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Inhibition of Cerebral Vascular Inflammation by Brain Endothelium-Targeted Oligodeoxynucleotide Complex

Jing Hu¹, Daniah Al-Waili¹, Aishlin Hassan³, Guo-Chang Fan², Mei Xin³, and Jiukuan Hao^{1,*}

¹Division of Pharmaceutical Sciences, James L. Winkle College of Pharmacy, University of Cincinnati, Cincinnati, OH 45267

²Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, OH 45267

³Department of Pediatrics, University of Cincinnati & Cincinnati Children's Hospital Medical Center

Abstract

The present study generated a novel DNA complex to specifically target endothelial NF- κ B to inhibit cerebral vascular inflammation. This DNA complex (GS24-NF κ B) contains a DNA decoy which inhibits NF- κ B activity, and a DNA aptamer (GS-24), a ligand of transferrin receptor (TfR), which allows for targeted delivery of the DNA decoy into cells. The results indicate that GS24-NF κ B was successfully delivered into a murine brain-derived endothelial cell line, bEND5, and inhibited inflammatory responses induced by tumor necrosis factor α (TNF- α) or oxygen-glucose deprivation/re-oxygenation (OGD/R) via down-regulation of the nuclear NF- κ B subunit, p65, as well as its downstream inflammatory cytokines, inter-cellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1). The inhibitory effect of the GS24-NF κ B was demonstrated by a significant reduction in TNF- α or OGD/R induced monocyte adhesion to the bEND5 cells after GS24-NF κ B treatment. Intravenous (i.v.) injection of GS24-NF κ B (15mg/kg) was able to inhibit the levels of phospho-p65 and VCAM-1 in brain endothelial cells in a mouse lipopolysaccharide (LPS)-induced inflammatory model *in vivo*. In conclusion, our approach using DNA nanotechnology for DNA decoy delivery could potentially be utilized for inhibition of inflammation in ischemic stroke and other neuro-inflammatory diseases affecting cerebral vasculature.

Keywords

NF- κ B; inflammation; oxygen-glucose deprivation; lipopolysaccharide; aptamer; endothelial cells

*To whom correspondence should be addressed: Jiukuan Hao, Division of Pharmaceutical Sciences, James L. Winkle College of Pharmacy, University of Cincinnati, Cincinnati, OH 45267, USA. Tel: +1-513-556-2041; +1-513-558- 0915 (O). Fax: +1-513-558-4372. haojn@ucmail.uc.edu.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

Introduction

Neuro-inflammation has been implicated in various brain diseases, e.g. stroke, multiple sclerosis, Parkinson's disease, and Alzheimer's disease. Vascular inflammation plays an important role in neurodegenerative diseases by triggering the release of cytokines and inflammatory mediators, such as CD14 LPS receptor (Lacroix et al., 1998), interleukin-1 (Quan et al., 1998b), TNF (Barone et al., 1997, Nadeau and Rivest, 1999, Yang et al., 1999), cyclooxygenase-2 (Quan et al., 1998a), inducible nitric oxide synthase (iNOS) (Wong et al., 1996), the adhesion molecules like ICAM-1 and VCAM-1 (Lindsberg et al., 1996, Henninger et al., 1997, Stanimirovic et al., 1997) etc. Up-regulation of above the substances damages the barrier function of the Blood-brain Barrier (BBB) (de Vries et al., 1996a, de Vries et al., 1996b), and facilitates adhesion and trans-endothelial migration of leukocytes into the brain (Nakajima and Kohsaka, 2001, Yilmaz and Granger, 2008). In response to these inflammatory signals, macrophages, microglia and other immune cells are activated and accumulate throughout the CNS. This process has been implicated in the development of neuronal injury and death (Aloisi, 2001, Nakajima and Kohsaka, 2001). Importantly, up-regulation of inflammatory mediators is closely associated with the activation of transcription factor NF- κ B, which is a master switch of inflammatory gene expression (Baldwin, 2001, Keifer et al., 2001). In mammals, the NF- κ B family has five proteins: NF- κ B1 (p50), NF- κ B2 (p52), RelA (p65), RelB, and c-Rel (Gilmore, 2006). NF- κ B is a protein complex made up by various combinations of homo- or heterodimers of the five proteins listed above. Normally, NF- κ B is in an inactive state, in which NF- κ B dimers are associated with I κ B proteins in the cytosol (Jacobs and Harrison, 1998, Schwaninger et al., 2006). Activation of NF- κ B, by removing I κ B, does not require new protein synthesis, which allows NF- κ B to act as a "first responder" to many cellular stimuli. When cells are perturbed, NF- κ B is rapidly activated causing the up-regulation of inflammatory gene expression. The key steps in the NF- κ B activation are the disassociation of I κ B proteins from NF- κ B dimers, and the translocation of free NF- κ B into the nucleus (Fig. 1), where NF- κ B can regulate gene expression by interacting with binding sites in the promoter regions of multiple inflammatory genes. Notably, activation of NF- κ B has been seen in the BBB endothelial cells under inflammatory conditions (Kooij et al., Pan et al., Quan et al., 1997, Laflamme et al., 1999). Based on the central role of NF- κ B in inflammatory responses, we hypothesize that the inhibition of NF- κ B activity in cerebral endothelial cells will have suppressive effects on cerebral vascular inflammation, because it targets multiple inflammatory signals instead of single inflammatory molecules. Therefore, cerebral endothelial transcription factor NF- κ B could be a novel therapeutic target for neuro-inflammatory diseases such as ischemic stroke, multiple sclerosis, and Parkinson's disease..

