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Evaluation of Antiangiogenic Potential of MMP_2 Antisense Oligonucleotide for the Management of Proliferative Diabetic Retinopathy Using Chicken Chorioallantoic Membrane

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Abstract

Proliferative diabetic retinopathy (PDR) is the advanced stage of diabetic retinopathy in which neovascularization occurs at the retina. Angiogenesis, the formation of new vessels from the existing one involves sequential step starting from the degradation of basement membrane by matrix metalloproteinases (MMPs). The elevated expression of MMP2 is considered as the one of the important parameter in the progress of PDR. Therefore, it is important to inhibit the activity of MMP2 in the retina of the PDR patients for the management of the diseases. In this concern we examined the anti angiogenic potential of antisense MMP2 oligonucleotide using chicken late CAM (chorio allantoic membrane) using various techniques. CAM has been used as a model for retinal research by ophthalmologist for many years due to its similarities with retina in the tissue thickness and blood vessels formation. MMP2 ASO inhibited blood vessels growth at the treated area and their length and size and reduced the thickness of the CAM compared to control. MMP2 ASO enhanced the accumulation of fibroblast and flattened endothelial cells and reduced the expression of MMP2 at gene and protein level. The chorionic epithelial layer of the CAM got flattened after the treatment with MMP2 ASO as an indication of lack of blood vessels formation and growth at the treated area. Altogether, our results demonstrates that MMP2 ASO is able to inhibit neovascularization effectively and therefore can be a suitable therapeutic molecule in the management of pathological neovascularization.

Keywords: Angiogenesis, neovascularization, antisense oligonucleotide MMP2.

Abbreviations: PDR: proliferative diabetic retinopathy; MMP2: matrix mettaloproteinases; CAM: chorio allantoic membrane; ECM: extra cellular matrix; EC: endothelial cells

Introduction

Angiogenesis refers as the formation of new capillaries from the existing vessels as a result of various complex events starts from the enzymatic degradation of basement membrane, matrix remodeling followed by endothelial cell (EC) proliferation, migration and differentiation [1]. Matrix metalloproteinases are the family of Zinc dependent proteolytic enzymes deal with the digestion and remodeling of basement membrane and extracellular matrix during angiogenesis. [2]. Diabetic retinopathy (DR) is a common microvascular disorder occurs in diabetic population that can lead to blindness. The proliferative diabetic retinopathy (PDR) develops when the oxygen demands of the inner retina exceeds resulting in the up regulation of angiogenic factors which promotes neovascularization [3,4]. Reported study of Das and Kousuke showed that fibro vascular tissue of PDR patients contains activated form of MMP2 [5,6]. In 2013, Murilo et al. reported that activator HIF-1 can induce MMP2 expression in DR by increasing the expression of VEGF165 by Muller cells in hypoxic condition [7].

So far various methods using laser and cryotherapy are available for the treatment of PDR in its advanced stage. Treatment with anti VEGF molecule along with laser treatment is found to be more effective especially in the case of macular edema [8]. Hence it is important to develop a pharmaceutical agent which work along with other conventional treatment to prevent pathologic angiogenesis to provide a better management for PDR at its various stages of progression. Since MMP2 expression is highly up regulated in PDR patients we hypothesized that inhibiting MMP2 activity by small molecule called antisense oligonucleotide will evoke a new effective therapeutic approach for the treatment of retinal neovascularization.

In the present work we try to understand the inhibitory effect of antisense MMP2 oligonucleotide on angiogenesis using chicken chorioallantoic membrane (CAM) assay as a model for the retinal vascular system. Antisense oligonucleotides referred as short, synthetic oligonucleotide that are complementary in sequence and upon specific hybridization to its cognate gene product induces inhibition of gene expression [9]. The chick CAM is a part of extra embryonic tissue and is analogous to the retinal vasculature with roughly comparable tissue thickness and also in the size of the blood vessels [10]. CAM is widely used to study retinal angiogenesis by ophthalmologist researchers in normal [11] and also for various retinal disorders like age related macular degeneration [12], diabetic retinopathy [13] and is also used as a model system for the study of the precision and safety of vitreous retinal micro surgical instruments and techniques [10].

In this research work we used commercially available MMP2 antisense oligonucleotide sodium salt of 15 nucleotide sequences (5'-CACACCTTGCCATCG-3'- ASO) for better effectiveness and its anti angiogenic potential was analyzed and compared under three different concentrations. The delivery of the chemical was done by gelatin sponges to have a regional stimulation without much or no inflammation [14]. The inhibitory effect of antisense MMP2 on angiogenesis was scored by various methods to understand the effect from the cellular to the molecular and protein level. Altogether our data

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suggested that therapy using MMP2 antisense oligonucleotide for the treatment of PDR in it early stage of proliferation followed by other conventional treatment will have a promising therapeutic inhibitory effect on PDR neovascularization.

