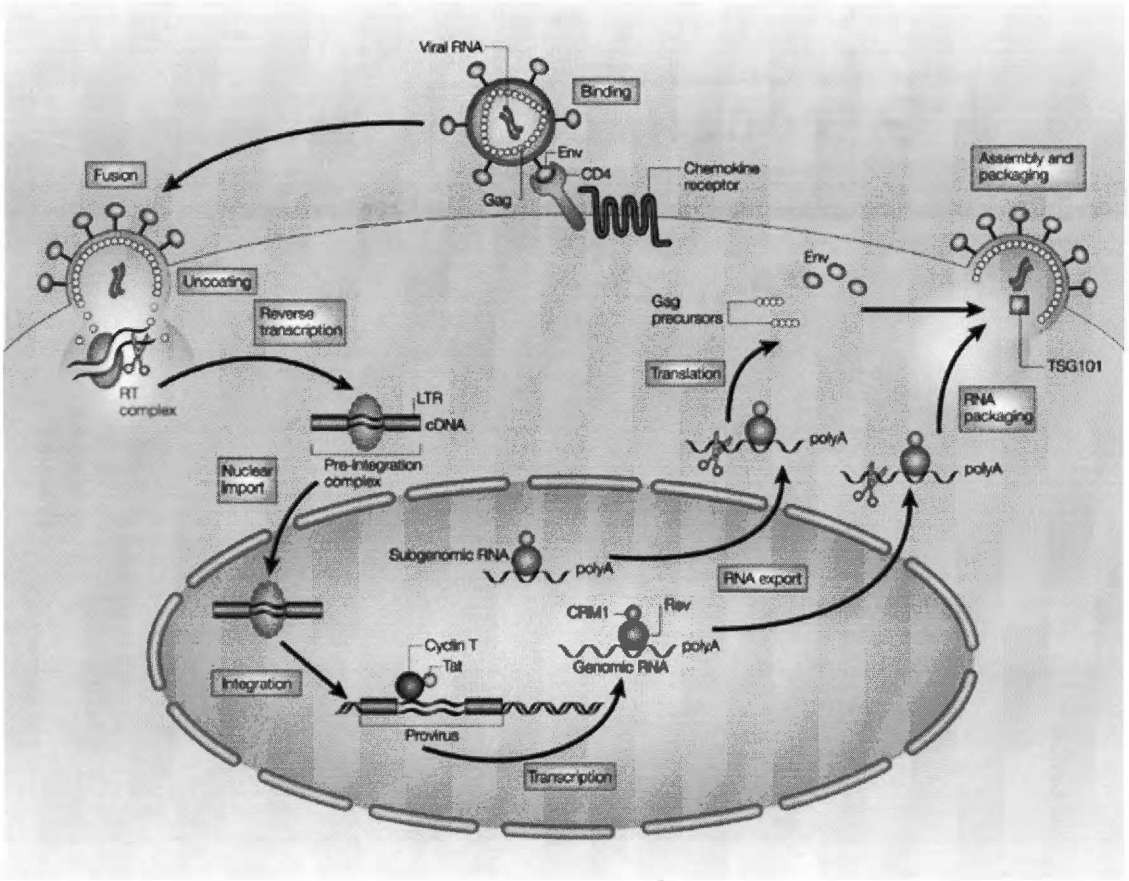

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Table1 Inhibition of viral replication by RNAi.

Virus	Target gene	Type of dsRNA	Cell type	Ref
HIV	tat, rev	plasmid based siRNA	293/EcR	57
HIV	LTR, vif, nef	siRNA	Macrophage, PBLs	60
HIV	gag, CD4	siRNA	Macrophage, HeLa	58
HIV	gag, pol	siRNA	T cell line	59
HIV	gag, LTR	siRNA	U87	56
HIV	tat, rev	siRNA	Jurkat, HPBLs	55
HIV	env	siRNA	PBMC	61
HIV	CXCR4,CCR5	siRNA	U87	64
HIV	tat, RT, NF-κB	siRNA	Macrophage	62
HIV	nef	long dsRNA	U937, MT-4	63
HIV	CCR5	lentiviral shRNA	Macrophage	47
HCV	NS3, NS5B	siRNA	Huh-7	65
HCV	capsid, NS4B	siRNA	Huh-7.5	66
HCV	5'UTR	siRNA	Huh-7	67
HCV	5'UTR, NS3, NS5B	siRNA Plasmid based siRNA	Huh-7	68
HCV	5'UTR	siRNA Plasmid based siRNA	Huh-7	69
Polio virus	capsid, polymerase	siRNA	HeLa S3, Mouse embryonic fibroblast	71
Influenza A virus	PB1, PB2, PA, NP, M, NS	siRNA	MDCK, chicken empryos	70
RSV	P and F proteins	siRNA	A549	72
HBV	X, core	retroviral shRNA	Huh-7	73
HBV	core, S, pol.,X	Plasmid based shRNA	Huh-7, mice	74
HBV	core	siRNA	Huh-7	75
HPV	E6, E7	siRNA	CASKi, siHa	77
HSV-1	gE	siRNA	keratinocytes	76

Table 2 Summary of in vivo RNAi experiments

Target gene	Target tissue	Type of dsRNA	Delivery	Ref
EGFP, Luciferase Placental alkaline phosphatase	liver, spleen, kidney lung, pancreas	siRNA	systemic injection.	106
AGRP	hypothalamus	plasmid based shRNA siRNA	local injection	91
NS5B	mainly liver	plasmid base shRNA siRNA	systemic injection.	89
β-glucuronidase	liver	adenoviral shRNA	systemic injection	90
trp53	hematopoietic stem cells	retroviral shRNA	*in vitro* transfection and reconstitution	95
fas	liver	siRNA	systemic injection	78
TNF-α	peritoneal cells	siRNA with DOTAP	ip injection	107
VEGF	retina	adenoviral shRNA	local injection	98
Caspase-8	liver	siRNA	systemic injection	102
VEGF	tumor	siRNA	local injection	80
S gene of HBV	liver	siRNA	systemic injection with pHBV	108
S gene of HBV	liver	plasmid based shRNA	systemic injection	109
Luciferase	implanted brain tumor	plasmid based shRNA with pegylated immuno-liposome	systemic injection	110
$P2X_3$	spinal cord	siRNA	local injection	103
Musk, rapsyn	muscle	siRNA	local injection	104
TGF-β	subconjuntival	siRNA	local injection	96
EGFR	brain tumor	plasmid based shRNA with pegylated immuno-liposome	systemic injection	94
Cxr, nrl	retina	plasmid based shRNA	in vivo electro-poration	97
NP, PA of Influenza A virus	lung	siRNA with oligofectamine	systemic inj. with local injection (i.n.)	105
ataxin-1	brain midline lobules IV/V	AAV mediated shRNA	local injection	92
tyrosine hydroxylase	midbrain neurons	AAV mediated shRNA	local injection	93

Part II

Reduction of murine VEGF gene expression and angiogenesis caused by CpG motif in mouse eyes using shRNA against VEGF

Abstract

Neovascularization in the cornea can lead to impaired vision. Although many molecules are involved in the neovascularization, vascular endothelial growth factor (VEGF) is the one of the most strongly implicated factors in pathological neovascularization of the eye. Therefore, VEGF is an attractive target for anti-angiogenic therapy to treat neovascular eye disease. Recent developments in RNA interference (RNAi) technology have made it possible to inhibit gene expression because the technology allows for specific and potent gene silencing. In this study, we investigated whether the plasmid DNA expressing shRNA against mVEGF (murine VEGF) can reduce mVEGF expression and angiogenesis in a mouse corneal neovascularization caused by CpG motif. Cells transfected with pshVEGF-EGFP, which expresses VEGF shRNA and EGFP bicistronically, showed reduced mVEGF, mRNA, and protein levels. In addition, the introduction of pshVEGF into the stromal cells of the mouse eyes by intrastromal injection reduced the angiogenic area as well as mVEGF production caused by CpG motif. Our results indicate that RNAi technology might be a useful approach to studying corneal stromal cell biology and can be used to treat a variety of corneal diseases induced by unwanted gene expression, such as neovascularization.

Introduction

Neovascularization in the cornea is a critical step in herpetic stromal keratitis (HSK), which is caused by ocular infection with the herpes simplex virus-1(HSV-1) (1, 2). The mechanisms by which ocular infection of HSV-1 induces neovascularization are not clear. However, it was demonstrated that bioactive CpG motif in HSV-1 DNA could contribute to angiogenesis in the eyes, and the angiogenesis was mediated by mVEGF production (3). In addition, the administration of the anti-mVEGF antibody prevents CpG-induced angiogenesis in mouse eyes (3). These results imply that mVEGF is an important molecule for neovascularization in mouse eyes and that inhibition of mVEGF production can reduce angiogenesis in mouse eyes.

RNAi is a powerful technique used to induce gene silencing sequence specifically (4). To investigate whether RNAi technology can inhibit mVEGF expression *in vitro* and *in vivo*, we designed a plasmid DNA expressing mVEGF shRNA.

RNA interference (RNAi) is a phenomenon in which the introduction of double-stranded RNA (dsRNA) into certain organisms and cell types induces degradation of homologous target mRNA (4). RNAi was first discovered in *C. elegans* and it has become clear that RNAi occurs in other organisms as well including fungi, *Drosophila*, plants, and mammals (5, 6, 7, 8). There are a few different small RNA inducing RNAi, including chemically synthesized siRNA and short hairpin RNA (shRNA) from the plasmid or viral vector, and micro RNA (8, 9, 10, 11, 12). Among the small RNA, synthetic siRNA is the most commonly used. However, synthetic siRNA requires cell lines to have high transfection efficiency, but unfortunately, many cell lines and primary

cells do not show high transfection efficiency. To overcome this limitation, we generated plasmid DNA expressing mVEGF shRNA and EGFP bicistronically (pshVEGF- EGFP), and sorted the EGFP positive cells after transfection to determine the inhibition of mRNA of mVEGF. In the present study, we demonstrated that mVEGF mRNA is highly reduced in pshVEGF-EGFP transfected cells, but not in pshGAPDH-EGFP (control plasmid) transfected cells. This result indicates that shRNA expressing a plasmid containing reporter gene is useful particularly when a cell line has a low transfection efficiency. We also demonstrated that the intrastromal injection of pshVEGF into mouse stromal cells reduced angiogenesis and mVEGF production induced by CpG motifs. These results imply that the eye is a suitable organ for the application of RNAi technology and can help to understand ocular biology and control ocular disease caused by unwanted gene expression in corneal stromal cells.

Materials and Methods

Mice

Five to six week old female BALB/c mice were used. All experiments were conducted in compliance with the guide for the care and use of Laboratory Animal Resource Council. The animal facilities of the University of Tennessee (Knoxville, TN), used are accredited by the American Association of Laboratory Animal Care. All ocular experimental procedures were conducted according to the Association for Research in Vision and Ophthalmology Resolution on the use and care of laboratory animals.