The concept of NF- κ B inhibition is supported by evidence from a number of studies examining generalized NF- κ B suppression. First, glucocorticoids are the most effective drugs for neuro-inflammatory diseases, like multiple sclerosis, and for immune suppression due to their actions in NF- κ B inhibition (Ray and Prefontaine, 1994, Auphan et al., 1995). Second, NF- κ B inhibition reduces infarct size in ischemic stroke (Huang et al., 2003, Wang et al., 2008) and the incidence of relapses in experimental autoimmune encephalomyelitis (EAE) (Vanderlugt et al., 2000). Third, inhibition of NF- κ B activity in the heart and lung

prevents expression of pro-inflammatory genes after LPS challenge (Liu et al., 1999a, Liu et al., 1999b). While NF- κ B is a very attractive drug target, there is some controversy about the outcomes of therapeutic approaches targeting the NF- κ B pathway in neuro-inflammatory diseases. For instance, some reports showed that NF- κ B activation was neuroprotective, and inhibition of NF- κ B increased neuronal death in ischemic stroke models (Beg and Baltimore, 1996, Hill et al., 2001, Duckworth et al., 2006). However, other studies indicated that the activation of NF- κ B contributed to neuronal damage after ischemic stroke, as inhibition of NF- κ B activity improved the outcomes of ischemic stroke in animal models (Xu et al., 2002, Shen et al., 2003, Nurmi et al., 2004, Zhang et al., 2005). This apparent dual effect of NF- κ B on ischemic stroke is due to the ability of NF- κ B to activate both a pro-inflammatory and an anti-apoptotic pathway. Activation of NF- κ B upregulates the anti-apoptotic regulators, such as Bcl-2, TRAF-1, and XIAP, which limit infarct size in ischemic stroke. At the same time, the NF- κ B-dependent up-regulation of inflammatory mediators, including iNOS, COX-2, ICAM-1, VCAM-1, IL-6, and MMP-9, contributes to ischemic tissue damage. Thus, the ultimate fate of neurons implicated in these diseases may depend on which NF- κ B pathway is activated, as well as where and when it is activated (Nijboer et al., 2008).

NF- κ B decoy is a short double-stranded DNA oligodeoxynucleotide (ODN), which consists of NF- κ B decoy-1 (5'-CCTTGAAGGGATTCCCTCC-3') and NF- κ B decoy-2 (3'-GGAAGTTCCCTAAAGG GAGG-5') (Morishita et al., 1997). NF- κ B decoy inhibits NF- κ B activity by binding to its "cis"- binding sequence (6-10bp), preventing the cis-trans interaction between NF- κ B and the promoter region of the target gene (Morishita et al., 1998). It has been shown that the NF- κ B decoy was effective in modulating gene expression under experimental conditions (Bielinska et al., 1990, Morishita et al., 1997, Tomita et al., 2000, Tomita et al., 2001, Matsuda et al., 2004). The effectiveness of these decoy ODNs against the expression of genes involved in inflammatory responses in brain endothelial cells has also been demonstrated by a number of experiments *in vitro* (Xu et al., 1997, Tomita et al., 1998, Hess et al., 2000). However, the effectiveness of NF- κ B decoy *in vivo* is dependent on overcoming drug delivery problems at the BBB. Most of the current approaches used for brain delivery of macromolecular drugs are invasive, like intra-cerebral injection, and can cause brain tissue damage and possible infection. Therefore, the development of non-invasive systemic delivery systems is critical to eventually achieve clinical applications for the DNA decoy approach. In the present study, we constructed a novel DNA complex for brain-targeted delivery of NF- κ B decoy with the goal of inhibiting cerebral vascular inflammation. We used a DNA aptamer (GS-24, a ligand of TfR) as a vector to deliver NF- κ B decoy into brain endothelial cells. The GS24 DNA aptamer (Fig. 2A) can specifically bind to the extracellular domain of mouse TfR (TfR-ECD) for cellular uptake. The TfR, a membrane glycoprotein, is involved in receptor-mediated uptake of transferrin-bound iron. TfR has become a well-known target for brain drug delivery due to the high expression of TfR on the BBB (Qian et al., 2002). GS24 aptamer interacts with TfR at a different binding site from that of transferrin (Chen et al., 2008), consequently avoiding competition with transferrin for the binding site. This limits potential side-effects of GS24 on the normal functions of TfR and reduces the challenges of drug delivery. GS24 has been successfully used to deliver a lysosomal enzyme into deficient cells to correct defective