Material and Methods

Chorioallantoic membrane assay

Fertilized White Leghorn chicken eggs (*Galus galus domesticus*) weighing 50 ± 2 g (Tamil Nadu Poultry Research Station Nandanam, Chennai, India) were incubated at 37°C in a humidified atmosphere (>60% relative humidity) as per the protocol for Hen's Egg Test - Chorioallantoic Membrane (HET-CAM) method [15]. On day 3rd of post incubation, 2 to 3 ml of albumin was withdrawn using a 21-gauge needle, through a small opening at the large blunt edge of the egg in order to minimize adhesion of the shell membrane with CAM. Then a square window of 1 cm^2 was opened in the egg shell at the opposite side of blunt edge and sealed with paraffin film to prevent dehydration. The eggs were returned for further incubation. On embryonic day 9, gelatin sponges (Johnson & Johnson Pvt Ltd, Mumbai, India) cut into a size of 1mm^3 were placed on the top of growing CAM under sterile conditions [14]. The sponges were soaked with 10 µl of 10, 30 and 50 µM concentration of phosphorothioate MMP2 ASO Sodium Salt (Calbiochem, EMD Biosciences, San Diego, CA). Phosphate-buffered saline (PBS, pH 7.4) served as negative control and was denoted as 0 µM concentration [13]. The window was closed with a transparent adhesive tape and the eggs were returned for further incubation till day 12 at which vascularization potential of the CAM reaches its maximum. *In ovo* CAM was photographed at 0, 24, 48 and 72 hours using digital camera of 12X5.0 Mega Pixel (Power Shot A95). Total length and size of the tubule complexes were measured as blood vessels length and size at the area of treatment with Image J and *AngioQuant* Toolbox, MATLAB 6.5 (R13) softwares [17-19]. For the experiments the eggs were categorized into 4 group, each containing 40 eggs each. After 72 hours of incubation with phosphorothioate MMP2 ASO, CAM tissues were flooded with Bouin's fixative for 24 hours, dehydrated and embedded in paraffin wax. Vertical tissue sections of 5 µm in thickness were taken using rotary microtome (Weswox, Hariyana, India). After staining with hematoxylin and eosin (HE), sections were mounted with DPX and observed using light microscope at 40X magnification (4X objective and 10X eyepiece). The images were recorded at 10X magnification using Nikon D70 DSLR (6.1 megapixel) camera attached to light microscope [20].

Morphometry

Thickness of the CAM (*d*CAM) was measured on H&E stained cross sections with a calibrated objective at 40X magnification, using 10x10 calibrated grid (16mm diameter) at the 10X ocular. Distance between the allantoic and chorionic epithelial layers of the CAM was measured at 6 different locations from 6 serial cross sections of the same sample and were averaged to yield mean CAM thickness [16]. In paraffin-embedded tissue, material shrinkage is estimated to be ~25% relative to the fresh material and all tissues were prepared similarly, thus, shrinkage corrections are unnecessary for the comparisons of tissue thickness [21].

Semi-quantitative reverse transcriptase–polymerase chain reactions (RT-PCRs)

Total RNA was isolated from CAM (10 samples from each group) after treatment using TRI*zol* reagent according to the manufacture's protocol (Sigma Aldrich, St. Louis, MO, USA). The quantity and the purity of the isolated RNA was checked with UV-Visible spectrophotometer and after running on 1% agarose gel electrophoresis, respectively. cDNA was synthesized for each group from 5 µg of total RNA using ImProm-11™ Reverse Transcriptase kit with Oligo (dt) (MWG, Eurofins, Stuttgart, Germany) based on manufacture's protocol (Promega, Madison, WI, USA). PCR amplification was performed using GoTaq Green Master Mix kit with 1.5 µl of cDNA from each group (Promega, Madison, USA). PCR reaction was set up based on manufacture's protocol. Variation in the mRNA expression of pro-angiogenic molecules namely VEGF165 [22], FGF2 [23], MMP2 [22] and MMP9 [23] were evaluated using PCR method with 100 Pico moles of chicken specific primers (Bioserve, Hydrabad, India) and the relative level of mRNA from each amplified transcripts were normalized with GAPDH as control [24]. PCR products (5 µl each) were subjected to electrophoresis on 1.5% agarose gels containing 0.5 µg/ml Ethidium Bromide and photographed using Cannon digital camera of 12X5.0 Mega Pixel (Power Shot A95). The base pair products were compared against DNA ladder of 100 base pair (Invitrogen, Carlsbad, CA, USA). The relative density of the bands per experiment was calculated using *Scion Image release α* 4.0 3.2 software. Specific primer sequences and PCR reaction set up was given in Tables 1 and 2, respectively.

Gelatin zymography

CAM was incubated with phosphorothioate MMP2 ASO for 72 hours and homogenized (100 mg/ml) using Tris buffer (0.5M Tris-HCL, pH 6.8 containing 10% sodium dodecylsulphate, glycerol and 0.01% bromophenol blue), and centrifuged at 15,294 *g* at 4°C for 10 minutes. The concentration of protein in the supernatant was determined using Bradford reagent (Bangalore Genei, Bangalore, India) and the gelatinase activity was examined on a 10% SDS-PAGE containing 1 mg/ml of gelatin (Medox, Chennai, India). Protein samples of 25 µg/40 µl were loaded per well along with 20 µl of pre-stained SDS-PAGE standard marker (Bio-Rad, Meyerside Drive Mississauga, Canada). After electrophoresis, the gels were washed with 2.5% Triton-X-100 and incubated in digestion buffer (100 ml 50 mM Tris HCl, pH 7.5, 100 mM CaCl2, 1 µM $ZnCl_2$, 1% Triton X-100, 0.02% NaN_3) for 16 to 18 hours at 37°C with gentle agitation. Gels were stained with staining solution (0.05% Coomassie blue in 50% of methanol and 10% of acetic acid) for 1 hour and destained with methanol/acetic acid mixture. Gelatinase activity of MMP2 was detected as clear bands against background. The gels were scanned and images were recorded using Bio-Rad Calibrated Densitometer Software