Plasmid construction

We designed shRNA to interfere with mVEGF expression, referring to technical information (Ambion, Austin, TX). The target sequence was confirmed to determine there is no homology to any other mouse genes by using a BLAST search. The 19-mer sense shRNA sequence and antisense shRNA sequence were linked with a nine nucleotide spacer (TTCAAGAGA) as a loop. Six Ts and 6As were added as a termination signal to the 3' end of the forward oligomer and 5'end of the reverse oligomers, respectively. Then 4 nucleotides corresponding to the EcoRI (AATT) and Apa1 (GGCC) sites were added to the 5' and 3' end of the reverse oligomer, respectively.

The oligomer sequences for pshVEGF are (forward) 5'-GCTACTGCCGTCCAATTG ATTCAAGAGATCAATTGGACGGCAGTAGCTTTTTT-3' (reverse) 5'-AATTAAAA AAGCTACTGCCGTCCAATTGATCTCTTGAATCAATTGGACGGCAGTAGCGGC C-3'.

The forward and reverse oligomers were incubated in annealing buffer (100mM K-acetate, 30mM HEPES-KOH (pH7.4) and 2mM Mg-acetate) for3 min at 90oC, followed by incubation for 1hr at 37oC. The annealed oligomer was legated with linearized pSilencer1.0-U6 vector (Ambion) at Apa1 and EcoRI sites. After transformation, the sequence was confirmed by DNA sequencing. pshGAPDH was purchased from ambion (Austin, TX). For pshVEGF-EGFP and pshGAPDH-EGFP, DNA fragment containing CMV promoter, EGFP gene, and polyA sequence was inserted at SacI site using blunt end ligation.

Cell culture and transfection

Cos-7 and MBE cells were cultured in DMEM (Mediatech, VA) supplemented with 10% heat-inactivated FBS and penicillin/streptomycin in 5% CO_2. Cells were reseeded at a confluency of 70-80% in the absence of antibiotics 1 day before transfection. Transfection with plasmids was carried out with Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications.

Reagents

Phosphorothioate ODNs were kindly provided by Dennis M. Klinman (Biologics Evaluation and Research, Food and Drug Administration, Washington, DC). The sequences of stimulatory ODNs used in this study were: 1466, TCAACGTTGA, and 1555, GCTAGACGTTAGCGT. Subsequent studies were performed using an equimolar mixture of ODNs 1466 and 1555. The control ODN 1471 has sequence TCAAGCTTGA.

Total RNA isolation and RT-PCR

Total RNA from cells was extracted by using RNeasy RNA extraction kit (Qiagen, Valencia, CA). Briefly, cells were lysed in lysis buffer, and RNA was purified following manufacturer's protocol. DNase treatment (Qiagen) was done to remove genomic DNA. For the RT-PCR, one-step RT-PCR was performed according to the manufacturer's protocol (Qiagen) with 80ng of total RNA. The following RT-PCR conditions were used: 1 cycle of 50°C for 30 min followed by 1cycle of 94°C for 2 min: 30 cycles of 94°C for 1 min, 56°C for 1min and 72°C for 1 min and a final cycle of 72°C for 5 min. The primer sequences for VEGF were 5'-GCGGGCTGCCTCGCAGTC-3' (forward) and 5'-TCACCGCCTTGGCTTGTCAC-3' (reverse). The primer sequences for GAPDH were 5'-CATCCTGCACCACCAACTGCTTAG (forward) and 5'-GCCTGCTTCACCACCTTCTTGATG-3' (reverse).

Quantitative Real-Time PCR

Total RNA was isolated from GFP positive cells after transfection using RNeasy RNA extraction kit (Qiagen) according to manufacturer's protocol. DNase(Qiagen) was treated to remove genomic DNA. To generate cDNA 1ug of total RNA was reverse transcriptased using reverse transcriptase (Invitrogen). All cDNA samples were aliquoted and stored at -20oC until use. Real time PCR was performed using a smart cycler system (Chepheid). PCR was performed using SYBR Green I reagent (Qiagen), according to the manufacturer's protocol. The semi-quantitative comparison between samples was calculated as follows: the data were normalized by subtracting the difference of the threshold cycles (Ct) between the gene of interest's Ct and the GAPDH's Ct (gene

of interest Ct – GAPDH Ct = ΔCt) for each sample. Then, ΔΔCt was calculated by subtraction (ΔΔCt = ΔCt, pshVEGF- ΔCt, pshGAPDH). To determine relative expression of VEGF mRNA following formula was used $2^{-\Delta\Delta Ct}$.

The primers used were GAPDH 5'-CATCCTGCACCACCAACTGCTTAG-3' (forward), 5'-GCCTGCTTCACCACCTTCTTGATG-3' (reverse), VEGF 5'-GGAGATCCTT-CGAGGAGCACTT-3' (forward), 5'-GGCGATTTAGCAGCAGATATAAGAA-3' (reverse).

Flow cytometry

Trypsinized cells were fixed and permeabilized using cytofix/cytoperm buffer (BD Biosciences, Mountain View, CA) for 30 minutes on ice. Labeling of intracellular VEGF was carried out with biotinylated VEGF antibody (R&D systems, Inc., Minneapolis, MN) followed by staining with streptavidin-PE. Finally, the cells were washed three times and samples were acquired on a FACScan (BD Bioscience). The data were analyzed using the CellQuest 3.1 software (BD Biosciences).

Corneal lysate VEGF Enzyme-Linked Immunosorbent Assay (ELISA)

Corneas were isolated and put into DMEM without serum and stored at -80°C. The corneas were homogenized using ultra sonicater (Heat systems-Ultrasonics, NY). The lysate was then clarified by centrifugation at 12000rpm for 5 minutes at 4°C. The supernatant was collected and stored at -80°C until further use. Lysate was assayed using a standard sandwich ELISA protocol. Anti-mouse VEGF capture and biotinylated detection antibody (R&D systems, Inc., Minneapolis, MN) was used. mVEGF164 protein

was used as a standard (R&D systems, Inc.). The color reaction was developed using ABTS (Sigma-Aldrich, St. Louis, MO) and measured with an ELISA reader (Spectramax 340; Molecular Devices, Sunnyvale, CA) at 405nm. Quantification was performed with Spectramax ELISA reader software version 1.2.

Intrastromal injection

Corneal intrastromal injection was performed as described before (13). Under stereomicroscopic observation, a small tunnel from the corneal epithelium to the anterior stroma was made with a one-half-inch 30-gauge needle. Another needle was passed through the tunnel into the corneal stroma. Two microliters of solution containing the 1ug plasmid was forcibly injected into the stroma.

Corneal Micropocket Assay

The corneal micropocket assay used in this study observed the general protocol of Kenyon and colleagues (14)

Pellets 0.4x 0.4x 0.2mm^3 composed of sucralfate and hydron polymer were prepared. Known amount of bioactive CpG or non-bioactive control CpG were added to these pellets. Each pellet contains 1ug of CpG ODNs. The micropockets were placed ~1 mm from the limbus under stereomicroscope (Leica Micro Systems, Wetzlar, Germany) and the pellet was inserted into the micropocket.

Measurement of angiogenic area

Angiogenesis area was measured at day4 after pellet implantation by using caliper

(Biomedical Research instrument, Rockville, MD) with a streomicroscope.

The length of the neovessels generated from the limbal vessel ring toward the center of the cornea and the width of the neovessels presented in clock hours (each clock hour is equal to 30^{o} at the circumference) was measured (15). The angiogenic area was calculated according to he formula for an ellipse. A= [(clock hours) x 0.4 x (vessel length in mm) x π]/2.

Statistical analysis

Significant differences between groups were evaluated using student's t-test. A p value of ≤ 0.05 was regarded as indicating a significant difference between two groups.

Results

Selection of candidate sequences and construction plasmid

To select candidate target sequences, we used siRNA target finder on the website of ambion. The program scans the mVEGF sequence for the 21 nucleotide sequence starting AA. From among them, we chose three sequences having less than 50% G/C content because siRNAs with low G/C content are more active than those with higher G/C contents, according to the technical information from ambion. After the selection candidate sequence, we inserted an oligonucleotide having sense, loop, and anti-sense sequence into a shRNA expressing vector (pshVEGF) (Fig. 1A). To make plasmid expressing shRNA and EGFP bicistronically, we inserted an EGFP gene containing CMV promoter and a poly A sequence (pshVEGF-EGFP) (Fig. 1B). With this pshVEGF-EGFP, we validated effective sequence.

Co-transfection of pshVEGF and pEGFP-VEGF reduced EGFP signal.

Validation of an effective shRNA requires determination of the mRNA and protein levels using RT-PCR, a northern blot, a western blot, or flow cytometry. To make this validation step simple, we designed pEGFP-VEGF (Fig. 1C) and co-transfected it with pshVEGF into Cos-7 cells. If mVEGF shRNA degrades its target mRNA, EGFP mRNA can not be translated either. Co-transfection of pshVEGF and pEGFP-VEGF showed a reduced EGFP signal in compared to the co-transfection of pshGAPDH, expressing GAPDH shRNA and pEGFP-VEGF (Fig. 2). From this experiment, we chose the most effective shRNA for the rest of the experiment.

To determine the Mean Fluorescence Intensity (MFI) after co-transfection of pEGFP-VEGF with pshVEGF or pshGAPDH, flow cytometry was performed. The MFI of the cells which were co-transfected with pEGFP-VEGF and pshGAPDH was 330.29 ± 29.69, while the MFI of the cells which were co-transfected with pEGFP-VEGF and pshVEGF was 110.56 ± 16.39 (Fig. 3). These results indicate that pshVEGF targets VEGF mRNA effectively. In addition, it was shown that the co-transfection of shRNA with the plasmid expressing reporter gene-target gene mRNA is a useful tool to validate a shRNA candidate. This strategy might be useful, especially in case when antibodies against target proteins are not available.

pshVEGF-EGFP reduced endogenous VEGF gene expression in transfected cells.

The MBE cell line revealed a very low transfection efficiency (5-10%). Therefore, we could not detect a significant reduction of gene expression after the transfection. To solve this problem, we designed pshVEGF-EGFP and pshGAPDH-EGFP expressing shRNA and EGFP bicistronically (Fig. 1B). After the transfection of the plasmid, we sorted the EGFP positive cells prior to performing RT-PCR and real time PCR. The pshVEGF-EGFP positive cells suppressed 70% of mVEGF mRNA compared to the control cells, which were pshGAPDH-EGFP positive cells (Fig.4, 5). We also determined whether pshVEGF reduces the VEGF protein in MBE cells. In cells transfected with pshVEGF-EGFP, the EGFP positive cells showed a reduced mVEGF protein level. The MFI of mVEGF from the pshVEGF-EGFP transfected cells (EGFP positive cells) was 42.6 ± 15.6, while the MFI of mVEGF from the pshGAPDH-EGFP transfected cells was 104.1± 16.6. The MFI of mVEGF from the untrasfected cells was similar to that of the

pshGAPDH-EGFP transfected cells (98.6± 15.7 and 99.6± 10.2 respectively) (Fig.6).