Gene Name	Primer Sequence	Base Pair
VEGF (165)	Sense-5'-GACCCTGGTGGACATTTTCC-3' Antisense-5'-TGCGCTCGTTTAACTCAAGC-3'	381 bp
bFGF2	Sense- 5'-TTCTTCCTGCGCATCAAC-3' Antisense-5'-GGATAGCTTTCTGTCCAG-3'	250 bp
MMP2	Sense- 5'-CCTACACCAAGAACTTCC-3' Antisense-5'-ACTCCATTCCAAGAATCC-3'	580 bp
MMP9	Sense-5'-GATGCCATTGATGATGATGAG-3' Antisense-5'-GGTCCATATTTCCRTCTTGA-3'	1400 bp
GAPDH	Sense- 5'- GAGGAAAGGTCGCCTGGTGGATCG-3' Antisense-5'-GTGAGGACAAGCAGTGAGGAACG-3'	300 bp

Table 1: Primer Sequences with base pair.

Gene Name	Denaturation	Annealing	Extension	Cycles
VEGF(165)	94°C/1min	59°C/1min	72°C/1min	40
bFGF2	94°C/1min	54°C/1min	72°C/1min	35
MMP2	94°C/30sec	60°C/30sec	72°C/1min	35
MMP9	94°C/30sec	48°C/30sec	72°C/1min	35
GAPDH	94°C/30sec	60°C/30sec	72°C/1min	35

Table 2: Amplification conditions.

(GS 800, Milford, MA, USA). Density of the bands was calculated using PD Quest Advances Software and was normalized with control [25].

Scanning electron microscopic (SEM) analysis

Treated area of the CAM was dissected out and washed with PBS after 72 hours of treatment with 50 µM concentration of phosphorothioate MMP2 ASO. It was dried at room temperature. The unfolded air-dried membranes were glued onto stubs with carbon, spattered with gold (10 min, 14-17 Ma, 0.07 mbar) and observed under a Hitachi S-3400N Variable Pressure Scanning Electron Microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 15-30 kV and the images were recorded at 100X magnification [26,19].

Immunohistochemistry

The deparafinized and dehydrated CAM treated with 50 µM concentration of phosphorothioate MMP2 ASO was allowed to undergo antigen retrieval process using 10 mM sodium citrate (pH 6.0) in a microwave oven for 20 minutes and then washed in double-distilled water for 3x5 min in PBS (pH 7.3). Normal Goat Serum Blocking Solution (containing 2% goat serum, 1% BSA, 0.1% cold fish skin gelatin, 0.1% Triton X-100, 0.05%, Tween- 20, 0.05% sodium azide in 0.01 M PBS, pH 7.2) of 50 to 75 µl was added immediately on the sections and then incubated for 1 h in a humidified chamber. After washing with PBS, primary antibody of MMP2 (1:200, rabbit polyclonal, was applied on the sections and after overnight incubation rinsed with PBS containing 0.05% Tween-20. FITC-conjugated secondary antibody (1:40 dilution) was applied for 1 h according to manufacturer's instruction (Goat anti-rabbit IgG, Bangalore Genei, Bangalore, India). Images were recorded at 40X magnification using BX51 Olympus Fluorescence Microscope at a wavelength of 515 nm with ASI FISH View 5.5 software [27].

Statistical analysis

All the experiments were performed in triplicate (n=3) unless otherwise specified. Data are presented as mean ± SEM and were analyzed by One-Way ANOVA analysis of variance test, Student's t-test and Tukey *post hoc* tests as appropriate using *Sigma Stat* 2.0. P value less than <0.001 was considered statistically significant.

Results

Antisense MMP2 inhibits angiogenesis

The effect of MMP2 ASO on angiogenesis was analyzed using CAM vascular bed. (Figure 1), shows the representative images of CAM recorded at 0 and 72 hours of treatment with 0, 10, 30 and 50 µM concentrations of MMP2 ASO. The MMP2 ASO able to inhibit new blood vessel formation effectively at the treated area and in all cases the inhibition seemed to be more prominent in and around of gelatin sponges which is suggestive of more localized effect of MMP2 ASO. CAM treated with 50 µM MMP2 ASO shows total inhibition of blood vessels formation around the gelatin sponge (Figure 1.o) and the same observation was further supported with *Angioquant* prune images in which blood vessels were marked as red line. The prune image of CAM treated with 50 µM MMP2 ASO (Figure 1.p) shows less or no blood vessels around the sponge when compared to 0 hour image (Figure 1. m). The inhibitory effect of MMP2 ASO on the growth of blood vessels was further confirmed by measuring the length and size of the vessels from the images of the treated CAM snapped at 0, 24, 48 and 72 hours of treatment using *Angioquant* software. (Figure 2), indicates that MMP2 ASO was able to inhibit the growth of blood vessels by reducing their length and size. MMP2 ASO at the concentrations of 10, 30 and 50 µM decreased the length and size of the blood vessels. The reduction

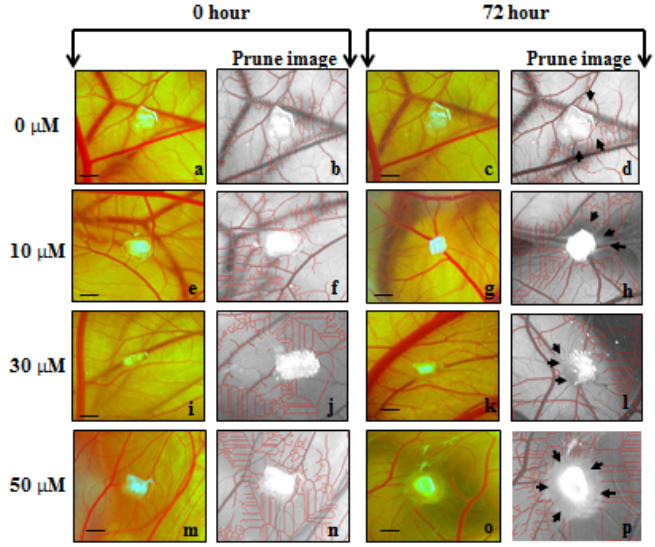

Figure 1. MMP2 ASO inhibit angiogenesis on CAM vascular bed. 1. Images of late CAM treated with 10, 30 and 50 µM concentration of MMP2 ASO and control treated with PBS for 72 hours. CAM incubated with 50 µM MMP2 ASO shows diminished blood vessels around the sponge compared to other concentrations (figure O). The same observation was further supported by skeletonized prune images by *Angioquant software* (figure p*)*. Images were taken using Cannon digital camera at 4X magnification and are representative of 3 independent experiments. Arrow: absence of blood vessels, bar - 10 µm.

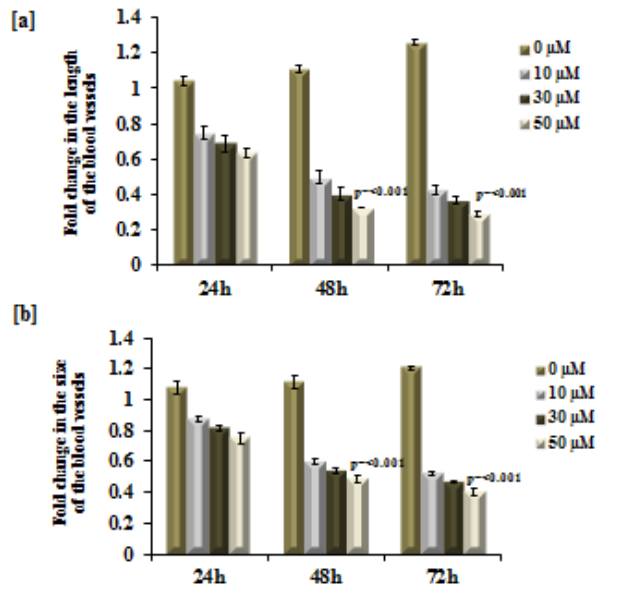

Figure 2. MMP2 ASO retard the growth of blood vessels on CAM. Total length and size (diameter) of the blood vessels was measured using *Angioquant* software from the images taken at 0, 24, 48 and 72 hours of incubation with various concentrations of MMP2 ASO. CAM treated with 50 µM MMP2 ASO shows significant reduction in the length **(Graph a)** and size **(Graph b)** of the vessels at 48 and 72 hours of incubation. Graph indicates that 50 µM concentration of MMP2 ASO significantly inhibited angiogenesis than other two concentrations from 48 hours of incubation. Experiments were performed in triplicate and data presented as mean ± SEM, * p <0.001versus control, n=3.

was statistically significant (p<0.001) at 50 µM concentration for 48 and 72 hours in which it shows 0.96 fold decrease in the size and 0.735 fold decrease for length of the blood vessel when compared to 0 hour value of the same concentration (Figure 2, graph a and b).

Antisense MMP2 changes the morphology of CAM

Effect of antisense MMP2 on angiogenesis was further analyzed by observing the morphological changes of CAM. The changes were assessed from haematoxylin and eosin stained cross sections of CAM treated with 10, 30 and 50 µM MMP2 ASO (Figure 3) and the tested samples were qualitatively compared with control CAM for morphological features. Control CAM shows uniform thickness at thin stratum along with fibroblast accumulation above the sub epithelial capillary network (SEC). The blood vessels are more prominent with small vessels around large vessels as a result of active neovascularization (Figure 3a). CAM treated with 10 µM MMP2 ASO shows normal chorionic and allantoic epithelial layers and diminished accumulation of fibrocytes at thin stratum. The number of blood vessels is reduced and existing large vessels show flattened appearance indicative of narrowing of vessels under treatment (Figure 3b). CAM treated with 30 µM MMP2 ASO shows flattened endothelial cells without sprouting appearances with few scattered flattened large blood vessels at primary stratum (Figure 3c). CAM treated with 50 µM MMP2 ASO shows very thin chorionic and allantoic epithelial layers without any bulging appearance suggestive of absence of new vessels at the treated area. Cells are clubbed together without sprouting from capillary plexus/ blood sinus. There are a few small flattened vessels forming a narrow structure indicating the inhibition of neovascularization (Figure 3d).