Intrastromal co-injection of pshVEGF and pEGFP-VEGF reduced EGFP signal in cornea

To determine that how much plasmid DNA is required to obtain optimal transfection, we injected 0.5, 1.0, 2.0, 4.0 or 8.0ug/2ul per eye with pEGFP. Intrastromal injection with 1.0 -4.0ug/2ul showed similar highest GFP signal. Therefore, we used 2ug/2ul dose for our experiments. In addition, we also investigated how long plasmid is expressed into stromal cells. We observed GFP signal from day 1 to day 10 after intrastromal injection. GFP signal was strong until day3. At day 4 the signal started decreasing. However, the signal was detected until day 8 or 9. Therefore, it seems that plasmid based shRNA is expressed long enough to see the effect *in vivo*.

To determine whether the shRNA against mVEGF degrades its target molecule in the cornea, we co-transfected pshVEGF and pEGFP-VEGF intrastromally. As a control, pshGAPDH and pEGFP-VEGF were co-injected. The co-injection of pshVEGF and pEGFP-VEGF showed a reduced EGFP signal compared to the co-injection of pshGAPDH and pEGFP-VEGF (Fig. 7). This result indicates that pshVEGF degrades its target mRNA in the stromal cells of the mouse cornea.

Intrastromal injection of pshVEGF reduced angiogenesis caused by CpG motif

To investigate whether pshVEGF can reduce angiogenesis in mouse eyes, pshVEGF or pshGFP was injected intrastromally. One day after injection, a pellet containing bioactive CpG or bioinactive CpG oligonucleotide was implanted to cause angiogenesis. Four days after implantation, an angiogenic area was measured. The pshVEGF-injected

eyes showed reduced angiogenesis compared to pshGFP-injected eyes (Fig. 8). The angiogenic area from the pshVEGF-injected eyes was 0.34 ± 0.11, while the area from the pshGFP-injected eyes was 0.49 ± 0.14 mm^2. The angiogenic area from control CpG-implanted eyes was 0.15 ± 0.11 mm^2 (Fig. 9).

The level of the mVEGF164 protein was measured by ELISA. An intrastromal injection of pshVEGF reduced the mVEGF164 protein level (126.67 ± 16.07pg / eye) in bioactive CpG-treated cornea in compared to the injection of pshGFP (183.33 ± 48.05pg / eye) (Table 1). Therefore, it is assumed that the reduction of the mVEGF protein results in the inhibition of angiogenesis in the mouse cornea.

Discussion

RNAi phenomenon was discovered very recently, but the RNAi technology has been used in many research areas because it is very powerful to reduce endogenous gene expression with specificity. The most popular purpose of the experiment using RNAi technology is to identify unknown biological gene function. The conventional way to identify the function of particular genes is the examination of mutant or knockout phenotype. However, all mutant and knockout animals are not available and making them is a time consuming process. On the other hand, it is relatively easy and quick to reduce particular gene expression using RNAi technology to determine function of the interested genes. Additionally, in *C. elegans*, high throughput screening format, in which many genes can be screened at a time to find genes having particular function, using siRNA has been developed and identified the genes involved in cell division and embryonic development (16, 17). This RNAi pathway is conserved from plants to mammals, so this technology can be used in many organisms.

To determine reduced protein level after transfection of siRNA or shRNA, antibodies against target proteins are required. However, some antibodies are not available. To overcome this problem, we designed co-transfection strategy in which plasmid expressing fusion protein (reporter protein-target protein) and shRNA are co-transfected. The result showed that co-transfection of pEGFP-VEGF and pshVEGF reduced EGFP signal (Fig.2). Therefore, this strategy might be useful to detect suppression of protein production indirectly when a antibody against target protein is not available.

Although the RNAi technology is very useful, it depends on high transfection

efficiency. Therefore, most experiments have been done with only cell lines having high transfection efficiency such as HeLa and HEK 293 cells. To overcome this limitation, we have designed plasmid expressing a shRNA and a reporter gene, EGFP, bicistronically (pshVEGF-EGFP) to identify which cells are transfected. After transfection of the plasmid into MBE cell line having low transfection efficiency, we sorted EGFP positive cells (pshVEGF-EGFP transfected cells) and showed down regulation of mVEGF mRNA in the sorted cell in comparison with the pshGAPDH-EGFP transfected cells (Fig.4, Fig. 5). In addition, flow cytometry experiment showed that pshVEGF-EGFP positive cells produced less mVEGF in comparison with pshVEGF-EGFP negative cells or pshGAPDH-EGFP positive cells. Therefore, these results imply that the plasmid expressing shRNA and EGFP is useful in cell lines having low transfection efficiency.

SiRNA degrades mRNA sequence specifically so, RNAi technology can be used for therapeutic purposes. Although down regulation of unwanted gene or mutated gene expression is an attractive method to treat disease and siRNA has the potential, the delivery of siRNA to proper cells or tissues is a problem for the therapeutic application of siRNA. Recently, the therapeutic potential of the siRNA was demonstrated in the mouse. Song et. al. (18) demonstrated that targeting of fas mRNA by fas siRNA decrease hepatocyte necrosis and protected mice from liver fibrosis and fulminant hepatitis. One of the critical reasons their *in vivo* experiment was successful is that the hydrodynamic injection delivers most siRNAs to the liver cells. However, this technique may not be used for large animals because the pressure may not reach to the liver in such animals. Direct injection into tissues is also used to deliver siRNA or viral vector mediated shRNA (19, 20). This method could achieve tissue or organ specific delivery of siRNA or shRNA.

However, it is used only in limited tissues or organs because direct injection is not available in many tissues. Viral vectors are being used by many researchers (21-25). Some viral vectors give good transduction efficiency but lack of tissue specific transduction could be a problem. Development of tissue specific promoter-derived shRNA might be the answer of the problem.

In this study, we showed that intrastromal co-injection of pshVEGF and pEGFP-VEGF reduced EGFP signal in comparison with co-injection of pshGAPDH and pEGFP-VEGF (Fig. 7). In addition, intrastromal injection of pshVEGF reduced angiogenesis and mVEGF protein production caused by CpG motif in eyes (Fig. 8, Fig.9, and Table 1). At this time, we do not know how many cells are transfected by pshIL-1RI. However we assumed that intrastromal injection transfected enough cells to reduce angiogenesis caused by the IL-1α treatment. Otherwise, shRNA might be spread to neighbor cells after expressed in transfected cells and inhibit target protein expression in untransfected cells as well as transfected cells. Although improvement of delivery system is necessary for more successful gene silencing, it seems that the eye might be a good target organ to use RNAi technology for such research and therapeutic purposes. Our results imply that RNAi technology may be helpful to understand corneal biology and hold promise for the development of gene specific therapeutics in eyes.

LIST OF REFERENCES

List of references

1. Zheng, M., Deshpande, S., Lee, S., Ferrara, F., and Rouse, BT. 2001. Contribution of vascular endothelial growth factor in the neovascularization process during the pathogenesis of herpetic stromal keratitis. J Viol. 75, 9828-9835.
2. Zheng, M., Schwarz, MA., Lee, S., Kumaraguru, U., and Rouse, BT. 2001. Control of stromal keratitis by inhibition of neovascularization. Am. J. Pathol. 159, 1021-1028.
3. Zheng, M., Klinman, D., Gierynska, M., and Rouse, BT. 2002. DNA containing CpG motifs induces angiogenesis. Proc. Natl. Acad. Sci. 99, 8944-8949.
4. Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391, 806-811.
5. Romano, N. and Macino, G. 1992. Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. Mol. Microbiol. 6. 3343-3352.
6. Vauchert, H., Beclin, C., and Fagard. M. 2001. Post-transcriptional gene silencing in plants. J. Cell Sci. 114, 3083-3091.
7. Zamore, P.D., Tuschl, T., Sharp, P.A., and Bartel, D.P., 2000. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21-23 nucleotide intervals. Cell 101, 25-33.
8. Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T.,

2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494-498.

9. Martinez, J., Patkaniowska, A., Urlaub, H., Luhrmann, R., and Tuschl, T., 2002. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell 100, 563

10. Stechschulte, S. U., Joussen, A. M., von Recum, H. A., Poulaki, V., Moromizato, Y., Yuan, J., D'Amato, R. J., Kuo, C., and Adamis, A. P. 2001. Rapid ocular angiogenic control via naked DNA delivery to cornea. Invest. Ophthalmol. Visual Sci. 42:1975

11. Kenyon, BM., Voest, E.E., Chen, C.C., Flynn, E., Folkman, J., D,Amato, R.J. 1996. A model of angiogenesis in the mouse cornea. Invest. Ophthalmol. Vis Sci. 37, 1625-1632.

12. Schwarz, M.A., Kandel, J., Brett, J., Li, J., Hayward, J., Schwarz, R.E., Chappy, O., Wautier, J., Chabot, J., Gerfo, P.L., Stern, D. 1999. Endothelial-monocyte activating polypeptide II, a novel antitumor cytokine that suppresses primary and metastatic tumor growth and induces apoptosis in growing endothelial cells. J. Exp. Med. 190, 341-353.

13. Hutvagner, G., and Zamore, P.D. 2002. RNAi: nature abhors a double-strand. Curr. Opin. Gene. and Dev. 12, 225-232.

14. Sui G., soohoo, C., Affar, E-B., Gay, F., Shi, Y., Forrester, W.C., and Shi, Y., 2002. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc. Natl. Acad. Sci. USA 99, 5515-5520.

15. Paddison, P.J., Caudy, A.A, Bernstein, E., Hannon G.J., and Conklin, D.S., 2002.

Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes and Dev. 16, 948-958.

16. Fraser, A.J., Kamath, R.S., Zipperten, P., Campos, M.M, Sohrmann, M., and Ahringer. J., 2000. Functional genomic analysis of *C. elegans* chromosome I by systemic RNA interference. Nature 408, 325-330.
17. Gonczy, P., Echerverri, C., Oegema, K., Coulron, A., Jones, S.J.M., Copley, R.R., Duperon, J., Oegema, J., Brehm, M., Carrin, E., Hannak, E., Kirkham, M., Pichler, S., Flohrs, K., Gossen, A., Liedel, S., Alleaume, A.M., Alleaume, A.M., Martin, C., Ozlu, N., Bork, P., and Hyman, A.A. 2000. Functional genomic analysis of cell division in *C. elegans* with RNAi of genes on chromosome III. Nature 408, 331-335.
18. Song, E., Lee, S.K., Wang, J., Ince, N., Ouyang, N., Min, J., Chen, J., Shankar P., and Libberman, J. 2003. RNA interference targeting Fas protects mice from fulminant hepatitis. Nat. Med. 9, 347-351.
19. Hommel, J.D., Sears, R.M., Georgescu, D., Simmons, D.L., and DiLeone, R.J. 2003. Local gene knockdown in the brain using viral-mediated RAN interference. Nat. Med.9, 1539-1543.
20. Nakamura, H., Siddiqui, S.S., Shen, X., Malik, A.B., Pulido, J.S., Kumar, N.M. and Yue, B.Y. 2004. RNA interference targeting transforming growth factor-β type II receptor suppresses ocular inflammation and fibrosis. Mol. Vis. 10, 703-711.