The potential of MMP2 ASO to reduce CAM thickness was analyzed by morphometry. The thickness of the CAM was measured from haematoxylin and eosin stained vertical cross sections after treating them with 0, 10, 30 and 50 µM MMP2 ASO. (Figure 4), shows that CAM treated with 10 (81.025 µm), 30 (79.46 µm) and 50 µM (75.27 µm) MMP2 ASO able to reduce CAM thickness effectively when compared to control CAM thickness value (83.99 µm). The thickness of the CAM was reduced significantly (*p=<0.001) under 50 µM MMP2 ASO indicative of reduction in the capillary sprouting and lesser endothelial cell proliferation and growth of blood vessels at the stroma region. The results demonstrate that MMP2 ASO has the potential to reduce tissue thickness through inhibiting the formation and growth of new blood vessels with potential effect at 50 µM concentration.

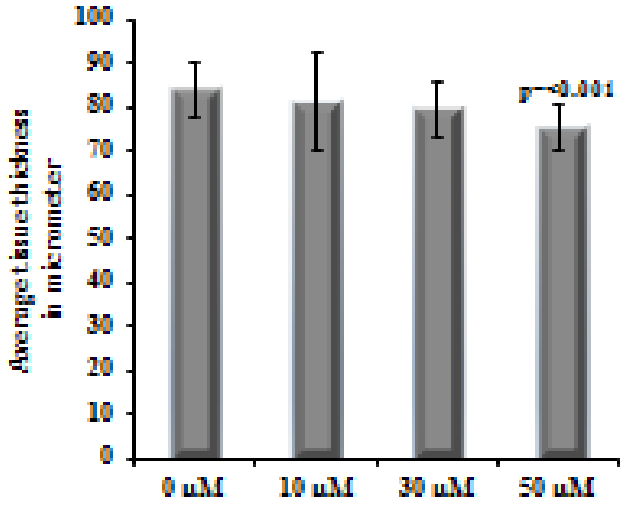

Figure 4. MMP2 ASO reduces the thickness of the CAM. Distances between chorionic and allantoic layers was measured from Haematoxylin and Eosin stained cross section after treated with 10, 30 and 50 µM concentration of MMP2 ASO and control treated with 0 µM concentration (1X PBS) for 72 hours . CAM treated with 50 µM MMP2 ASO shows significant reduction in the tissue thickness than other two concentrations. Material shrinkage is estimated to be ~25% relative to the fresh material in all cases. Each value is the mean ± SEM, *p= <0.001 versus control, n =6.

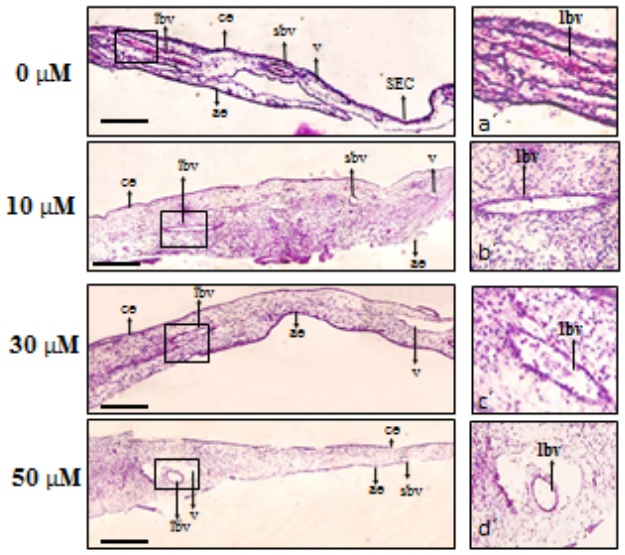

Figure 3. MMP2 ASO alter the cellular morphology of CAM. A. Images of CAM sections stained with H&E after treated with 10, 30 and 50 µM concentration of MMP2 ASO and control treated with 0 µM concentration (1X PBS) for 72 hours. Control CAM shows normal morphological structure of thin stratum with thin sub epithelial capillary network (SEC). CAM treated with 10 µM MMP2 ASO shows diminished accumulation of fibroblast at thin stratum with thinner chorionic and allantoic epithelial layer. CAM treated with 30 µM MMP2 ASO shows lesser number of blood vessels and more numbers of none proliferate flattened endothelial cells at primary stratum. CAM treated with 50 µM MMP2 ASO shows very thin allantoic and chorionic epithelia with clubbed endothelial cells at the capillary plexus with flattened blood vessels without sprouting. Gelatin sponges did not cause wound or inflammation. Figure a´, b´, c´ and d´ are enlarged version of the squared area of the figure a, b, c and d respectively. ibv- large blood vessel, sbv- small blood vessel, v- vein, ce and ae - chorionic and allantoic epithelial layers and SEC - sub epithelial capillary network. Representative sections are 5 µm in thickness and were taken at 40X magnification and bar is 50 µm. Images are the results of 3 set of experiments.

Antisense MMP2 is able to reduce angiogenesis by inhibiting targeted MMP2 gene expression along with other major angiogenic growth factors

The potential role of MMP2 ASO on angiogenesis inhibition through diminishing the expression of major angiogenic growth factors like VEGF165, FGF2 and MMP9 along with targeted MMP2 was analyzed by semi quantitative Reverse Transcription PCR. (Figure 5) shows gel images of PCR products after treatment with 0, 10, 30 and 50 µM MMP2 ASO. The results show that MMP2 ASO has the potential to down regulate the mRNA level expression of angiogenic growth factors. mRNA level expression of VEGF165, FGF2, MMP2 and MMP9 has decreased significantly (*p=<0.001) under 50 µM MMP2 ASO. With 30 µM MMP2 ASO a significant decrease in the expression of main targeted gene MMP2 (*p=<0.001) was observed after 72 hours of treatment.

Antisense MMP2 inhibits angiogenesis specifically by decreasing the gelatinase activity of MMP2 at the protein level

Specificity of MMP2 ASO to inhibit angiogenesis *via* inhibiting the gelatinase activity of targeted MMP2 protein was measured. The gelatinase activity of MMP2 was analyzed by zymography after treating the CAM with MMP2 ASO (10, 30 and 50 µM) for 72 hours

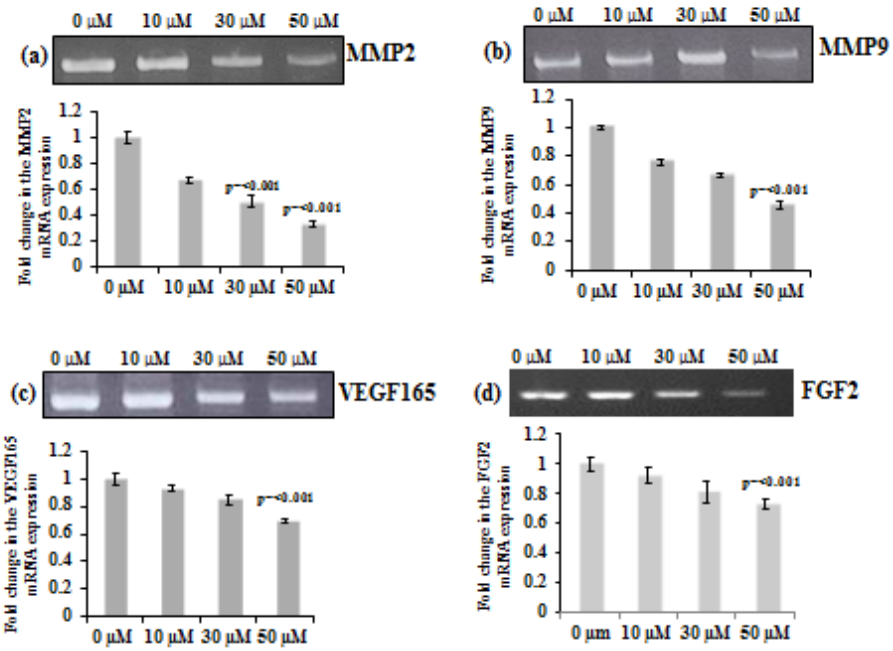

Figure 5. MMP2 ASO down regulates the expression of MMP2 gene along with MMP9, VEGF165 and FGF2 on CAM. Images of Reverse Transcriptase - PCR products of MMP2 (fig **a**), MMP9 (fig **b**), VEGF165 (fig **c**) and FGF2 (fig **d**) from CAM after treated with 10, 30 and 50 µM concentration of MMP2 ASO and control with 0 µM concentration (1X PBS) for 72 hours. Graph for OD value ratio of mRNA transcripts coded for MMP2, MMP9, VEGF165 and FGF2 after normalizing with GADPH OD value of the same. The OD value was measured using *Image J* software and the graph represents the intensity of the bands measured as relative OD. The relative level expression of MMP2 was reduced significantly for both 30 and 50 µM concentration of MMP2 ASO (graph **a**). Significant reduction in the expression of MMP9 (graph **b**), VEGF165 (graph **c**) and FGF2 (graph **d**) was observed under 50 µM concentration of MMP2 ASO after 72 hours of incubation. Each value is the mean ± SEM, *p= <0.001 versus control, n = 3. Images are the representatives of 3 set of experiments.