21. Svoboda, J., Heinar,, J., Geryk, J., Elleder, D., and Vernerova, Z. 2000. Retroviruses in foreign species and the problem of provirus silencing. Gene 261, 181-188.

22. Rubinson, D.A. et al. 2003.A lentivirus-based system to silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nature Genet. 33, 401-406.

23. Qin, X.F., An, D.S., Chen, I.S., and Baltimore, D. 2003. 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.

24. Bain, J.R., Schisler, J.C., Takeuchi, K., Newgard, C.B., Becker, T.C. 2004. An adenovirus vector for efficient RNA interference-mediated suppression of target genes in insulinoma cells and pancreatic is of langerhans. Diabetes 53, 2190-2194.

25. Tomar, R.S., Matta, H., and Chaudhary, P.M. 2003. Use of adeno-associated viral vector for delivery of small interfering RNA Oncogene 22, 5712-5715

APPENDIX

Fig. 1 plasmid constructs 1.

A. pshVEGF expresses shRNA against mVEGF

B. pshVEGF-EGFP expresses shRNA against mVEGF and EGFP bicistronically

C. pEGFP-VEGF expresses EGFP-VEGF fusion protein

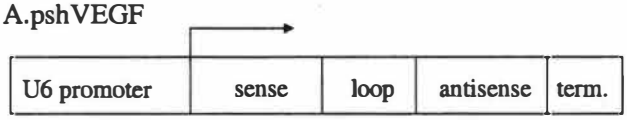
Sense: sense sequence, Antisense: antisense sequence,

Term: RNA polymerase III transcription termination sequence

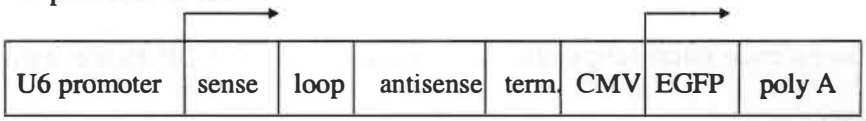
CMV: CMV promoter, Poly A: Poly A sequence

Arrows represent transcription start site and direction

A.pshVEGF



B. pshVEGF-EGFP



C. pEGFP-VEGF

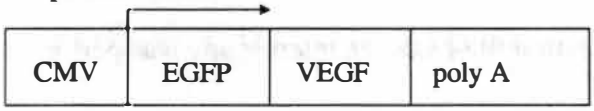
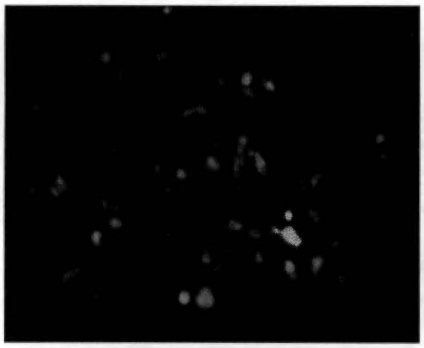


Fig.1

Fig. 2 Fluorescence microscopy after co-transfection of pVEGF-EGFP with pshVEGF or pshGAPDH.

0.5ug of pEGFP-VEGF with 2ug of pshGAPDH (A) or 2ug of pshVEGF (B) are co-transfected. 24hrs after transfection fluorescence microscopy was performed.

A. pEGFP-VEGP + pshGAPDH



B. pEGFP-VEGF + pshVEGF

Fig.2

Fig.3 Inhibition of exogenous mVEGF protein.

24-36 hrs after co-transfection of pEGFP-VEGF with pshGAPDH (A) or pshVEGF (B) into cos-7 cells, flow cytometry was performed.

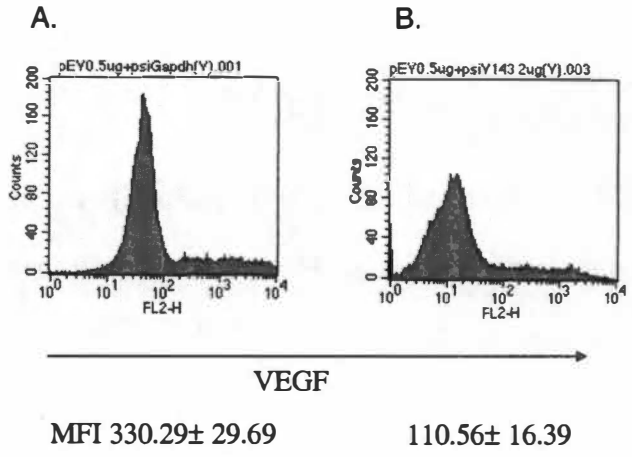

A.
B.
pEY0.5ug+psiGapdh(Y).001
pEY0.5ug+psiY143 2ug(Y).003
Counts
FL2-H
VEGF
MFI 330.29± 29.69
110.56± 16.39

Fig. 3

Fig. 4 Inhibition of endogenous mRNA of mVEGF.

pshGAPDH-EGFP positive and pshVEGF-EGFP positive cells were sorted .

Total RNA from the sorted cells was isolated and RT-PCR was performed.

Beta actin primers were used as a control.

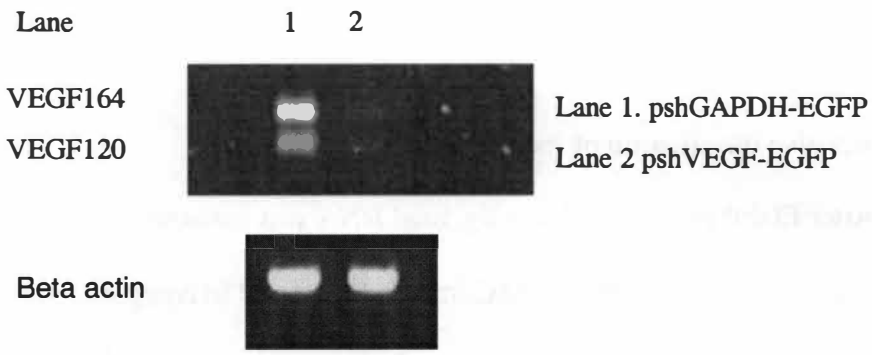

Lane
1
2
VEGF164
VEGF120
Beta actin
Lane 1. pshGAPDH-EGFP
Lane 2 pshVEGF-EGFP

Fig.4

Fig. 5 Relative quantitation of reduced mRNA of mVEGF.

After EGFP positive cell sorting, total RNA was isolated, and then cDNAs were made from the total RNA. ΔΔCt method was used to compare the relative expression of mRNA between the cells

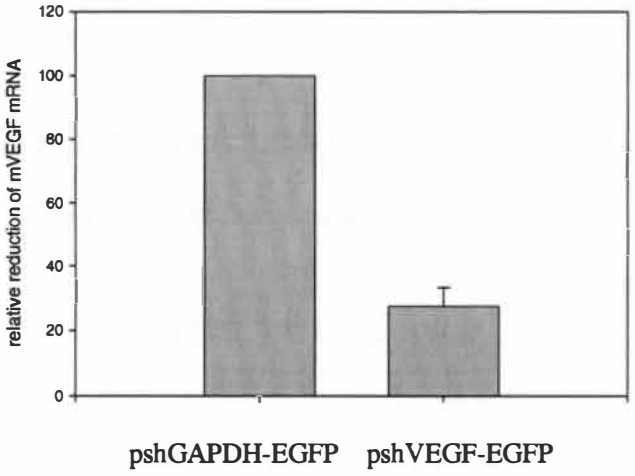
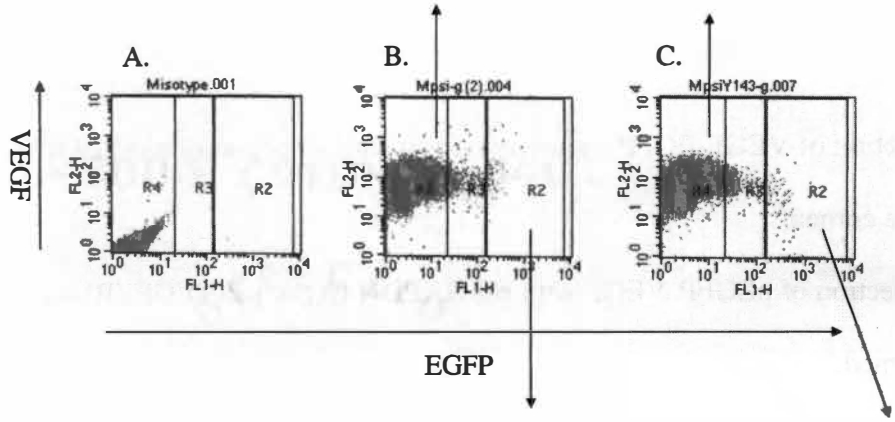

120
100
80
60
40
20
0
relative reduction of mVEGF mRNA
pshGAPDH-EGFP
pshVEGF-EGFP

Fig. 5

Fig. 6 Inhibition of endogenous mVEGP protein.

24hr after transfection of pshGAPDH-EGFP (B) or pshVEGF-EGFP (C), cells were harvested for intracellular staining to detect mVEGF protein. (A): isotype control antibody was treated.

MFI 96.8± 15.7 99.6± 10.2



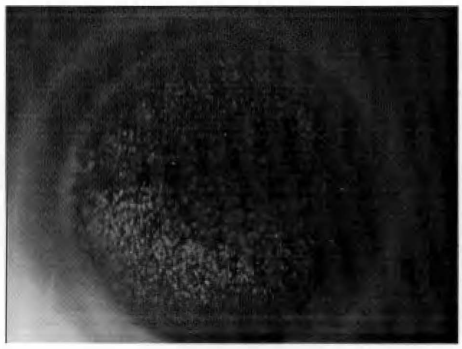
MFI 104.1± 16.6 42.6± 15.6

Fig. 6

Fig. 7 Reduction of VEGF-EGFP signal by intrastromal injection of pshVEGF in mouse cornea.

Co-injection of pEGFP-VEGF with pshGAPDH (A) or pshVEGF (B) was performed.

A. pEGFP-VEGF +pshGAPDH



B. pEGFP-VEGF + pshVEGF

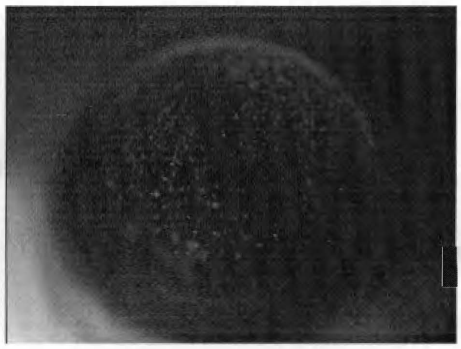


Fig. 7

Fig. 8 Reduction of angiogenesis caused by CpG motifs by pshVEGF in cornea. Intrastromal injection of pshVEGF (B) reduced angiogenesis induced by CpG motifs. pshGFP (A) was used as a control plasmid. Bioinactive CpG was used as a control pellet (C)

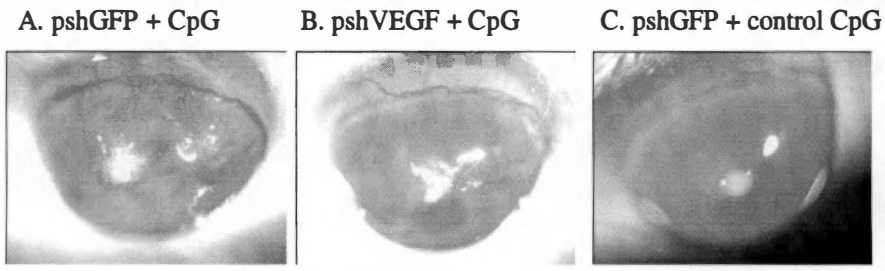


Fig.8

Fig. 9 Angiogenesis area at day 4 after CpG treatment following pshGFP or pshVEGF Injection.

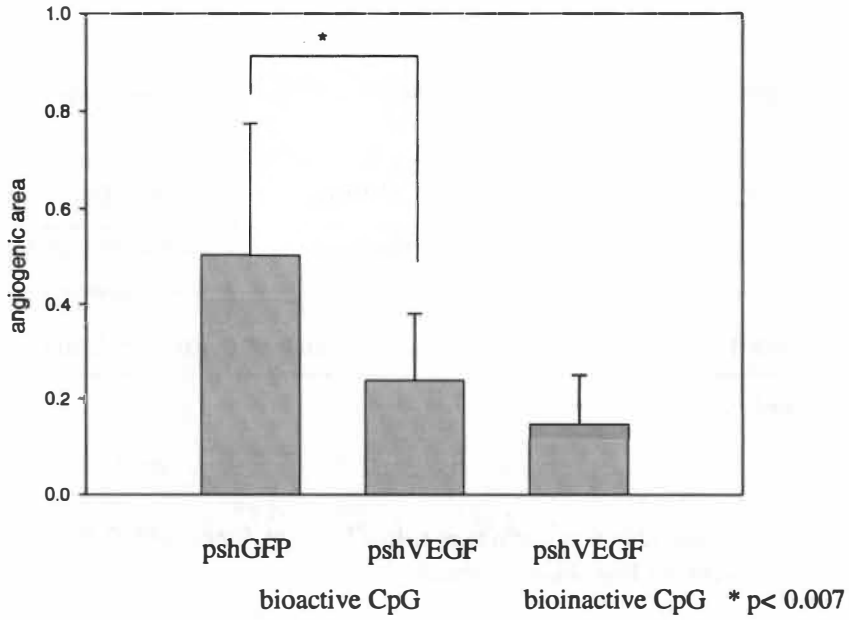

1.0
0.8
0.6
0.4
0.2
0.0
angiogenic area
*
pshGFP
pshVEGF
pshVEGF
bioactive CpG
bioinactive CpG
* p< 0.007

Fig. 9

Table 1. Measurement of the amount of mVEGF in corneas using ELISA

Plasmid	pshGFP	pshVEGF	pshGFP
CpG	bioactive	bioactive	bioinactive (control)
VEGF	183.33± 48.05	*126.67± 16.07 *	101.67 ± 23.09

pg/ eye

*p< 0.05

Four days after CpG treatment following pshGFP or pshVEGF injection ELISA was performed.

PART III

Inhibition of angiogenesis caused by IL-1α in mouse cornea using shRNA against IL-1 receptor type I

Abstract

Ocular infection with herpes simplex virus-1 (HSV-1) induces IL-1, which is a pro-inflammatory cytokine. Subsequently, the IL-1 protein induces the production of many other molecules, such as IL-6 and vascular endothelial growth factor (VEGF), a potent angiogenic factor, which results in neovascularization in the cornea. Therefore, the inhibition of IL-1 signaling is a useful strategy to reduce angiogenesis caused by IL-1 in the cornea.

In this study, we demonstrated that the intrastromal injection of plasmid-based shRNA against IL-1 receptor type I (IL-1RI) reduced angiogenesis caused by IL-1α in the cornea when it was compared to control shRNA-treated cornea. We also showed that the intrastromal injection of IL-1RI shRNA reduced VEGF production which is induced by IL-1α. This result implies that reduced angiogenesis by IL-1RI shRNA might be mediated by reduced VEGF expression. Although the IL-1 receptor antagonist (IL-1Ra) is widely used to interrupt IL-1 and IL-1RI interaction, our results indicated that the inhibition of IL-1RI expression using IL-1RI shRNA might be another valuable approach to treat the diseases caused by IL-1.

Introduction

Herpes simplex virus-1 (HSV-1) infection of the mouse eyes causes herpetic stromal keratitis (HSK), which results in blindness. After an HSV-1 infection, many cellular events are involved in the pathogenesis of HSK (1). Among them, neovascularization is a critical step in HSK pathogenesis, and the VEGF protein is a major angiogenic factor. It was shown that the inhibition of VEGF using a neutralizing antibody reduced HSK severity as well as angiogenesis caused by the HSV-1 infection (2, 3). In other experiments, it was shown that the HSV-1 infection induced IL-1 and IL-6, and the increased expression of the cytokines stimulated angiogenesis through up-regulation of VEGF (4). In addition, the transgenic mouse that over-expresses the IL-1 receptor antagonist (IL-1Ra) showed reduced HSK severity and angiogenesis in comparison to the wild type mice (4). IL-1Ra binds to the IL-1 receptor type I without transmitting an activation signal (5). Thus it represents a physiological inhibitor of IL-1 activity. These results imply that IL-1 production is a critical event in the development of HSK and inhibition of the IL-1 and IL-1 receptor interaction can be a useful approach to control angiogenesis and HSK.

There are two forms of IL-1 proteins, IL-1α and IL-1β, which are the products of distinct genes (6, 7). In most studies, their biological activities are indistinguishable, and they bind to the same receptors: IL-1 receptor type I (IL-1RI) and IL-1 receptor type II. (IL-1R II) (5). Type I receptor is an 80kDa transmembrane protein with an IL-1 signaling function. On the other hand, IL-1R type II is a 68kDa membrane protein with a short cytoplasmic tail and has no signaling function (5).

In this study, we demonstrated that the inhibition of IL-1R type 1 (IL-1RI) expression using RNAi (RNA interference) technology can reduce angiogenesis caused by the IL-1α protein in mouse eyes.

RNAi is a sequence-specific gene silencing mechanism initiated by the introduction of double-stranded RNA (dsRNA) into target cells (8). RNAi is mediated by small interfering RNAs (siRNAs) produced from long dsRNAs of exogenous or endogenous origin by an endonuclease called Dicer. The resulting siRNAs are about 21 to 23 nucleotides long and are incorporated into a RNA-induced silencing complex, which then targets and cleaves the mRNAs that are complementary to the siRNAs (9,10). In mammalian cells, the introduction of 19-21 nucleotide siRNA duplex is required to initiate RNAi without non-specific gene silencing (11). DNA plasmids expressing shRNAs have been shown to induce RNAi successfully (12-14).

We demonstrated that transfected cells with the plasmid expressing shRNA against IL-1RI reduced IL-1RI expression *in vitro*. In addition, intrastromal injection of the pshIL-1RI into mouse eyes showed reduced angiogenesis and VEGF production in comparison to control plasmid-injected eyes. Our results imply that targeting IL-1RI using shRNA can be a valuable approach to treat the disease caused by unwanted IL-1 production, such as HSK.

Materials and Methods

Mice

Five to six week old female BALB/c mice were used. All experiments were conducted in compliance with the guide for the care and use of Laboratory Animal Resource Council. The animal facilities of the University of Tennessee (Knoxville, TN), used are accredited by the American Association of Laboratory Animal Care. All ocular experimental procedures were conducted according to the Association for Research in Vision and Ophthalmology Resolution on the use and care of laboratory animals

Plasmid construction

We designed shRNA to interfere with IL-1RI expression, referring to technical information (Ambion, Austin, TX). The target sequence was confirmed to determine there is no homology to any other mouse genes by using a BLAST search. The 19-mer sense shRNA sequence and antisense shRNA sequence were linked with a nine nucleotide spacer (TTCAAGAGA) as a loop. Six Ts and six As were added as a termination signal to the 3' end of the forward oligomer and 5'end of the reverse oligomers, respectively. Then 4 nucleotides corresponding to the EcoRI (AATT) and Apal (GGCC) sites were added to the 5' and 3' end of the reverse oligomer, respectively.

The oligomer sequences for pshIL-1RI are (forward) 5'-GAAAGACCACAGTCTGC AATT CAAGAGATTGCAGACTGTGGTCTTTCTTTTTT-3', (reverse)5'-AATTAAA AAAG AAGACCACAGTCTGCAATCTCTTGAATTGCAGACTGTGGTCTTTCGGC C-3'.

The forward and reverse oligomers were incubated in annealing buffer (100mM K-acetate, 30mM HEPES-KOH (pH7.4) and 2mM Mg-acetate) for3 min at 90oC, followed by incubation for 1hr at 37oC. The annealed oligomer was legated with linearized pSilencer1.0-U6 vector (Ambion) at Apa1 and EcoRI sites. After transformation, the sequence was confirmed by DNA sequencing. pshGAPDH was purchased from ambion (Austin, TX). For pshVEGF-EGFP and pshGAPDH-EGFP, DNA fragment containing CMV promoter, EGFP gene, and polyA sequence was inserted at SacI site using blunt end ligation.

Cell culture and transfection

Cos-7 and LEII cells were cultured in DMEM (Mediatech, VA), supplemented with 10% heat-inactivated FBS and penicillin/streptomycin in 5% CO_2. Cells were reseeded at a confluency of 70-80% in the absence of antibiotics 1day before transfection. Transfection with plasmids was carried out with Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications.

Total RNA isolation and RT-PCR

Total RNA from the cells was extracted by using an RNeasy RNA extraction kit (Qiagen, Valencia, CA). Briefly, cells were lysed in a lysis buffer, and the RNA was purified following the manufacturer's protocol. DNase treatment (Qiagen) was performed to remove genomic DNA.

For the RT-PCR, one-step RT-PCR was performed according to the manufacturer's protocol (Qiagen) with 80ng of total RNA. The following RT-PCR conditions were used:

1 cycle of 50°C for 30 min followed by 1cycle of 94°C for 2 min: 30 cycles of 94°C for 1 min, 56°C for 1min and 72°C for 1 min and a final cycle of 72°C for 5 min. The primer sequences for IL-1RI were 5'-ACCCCCATATCAGCGGACCG-3' (forward) and 5'-TTGCTTCCCCCGGAACGTAT-3' (reverse). The primer sequences for GAPDH were 5'-CATCCTGCACCACCAACTGCTTAG (forward) and 5'-GCCTGCTTCACCACCTTCTTGATG-3' (reverse).