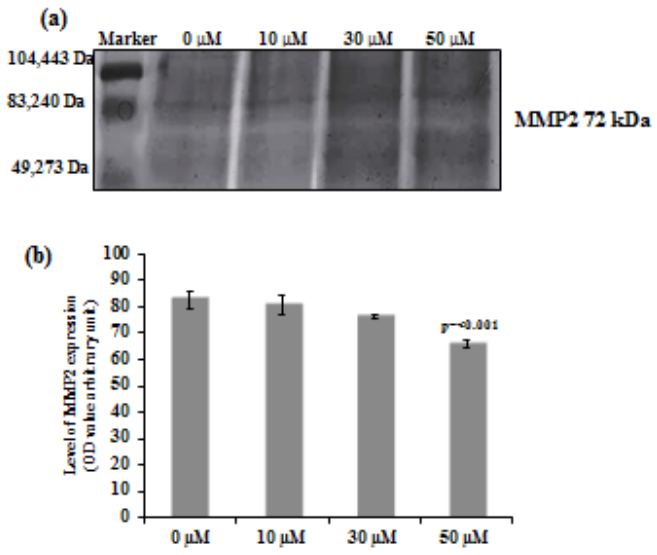

Figure 6. MMP2 ASO decreases the gelatinase activity of MMP2 on CAM. **(A)** represent the gelatin zymography of MMP2 activity from CAM after treated with 10, 30 and 50 µM concentrations of MMP2 ASO and control with 0 µM concentration (1X PBS) for 72 hours. The image shows that the gelatinase activity was reduced at 50 µM concentration of antisense MMP2 which was visible as white color in black background. **(B)** - The OD value of MMP2 gelatinase activity on CAM after treated with MMP2 ASO. CAM incubated with 50 µM concentration of MMP2 ASO shows decreased gelatinase activity than other two concentrations and is significant when compared with control. Each value is the mean ± SEM, *p= <0.001 versus control, n=3. Image is the result of 3 set of experiments.

and the effect was compared with that of control. (Figure 6a) shows that the gelatinase activity of MMP2 is reduced under all studied concentrations of antisense MMP2. The intensity measured in terms of OD shows that the gelatinase activity of MMP2 has decreased significantly (*p=<0.001) with 50 µM MMP2 ASO after 72 hours (Figure 6b). The gelatin zymogram analysis of MMP2 activity indicated that antisense MMP2 has the potential to inhibit basement membrane degradation accelerated by the gelatinase activity of MMP2 with effect being significant at 50 µM concentration. Thus, MMP2 ASO has the specificity by inhibiting MMP2 expression at the protein level.

Antisense MMP2 inhibits angiogenesis by inhibiting the expression of MMP2 protein

The antiangiogenic effect of 50 µM MMP2 ASO *via* reducing the angiogenic potential of MMP2 was further confirmed by inspecting the down regulation of MMP2 protein using immunohistochemical method. (Figure 7) shows that when treated with 50 µM MMP2 ASO the expression of MMP2 is considerably reduced in the chorionic layer and also in the stroma region due to lesser sprouting of blood vessels and or inhibition of neovascularization at this concentration. Higher expression pattern for MMP2 is observed in control CAM throughout the chorionic layer and also at the stroma region where formation of blood vessels occurs periodically by means of sprouting as per demand for the development of embryo [8].

Antisense MMP2 induce variation in the morphology of blood vessels

The effect of MMP2 ASO on angiogenesis was confirmed in detail by observing the microvascular morphology of CAM using SEM after

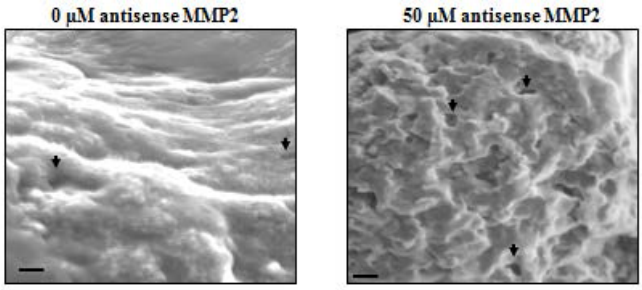

Figure 7. MMP2 ASO reduces the expression of MMP2 protein on CAM. Immunohistochemical images of CAM treated with 50 µM concentration of MMP2 ASO and control with 0 µM concentration (1X PBS) for 72 hours. CAM treated with 50 µM concentration of MMP2 ASO shows less expression of MMP2 at the stroma region than compared to control. Arrow indicates the presents of protein at the stroma and epithelial layer, magnification is 40X and bar is 50 µm. ae- allantoic epithelial layer, ce- chorionic epithelial layer.

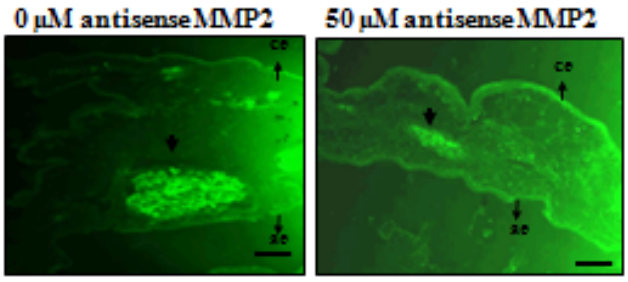

Figure 8. MMP2 ASO alter the micro vascular morphology of CAM. Micro vascular SEM images of CAM incubated with 50 µM concentration of MMP2 ASO and control with 0 µM concentration (1X PBS) for 72 hours. Control shows minimum flat appearance of capillary network, bulging blood vessel from capillary plexus and large angiogenic holes. CAM treated with 50 µM antisense MMP2 shows flat capillary network without sprouting vessels along with large and numerous angiogenic holes as an indication of less proliferation of blood vessels. Arrow indicates the presents of angiogenic holes, bars = 200 µm and images are representative of 3 set of experiments.