Flow cytometry

Trypsinized cells were fixed and permeabilized using cytofix/cytoperm buffer (BD Biosciences, Mountain View, CA) for 30 minutes on ice. Labeling intracellular mVEGF was carried out with the biotinylated mVEGF antibody (R&D systems, Inc., Minneapolis, MN)and followed by staining with streptavidin-PE. Finally, the cells were washed three times and samples were acquired on a FACScan (BD Bioscience). The data were analyzed using CellQuest 3.1 software (BD Biosciences).

Corneal lysate VEGF Enzyme-Linked Immunosorbent Assay (ELISA)

Corneas were isolated and put into DMEM without serum and stored at -80°C. The corneas were homogenized using an ultra sonicater (Heat systems-Ultrasonics, NY). The lysate was then clarified by centrifugation at 12000rpm for 5 minutes at 4°C. The supernatant was collected and stored at -80°C until further use. Lysate was assayed using a standard sandwich ELISA protocol. Anti-mouse VEGF capture and biotinylated detection antibody (R&D systems, Inc., Minneapolis, MN) were used. The mVEGF164 protein was used as a standard (R&D systems, Inc.). The color reaction was developed

using ABTS (Sigma-Aldrich, St. Louis, MO) and measured with an ELISA reader (Spectramax 340; Molecular Devices, Sunnyvale, CA) at 405nm. Quantification was performed with Spectramax ELISA reader software version 1.2.

Intrastromal injection

Corneal intrastromal injection was performed as described before (15). Under stereomicroscopic observation, a small tunnel from the corneal epithelium to the anterior stroma was made with a one-half-inch 30-gauge needle. Another needle was passed through the tunnel into the corneal stroma. Two microliters of solution containing 1ug plasmid was forcibly injected into the stroma.

Corneal Micropocket Assay

The corneal micropocket assay used in this study observed the general protocol of Kenyon and colleagues (16).

Pellets 0.4x 0.4x 0.2mm^3 composed of sucralfate and hydron polymer were prepared. A known amount of bioactive CpG or non-bioactive control CpG was added to the pellets. Each pellet contained 1ug of CpG ODNs. The micropockets were placed ~1 mm from the limbus under a stereomicroscope (Leica Micro Systems, Wetzlar, Germany) and the pellet was inserted into the micropocket.

Measurement of the angiogenic area

The angiogenesis area was measured at day4 after pellet implantation by using caliper (Biomedical Research instrument, Rockville, MD) under a streomicroscope.

The length of the neovessels generated from the limbal vessel ring toward the center of the cornea, and the width of the neovessels presented in clock hours (each clock hour is equal to 30^{o} at the circumference) was measured (17). The angiogenic area was calculated according to the formula for an ellipse. A= [(clock hours) x 0.4 x (vessel length in mm) x π]/2.

Statistical analysis

Significant differences between groups were evaluated using student's t-test. A p value of ≤ 0.05 was regarded as indicating a significant difference between two groups.

Results

Selection of candidate sequences and construction plasmid

To select candidate target sequences, we used siRNA target finder on the website of ambion. The program scans the IL-1RI sequences for the 21 nucleotide sequences starting AA. From among them, we chose three sequences having less than 50% G/C content because siRNAs with low G/C content are more active than those with higher G/C contents, according to the technical information from ambion. After the selection candidate sequence, we inserted an oligonucleotide having sense, loop, and anti-sense sequence into a shRNA expressing vector (pshVEGF) (Fig. 1A). To make plasmid expressing shRNA and EGFP bicistronically, we inserted an EGFP gene containing CMV promoter and a poly A sequence (pshVEGF-EGFP) (Fig. 1B). With this pshIL-1RI-EGFP, we validated effective sequence.

Reduction of the IL-1RI protein in transfected cells with pshIL-1RI-EGFP

pshIL-1RI-EGFP or pshGAPDH-EGFP was transfected into LEII cells. 24-36 hrs after transfection, the cells were labeled with a biotinylated anti-IL-1RI antibody and then stained with streptoavidin-PE. Transfected cells with pshIL-1RI-EGFP (EGFP positive cells) showed a reduced level of IL-1RI (MFI: 53.32 ± 7.38), while the cells that were transfected with pshGAPDH-EGFP showed a higher level of IL-1RI (MFI: 162.84 ± 17.20). This value is similar to that of the untransfected cells (MFI: 171.68 ± 7.54 and 126.84 ± 6.40) (Fig. 2). This result demonstrated that pshIL-1RI reduced endogenous IL-1RI in transfected cells. From this experiment we chose one pshIL-1R for the rest of the

experiment.

Reduction of IL-1RI mRNA in the transfected cells with pshIL-1RI-EGFP

To sort the transfected cells with a plasmid, we transfected LEII cells with pshIL-1RI-EGFP. 24-36 hours after transfection of the plasmid, we sorted the EGFP positive cells, and then performed RT-PCR. The pshIL-1RI-EGFP positive cells showed reduced IL-1RI mRNA compared to the control pshGAPDH-EGFP positive cells (Fig. 3)

Inhibition of angiogenesis induced by IL-1α using pshIL-1RI in eyes

Before we start *in vivo* experiment, we determined how much plasmid DNA should be injected. For this purpose, we injected pEGFP 0.5, 1, 2, 4, or 8μg per 2μl intrastromally, and found that injection with 1-4μg/2μl induced higher transfection. In addition, we investigated how long transfected pEGFP is expressed. Single injection of 2μg/2μl of EGFP into stromal cells induced high expression up to day 3. At day 4, signals were decreased, but they were detected up to day 8 and 9. In this study, we injected 2μg/2μl of psiIL-1RI or psiGFP.

One day after the intrastromal injection of pshIL-1RI or pshGFP, a pellet containing IL-1α was implanted. Four days after implantation, angiogenesis was observed. The pshIL-1RI injected eyes showed reduced angiogenesis compared to the pshGFP-injected eyes (Fig. 4). The angiogenic area of the eyes injected with pshIL-1RI was 1.34 ± 0.39 mm^2, and the eyes injected with pshGFP had an angiogenic area of 0.54 ± 0.22 mm^2 (Fig. 5). These results indicated that pshIL-1RI can reduce angiogenesis caused by IL-1α in mouse eyes.

Intrastromal injection of pshIL-1RI reduced mVEGF production induced by IL-1α treatment in eyes

To investigate whether the reduction of angiogenesis in the eyes injected with pshIL-1RI was mediated by inhibition of mVEGF production, we measured the VEGF protein level from the plasmid-injected eyes. Eyes injected with pshIL-1RI showed a highly reduced VEGF production compared to the pshGFP injected control eyes (142.50± ± 51.19 pg/eye vs 661.00 ± 199.57 pg/eye) (Table 1). This result indicated that reduced angiogenesis resulted from reduced VEGF production, and confirmed that the interruption of IL-1 and IL-1RI can reduce VEGF production and angiogenesis.

Discussion

Previously it was proposed that IL-1 production after ocular infection of HSV-1 induces IL-6 and VEGF, which causes angiogenesis and HSK (4). The IL-1Ra Tg mice, in which IL-1 and IL-1RI interaction is interrupted, showed reduced IL-6, VEGF, and its receptor VEGFR-2 after HSV-1 infection. It was also reported that IL-1 is required for both angiogenesis and tumor invasiveness using IL-1β or IL-1α knockout mice (18). A different study showed that IL-1α induces angiogenesis *in vivo* through the VEGF receptor pathway, possibly by inducing VEGF synthesis (19). These results suggest that the inhibition of IL-1 and IL-1R interaction might be useful as a therapeutic strategy to reduce angiogenesis.

There are several possible strategies to reduce the interaction between IL-1 and IL-1 receptor. Treatment of recombinant IL-1Ra is the most popular because it binds to the IL-1 receptor with a high affinity and prevents the binding of IL-1 to its receptor via classic competitive receptor antagonism (20). In addition, the IL-1RI antibody, antibodies to the IL-1 receptor accessory protein, and anti-IL-1 neutralizing antibodies can also be used to reduce IL-1 activity.

In this study, we investigated whether IL-1 activity could be reduced using RNAi technology. Our results demonstrated that pshIL-1RI transfected cells produced a lower level of IL-1RI at the mRNA and protein levels (Fig. 2, and Fig.3). Moreover, intrastromal injection of pshIL-1RI reduced the angiogenesis and VEGF production caused by IL-1α protein in mouse eyes (Fig. 4, Fig. 5, and Table 1). Originally, we tried to induce IL-1α production and angiogenesis by HSV-1 infection in eyes. However,

intrastromally-injected eyes with plasmid DNA are not well infected, so we implanted pellets containing IL-1α protein to trigger IL-1α and its receptor interaction, and angiogenesis. Although the angiogenesis is not induced by HSV-1 infection, our results showed that the potential of shRNA against IL-1RI to be used for therapeutic purposes.

Recently, another mechanism by which IL-1β induces angiogenesis was proposed. Cox-2 overexpression increased the expression of angiogenic factors, such as VEGF, and bFGF and the cox-2 inhibitor significantly reduced VEGF and bFGF production as well as angiogenesis (21, 22). Another experiment showed that IL-1β enhanced PEG2 and TXA2 production and induced angiogenesis (23). Induced production of prostanoids by IL-1β was blocked by the cox-2 selective inhibitors DFU and JTE522 and administration of the cox-2 inhibitors resulted in the suppression of IL-1β induced angiogenesis *in vivo*. Furthermore, TXA2 antagonist and EP4 antagonist inhibited IL-1β induced angiogenesis in mouse eyes (23). Therefore, treatment of siRNA targeting cox-2, which produces PGE2 and TXA2 from arachidonic acid, is also an attractive strategy to inhibit angiogenesis induced by IL-1.

RNAi technology is becoming popular because of the specificity and potency of gene silencing. In particular, it is widely used to determine unknown gene functions. On the other hand, some researchers are trying to use this technology for therapeutic purposes. However, enough cells must be transfected by siRNA or shRNA in order to achieve enough suppression of unwanted gene expression *in vivo*. At this time, we do not know how many cells are transfected by pshIL-1RI, although it was already shown that intrastromal injection with a plasmid can transfect stromal cells (15, 24). One possible experiment with which transfection efficiency after intrastromal injection with a plasmid

could be determined is the confocal image analysis of wholemount cornea (24). After excision and fixation of pEGFP-injected corneas, they are flatmounted on a slide with medium containing DAPI and placed for 48hours to allow DAPI to penetrate. Using a spectral laser scanning confocal microscope with the corresponding laser for DAPI and EGFP, corneas could be analyzed from z-stacks to see how many cells are transfected in stromal cells.

Our results showed that intrastromal injection with psiIL-1RI reduced angiogenesis and VEGF production induced by IL-1α. Therefore, we assumed that intrastromal injection transfected enough cells to reduce angiogenesis caused by the IL-1α treatment. Otherwise, shRNA might be spread to neighbor cells after expressed in transfected cells and inhibit gene expression in untransfected cells as well as transfected cells. In fact, plant and *C.elegans* have systemic distribution of siRNA when siRNA is injected locally (25, 26). In addition, global decrease in HCV protein expression was observed in S1179I cells, suggesting that either all of the cells were transfected with the siRNA or that RNAi may have spread from cell to cell (27). For the transfection of a plasmid into stromal cells, we injected plasmid DNA with pressure to increase transfection, so transient disruption of cell membrane might be important for the plasmid transfection. However, shRNAs are much smaller than plasmid DNA. Therefore, it may be possible to be spread (possibly through gap junction) to next cells after shRNAs are expressed in transfected cells.

Even though we do not know which one is true, it seems that the eye is a suitable organ to use RNAi technology for *in vivo* experiments because we and other researchers have shown that the injection of siRNAs into eyes may inhibit their target gene expression as well as reduce symptoms (28-30). Previous *in vivo* experiments targeting

eyes used slightly different delivery method and different types of dsRNA. Nakamura et al. injected synthetic siRNA using subconjunctival injection. In other experiments, subretinal injection or *in vivo* eletroporation were used to transfect retina with adenoviral shRNA or plasmid-based shRNA. In our study, we used intrastromal injection with plasmid-based shRNA.

Each type of dsRNA has its advantages and disadvantages. Synthetic siRNAs are more convenient and have a higher transfection efficiency, but they are more expensive and less stable. Virus-mediated shRNAs have a high transfection efficiency and are stable, but viral infection causes inflammation. On the other hand, plasmid-based shRNAs are stable, which makes shRNAs active for longer than siRNAs, inexpensive and non-toxic, but their transfection efficiency is lower than other dsRNAs. Therefore, the choice of delivery system and dsRNA depends on the purpose of the experiment. In this study, we transfected plasmid-based shRNA using intrastromal injection to inhibit the target gene in stromal cells for longer without causing any inflammation.

Although RNAi technology has limited use *in vivo* because of delivery problems, our study shows that RNAi technology may be an attractive strategy when used in mouse corneas to study corneal biology as well as corneal disease.

LIST OF REFERENCES

List of references

1. Thomas, J.T. and Rouse, B.T. 1997. Immunopathogenesis of herpetic ocular disease. Imm. Research. 16/4, 375-386.
2. Zheng, M., Deshpande, S., Lee, S., Ferrara, F., and Rouse, BT. 2001. Contribution of vascular endothelial growth factor in the neovascularization process during the pathogenesis of herpetic stromal keratitis. J. Viol. 75, 9828-9835.
3. Zheng, M., Schwarz, MA., Lee, S., Kumaraguru, U., and Rouse BT. 2001. Control of stromal keratitis by inhibition of neovascularization. Am. J. Pathol. 159, 1021-1028.
4. Biswas, PS., Banerjee, K., Kim, B., and Rouse, BT. 2004. Mice transgenic for IL-1 receptor antagonist protein are resistant to herpetic stromal keratitis: possible role for IL-1 in herpetic stromal keratitis pathogenesis. J. Immunol. 172, 3736-3744.
5. Dinarello, C.A. 1997. Interleukin-1. Cytokine& Growth Factor Reviews. 8, 253-265.
6. March, C.J., Mosley, B., Larsen, A., Cerretti, D.P, Cerretti, D.P., Braedt, G., Price, V., Gillis, S., Henney, C.S., Kronheim S.R., Grabstein, K., Conlon, P.J., Hopp, T.P. and Cosman, D. 1985. Cloning sequence and expression of two distince human interleukin 1 complementary DNAs Nature 315, 641-644.
7. Lomedico, P.T., Kilian, P.L., Gubler, U., Stern, A.S. and Chizzonite, R. 1986. Molecular biology of interleukin 1. Cold Spring Harbor Symp. Quant. Biol. 51,

631

8. Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806-811.
9. Vauchert, H., Beclin, C., and Fagard. M. 2001. Post-transcriptional gene silencing in plants. J. Cell Sci. 114, 3083-3091.
10. Zamore, P.D., Tuschl, T., Sharp, P.A., and Bartel, D.P., 2000. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21-23 nucleotide intervals. Cell 101, 25-33.
11. Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T., 2001. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494-498.
12. Brummelkamp, T.R., Bernards, R., and Agami, R. 2002. A system for stable expression of short interfering RNAs in mammalian cells. Science 296, 550-553.
13. Paddison, P.J., Caudy, A.A, Bernstein, E., Hannon G.J., and Conklin, D.S., 2002. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes and Dev. 16, 948-958.
14. Sui, G., Soohoo, C., Affar, E.B., Gay, F., Shi, Y., and Forrester, C. 2002. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc. Natl. Acad. Sci. USA 99, 5515-5520.
15. Stechschulte, S. U., Joussen, A. M., von Recum, H. A., Poulaki, V., Moromizato, Y., Yuan, J., D'Amato, R. J., Kuo, C., and Adamis, A. P. 2001. Rapid ocular angiogenic control via naked DNA delivery to cornea. Invest. Ophthalmol. Visual

Sci. 42:1975

16. Kenyon, BM., Voest, E.E., Chen, C.C., Flynn, E., Folkman, J., D,Amato, R.J. 1996. A model of angiogenesis in the mouse cornea. Invest. Ophthalmol. Vis Sci. 37, 1625-1632.

17. Schwarz, M.A., Kandel, J., Brett, J., Li, J., Hayward, J., Schwarz, R.E., Chappy, O., Wautier, J., Chabot, J., Gerfo, P.L., Stern, D. 1999. Endothelial-monocyte activating polypeptide II, a novel antitumor cytokine that suppresses primary and metastatic tumor growth and induces apoptosis in growing endothelial cells. J. Exp. Med. 190, 341-353.

18. Voronov, E., Shouval, D. S., Krelin, Y., Cagnano, E., Benharroch, D., Iwakura, Y., Dinarello, C. A., Apte, R. N. 2003. IL-1 is required for tumor invasiveness and angiogenesis. *Proc. Natl. Acad. Sci. USA* 100, 2645-2650

19. Salven, P., Hattori, K., Heissig, B., Rafii, S. 2002. Interleukin-1α promotes angiogenesis in vivo via VEGFR-2 pathway by inducing inflammatory cell VEGF synthesis and secretion. *FASEB J.* 16,1471-1473

20. Granowitz, E.V., Porat, R., Mier, J.W., Pribble, J.P., Stiles, D.M., Bloedow, D.C., Catalano, M.A., Wolff, S.M., and Dinarello, C.A. 1992. Phamacokinetics, safety, and immunomodulatory effects of human recombinant interleukin-1 receptor antagonist in healthy humans. Cytokine 4, 353-360.

21. Sawaoka, H., Tsuji, S., Tsujii, M., Gunawan, E. S., Sasaki, Y., Kawano, S., Hori, M. 1999. Cyclooxygenase inhibitors suppress angiogenesis and reduce tumor growth in vivo. Lab. Invest. 79,1469-1477

22. Majima, M., Hayashi, I., Muramatsu, M., Katada, J., Yamashina, S., Katori, M.

2000. Cyclo-oxygenase-2 enhances basic fibroblast growth factor-induced angiogenesis through induction of vascular endothelial growth factor in rat sponge implants. Br. J. Pharmacol. 130,641-649

23. Kuwano, T., Nakao, S., Yamanoto, H., Tsuneyoshi, M., Yamamoto, T., Kuwano, M., and Ono, M. 2004. Cyclooxygenase 2 is a key enzyme for inflammatory cytokine-induced angiogenesis. Faseb J. 18, 300-310.
24. Carlson, E.C., Liu, C-Y., Yang, X., Gregory, M., Ksander, B., Drazba, J. and Perez, V.L. In vivo gene delivery and visualization of corneal stromal cells using an adenoviral vector and keratocyte-specific promoter. Invest Ophthalmol Vis Sci. 45, 2194-2200. (2004).
25. Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806-811 (1998).
26. Palauqui, J.C., Elmayan, T., Pollien, J.M. and Vaucheret, M. EMBO J 16, 4738-4745. (1997).
27. Kapadia, S.B., Brideau-Andersen, A., Chisari F.V. 2003. Interference of hepatitis C virus RNA replication by short interfering RNAs Proc. Natl. Acad. Sci USA 100 2014-2018.
28. Nakamura, H., Siddiqui, S.S., Shen, X., Malik, A.B., Pulido, J.S., Kumar, N.M., Yue, Y.J.T. 2004. RNA interference targeting transforming growth factor-β type II receptor suppresses ocular inflammation and fibrosis. Mol Vis. 10, 703-710.
29. Matsuda, T. and Cepko, C. 2004. Eletroporation and RNA interference in the rodent retinal in vivo and in vitro. Proc. Natl. Acad. Sci. USA. 101, 16-22.

30. Riech, S.J., Fosnot, J., Kuroki, A., Tang, W., Yang, S., Maguire, A.M., Bennett, J., and Tolentino, M.J. 2003. Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. Mol Vis 9, 210-216

APPENDIX

Fig. 1 Plasmid constructs 2.

A. pshIL-1RI expresses shRNA against IL-1 receptor type 1(IL-1RI)

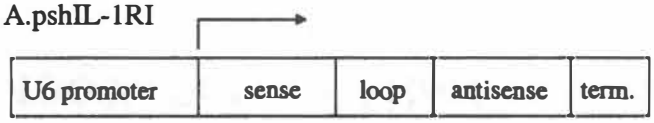
B. pshIL-1RI-EGFP expresses shRNA against IL-RI and EGFP bicistronically

Sense: sense sequence, Antisense: antisense sequence,

Term.: RNA polymerase III transcription termination sequence,

CMV: CMV promoter, Poly A: poly A sequence

A.pshIL-1RI



B. pshIL-1RI-EGFP

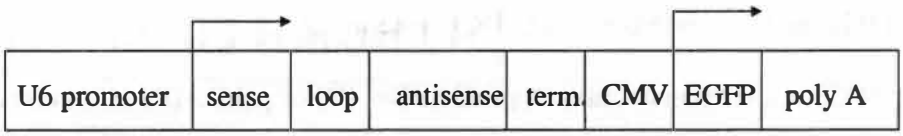


Fig. 1

Fig. 2 Inhibition of endogenous IL-1R I protein.