72 hours of treatment and compared with control. Figure 8, indicates that CAM treated with 50 µM MMP2 ASO shows flattened morphology of the membrane with clubbing effect (grouping effect). The surface contains large angiogenic holes without sprouting appearance. The morphology of microvasculature seems to be altered compared to control because of the absence of proliferated blood vessels. Control CAM shows the presence of large angiogenic holes with minimal bulging effect of blood vessel from the capillary plexus. SEM image analysis shows that 50 µM MMP2 ASO has the potential to inhibit angiogenesis by distracting the normal morphological structure of blood vessels.

Discussion

The present work reveals that phosphorothioate MMP2 ASO is able to inhibit neovascularization at the treated area as an indication of localized inhibitory effect of MMP2 ASO on angiogenesis. Retarded growth of the vessels was observed at an early period of incubation and is able to sustain the effect for a prolonged treatment period. This shows the long term stabile effect of phosphorothioate ASO at the tissue level. The antiangiogenic potential of MMP2 ASO is because of its functional inhibitory effect on MMP2 gene is directly indicates its specific and potential inhibitory activation at the mRNA level. Thus, MMP2 ASO shows concentration dependent inhibitory effect even at 50 µM concentration with significant inhibitory potential on angiogenesis within short interval of treatment. This observation is in concordant with the fact that after a single application to tissue the antisense effect of the phosphorothioate can be observed for over 48 hours [28]. MMP2 ASO not only inhibiting the growth of the vessels but also interact with the cellular compartment of angiogenic process to exert its antiangiogenic effect. Histological evaluation revealed that under MMP2 ASO treatment cellular morphology of EC and fibroblast cells has changed which resulted in the altered histological structure of the membrane. These cellular changes resulted in the alteration of morphological appearance of the membrane indicative of reduced proliferation and retarded growth of the vessels under MMP2 ASO. Previously Fang et al. showed that suppression of MMP2 activity by phosphorothioate MMP2 ASO resulted in the loss of angiogenic phenotype both in *in vitro* and also in the tumor nodules placed on CAM [27]. Our data support the basic conception of the above experiment by showing the retarded growth of the blood vessels at the treated area.

During embryonic development the thickness of CAM gradually increases due to the growth and migration of blood vessels from stroma region to the inner shell membrane (ISM) [21]. Earlier reports suggest that angiogenic or antiangiogenic effect of any compound can be easily analysed by measuring the tissue thickness of CAM [20]. The morphology of the cellular compartment of CAM alters due to the effect of the test compounds which will results a variation in the CAM thickness [28,29]. In this study we found that MMP2 ASO able to reduce the tissue thickness significantly under 50 µM concentration. This effect is considered as the inhibition of neovascularization and diminished growth of blood vessels under MMP2 ASO. This experimental data suggested that the treatment based on MMP2 ASO might help to reduce the neovascularization at the pathological condition of diabetic proliferative retinopathy where the progression of the disease causes increased retinal tissue thickness resulting in blindness [30].

Other convincing finding of this work is that the ability of phosphorothioate MMP2 ASO to inhibit neovascularization at the molecular level is mediated by down regulation of the expression of MMP2 gene along with other key angiogenic molecules. Down regulation of MMP2 gene under 30 and 50 µM concentration at 48 and 72 hours of treatment indicates its specific, rapid and potentially effective inhibitory effect on specified gene [25]. Interestingly it was noticed that the down regulation of other major angiogenic growth factors such as VEGF165, FGF2 and MMP9 also found to reduce significantly under 50 µM concentration indicative of its sustained and prolonged inhibitory effect on vascularisation. This molecular data suggested that the MMP2 ASO might be an effective therapeutic molecule to target the inhibition of neovascularization in which the elevated MMP2 expression favours the activation of VEGF165, a main cause for pathological angiogenesis in various disease [8]. Reduced gelatinase activity and less expression of MMP2 at protein level directly indicates the specificity of phosphorothioate MMP2 ASO to inhibit the expression and the functional activation of targeted gene even up to protein level. This finding directly indicates that the retarded proliferation and growth of the vessels under MMP2 ASO is because of the reduced degradation of basement membrane. The persistence of the inhibitory effect of phosphorothioate MMP2 ASO on angiogenesis indicates its long term stability and specificity at the tissue level. Further investigation must undergo to prove whether these observed biological effects has indeed been produced by the antisense mechanism or whether it is due to a complex combination of non- sequences specific effects.

Conclusion

The present work demonstrates the potential inhibitory effect of MMP2 ASO on neovascularization on CAM. The antisense oligonucleotide for MMP2 might possibly bind to MMP2 gene, the down regulation of which could possibly results in inhibiting angiogenesis at the treated area. Significant reduction in the expression of VEGF165, FGF2 and MMP9 at molecular level shows the stabile antiangiogenic effect of phosphorothioate MMP2 ASO. The present *in vivo* model elucidates that the phosphorothioate MMP2 ASO shows concentration dependent antiangiogenic effect of which 50 µM MMP2 ASO shows noticeable antiangiogenic effect after 72 hours of incubation. Altogether our preliminary data indicates that MMP2 ASO could inhibit neovascularization and can be an effective therapeutic molecule in the treatment of pathological diseases. Studies using various cell lines and also other animal models are welcomed to understand the mechanism of action of the inhibitory effect of phosphorothioate MMP2 ASO on angiogenesis in details.

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