24-36hr after transfection of pshGAPDH (B) or pshIL-1RI (C), cells are harvested and IL-1RI was stained

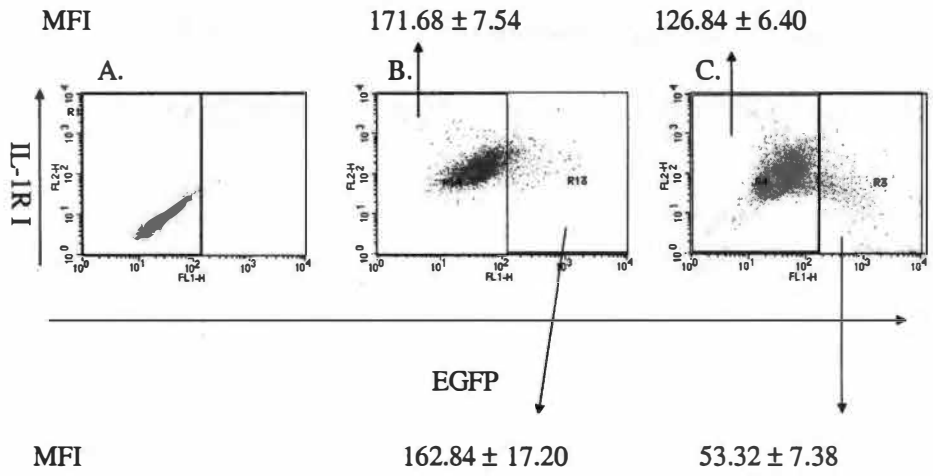

MFI
171.68 ± 7.54
126.84 ± 6.40
A.
B.
C.
IL-1R I
EGFP
MFI
162.84 ± 17.20
53.32 ± 7.38

Fig. 2

Fig. 3 Inhibiton of endogenous mRNA of IL-1R I.

After sorting of EGFP positive cells (lane 1: pshVEGF-EGFP$^+$, lane 2: pshIL-1RI-EGFP$^+$), tatal RNA was isolated and RT-PCR was performed.

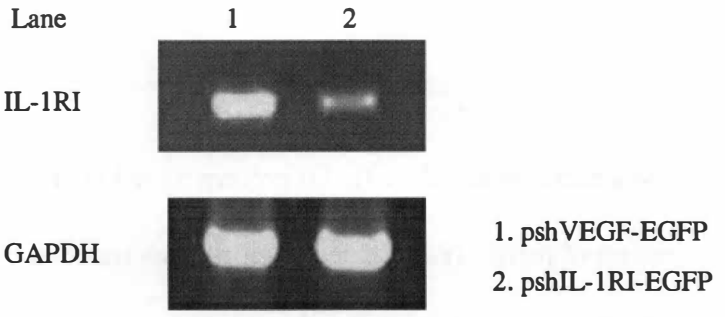
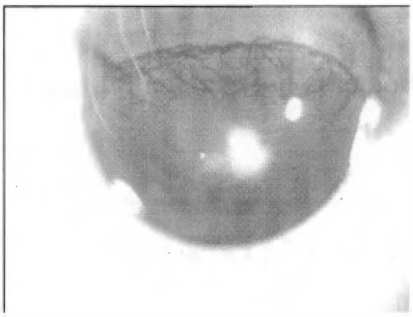


Fig. 3

Fig. 4 Reduction of angiogenesis induced by IL-1α protein by pshIL-1RI.

Intrastromal injection of pshIL-1RI reduced angiogenesis induced by IL-1α protein. pshGFP was used as a control plasmid.

A. pshGFP



B. pshIL-1R

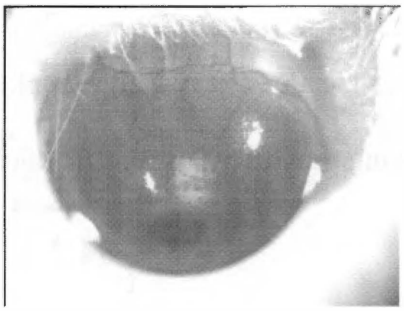


Fig. 4

Fig. 5 Angiogenic area at day 4 after implantation of pellet containing IL-1α following pshGFP or pshIL-1RI injection.

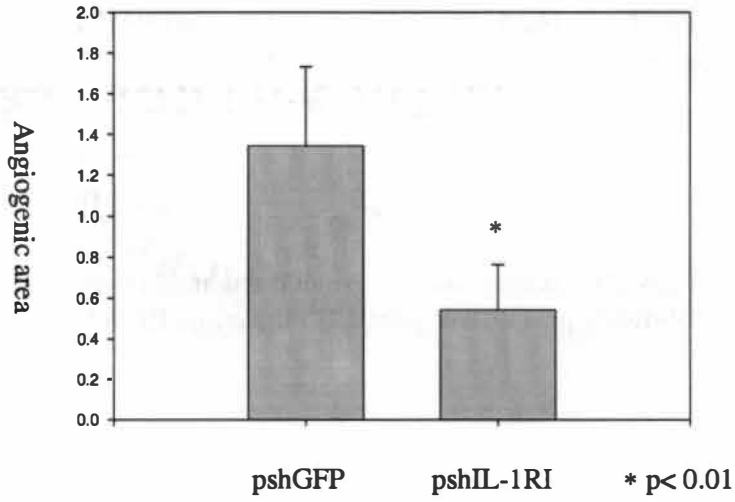

Angiogenic area
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
pshGFP
pshIL-1RI
* p< 0.01

Fig. 5

Table 1. Measurement of the amount of IL-1RI in cornea using ELISA

	pshGFP	pshIL-1RI
VEGF pg/ eye	142.5± 50.19 *	661.0±199.57

* $p < 0.03$

4 day after implantation of pellet containing IL-1α protein following pshGFP or pshVEGF injection, ELISA was performed.

Concluding remarks

The aim of our investigations was to exploit the novel approach of shRNA to control ocular angiogenesis *in vivo*. Previous experiments demonstrated that angiogenesis in the cornea is a critical step in HSK, which is caused by ocular infection with HSV-1. Although the mechanisms of angiogenesis induced by ocular infection of HSV-1 are unclear, it was shown that bio-active CpG motifs in HSV-1 DNA could contribute to angiogenesis in the eyes, and the angiogenesis was mediated by VEGF production. From additional experiments, it was demonstrated that treatment of the anti-mVEGF antibody prevents CpG induced angiogenesis in mouse eyes. Thus, these results indicate that mVEGF is a critical factor for angiogenesis caused by CpG motif in mouse eyes, and mVEGF might be a good target molecule to reduce angiogenesis and HSK severity.

From different experiments, another mechanism of angiogenesis induced by ocular infection of HSV-1 has been proposed. The experiments showed that ocular infection of HSV-1 induced IL-1 and IL-6, and the increased expression of the cytokines stimulated angiogenesis through up-regulation of mVEGF. These results indicated that cytokine production caused by ocular infection of HSV-1 is a main reason of production of mVEGF and induction of angiogenesis. This result was confirmed by the experiments using IL-1 receptor antagonist (IL-1Ra) transgenic mice. IL-1Ra binds to the IL-1 receptor type I without transmitting activation signal, so it inhibits IL-1 activity. The IL1Ra transgenic mice showed reduced HSK severity and angiogenesis compared to wild type mice when their eyes are infected with HSV-1. This result indicates that inhibition of the interaction between the IL-1 and IL-1 receptor can reduce HSK severity and angiogenesis, and IL-1 or IL-1 receptor may be good candidate molecules to be targeted.

From these previous experiments, we hypothesized that IL-1 and mVEGF are key molecules for angiogenesis caused by ocular infection of HSV-1 in mouse eyes, and investigated whether inhibitions of mVEGF and IL-1 receptor gene expressions using RNAi technology could reduce angiogenesis in mouse eyes. RNAi is a sequence specific gene silencing phenomenon by introduction of dsRNA. There are a few different dsRNA inducing RNAi. In this study we used plasmid based- shRNA to inhibit target gene expression because it is effective for longer than siRNA (this feature is required for our experiments because we observed eyes at day 5 after injection.), inexpensive, and non-toxic in eyes.

In vitro experiments showed that the transfected cells with a plasmid DNA expressing mVEGF shRNA or IL-1RI shRNA reduced exogenous and endogenous target gene expressions. For these experiments, we constructed plasmid expressing EGFP and shRNA bicistronically to identify transfected cells. The transfected cells, which are EGFP positive, were obtained by FACS sorting and were used to detect mRNA levels by RT-PCR or real time PCR. To detect protein level flow cytometry was performed and the results showed that EGFP positive cells reduced production of target proteins.

After confirmation of the effectiveness of the plasmid based-shRNA, we investigated whether intrastromal injection with the plasmid could inhibit angiogenesis caused by CpG motifs or IL-1α. The results showed that either plasmid expressing mVEGF shRNA or IL-1RI shRNA reduced angiogenic area and mVEGF production. These results imply that RNAi technology can be used in mouse eyes and help to understand corneal biology and to treat corneal disease induced by unwanted gene expression.

Recently, another mechanism by which IL-1β induces angiogenesis was proposed in eyes. The experiments showed that treatment of IL-1β increased cox-2 production, and the cox-2 production induced angiogenic factors, such as VEGF and bFGF. In addition, treatment of cox-2 inhibitor reduced VEGF and bFGF production as well as angiogenesis. Therefore, it will be interesting to determine whether angiogenesis can be decreased after targeting of cox-2 mRNA using RNAi technology and to compare the results with those from the experiments with shRNA against IL-1 receptor.

RNAi technology requires sufficient transfection. At this point we do not know how many cells are transfected after intrastromal injection with plasmid. In the future, this question should be solved. One possible experiment to solve the question might be confocal image analysis of wholemount cornea which is injected with a plasmid expressing EGFP.

We and other researchers showed that RNAi is induced in mouse eyes, although we do not know how many cells are transfected. Therefore, it seems that the injection delivered plasmid into enough number of cells to induce RNAi. Otherwise, expressed shRNA in transfected cells might be spread to neighbor cells and induce RNAi in un-transfected cells, as well as to transfected cells. It is very important to know how many cells could be transfected with siRNA or shRNA *in vivo*, and how many cells should be transfected in order to see physiological effects *in vivo*. Another important issue in RNAi technology is off-target effect. Some researchers reported that siRNAs degraded, not only target mRNAs, but also other mRNAs. Now, we do not know why off-target effects occur. We need to know about RNAi machinery more in detail to solve the problems.

RNAi phenomenon was discovered in 1998, and RNAi was started to use in mammalian cells in 2002. Although this technology has very short history, application of this technique spread very fast especially for the *in vitro* experiments to determine unknown gene function. Recently, siRNA libraries were developed to screen many genes at a same time. Therefore, this technology will help us to understand many unknown gene functions. RNAi also has potential as a therapeutic strategy because of its specificity and potency. However, there are many questions including delivery systems to be solved before this technology can be used for clinical purposes. Although there are some limitations in RNAi-based therapies, significant work is ongoing to solve the problems. Even though analyzing gene functions using RNAi already impacted many biological sciences, better understanding of the nature of siRNAs and RNAi mechanisms will make this technology more useful.

VITA

Yunsang Lee was born in Seoul, Korea in May 1967. In 1986 he entered Inha University, Korea and was awarded the Bachelor of Science Degree in Biology with honor in 1990. In March of that year, he entered Seoul National University, Korea and received Master's Degree in Molecular Biology in 1992. After graduation, he joined military service for about 2 years. In 1995, he began graduate studies at The University of Tennessee, Knoxville and received the Degree of Master of Science in Biochemistry and Cellular and Molecular Biology in 2000. In 2001, he entered Ph.D program in Comperative and Experimental Medicine at The University of Tennessee, Knoxville and will graduate December 2004. He plans to pursue postdoctoral training in the U.S.