glycosaminoglycan degradation in the cells (Chen et al., 2008). In the present study, we have evaluated delivery of GS24-NF κ B *in vitro* and *in vivo*, and investigated the anti-inflammatory effect of GS24-NF κ B under TNF- α induced inflammatory conditions, OGD/R condition *in vitro*, and inflammation induced by LPS in mouse *in vivo*.

Methods and Materials

Cell culture

The brain endothelial cell line (bEND5) derived from mouse brain and immortalized with polyoma middle T oncogene was a gift from Dr. Ulrich Bickel, Texas Tech University. bEND5 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Atlanta Bio Inc. GA, USA), 1% (v/v) non-essential amino acids and 1% (v/v) 10000IU/ml penicillin/ 10000 μ g/ml streptomycin (all from ATCC, Manassas, VA, USA). U937 cells (a monocyte cell line from ATCC, Manassas, VA, USA) were cultured in RPMI 1640 medium (Hyclone, Logan, UT, USA) supplemented with 2 mM L-glutamine (Mediatech Inc. Cellgro, Manassas, VA, USA), 10% heat-inactivated FBS and 1% (v/v) 10000IU/ml penicillin/ 10000 μ g/ml streptomycin. Both cell lines were maintained at 37 °C, 5% CO₂ and 95% relative humidity.

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee at University of Cincinnati and they complied with pertinent NIH guidelines for care and use of animals. CD1 male mice (body weight ~25g) supplied by Charles River (Wilmington, MA, USA) were kept under standardized light/dark (12 hours), temperature (22°C), and humidity (70%) conditions, with free access to water and standard pelleted food.

Preparation of GS24-NF κ B complex

The GS24-NF κ B consists of GS24-NF κ B-decoy-1 and NF- κ B decoy-2 strands. The sequence of GS24-NF κ B-decoy-1 strand (Strand-1): 5'- GCG TGT GCA CAC GGT CAC TTA GTA TCG CTA CGT TCT TTG GTT CCG TTC GGC CTTGAA GGGATT TCC CTC C -3'. The sequence of NF- κ B decoy-2 strand (strand-2): 5'- GGAGGGAAATCCCTTCAAGG-3'. The sequence of scramble GS24-NF κ B: Scramble GS24-NF κ B -decoy-1 strand: 5'- AAG AGA GTA AAT CCT GGG ATC ATT CAG TAG TAA CCA CAA ACT TAC GCT GGC CTT GAA GGG ATT TCC CTC C-3'. Scramble NF- κ B decoy-2 strand: 5'- GGAGGGAAATCCCTTCAAGG-3'. The sequences of NF κ B-decoy are: the NF κ B-decoy-1 strand: 5'-CCT TGAAGGGATTTCCTCC-3', and the NF- κ B decoy-2 strand: 5'- GGAGGGAAATCCCTTCAAGG-3'. The designed sequences of the ODN strands listed above were purchased from Integrated DNA Technologies Inc (Coralville, Iowa, USA). The ODN complex was constructed by DNA denaturation and annealing processes. Briefly, the mixture of two complementary strands (mole ratio 1:1) was incubated at 95°C for 5 min, and then cooled at the room temperature for 45-60 min. The ODN complexes were analyzed by 8% native-PAGE gel.

Cellular uptake of GS24-NFκB by bEND5 cells

Quantitative cellular uptake of ^{32}P -labeled ODNs: GS24-NFκB and its scramble control ODNs were 5'-end labeled with [γ - ^{32}P] ATP (PerkinElmer Inc., Waltham, Massachusetts, USA) using T4 Polynucleotide Kinase (Fermentas, USA) according to the manufacturer's instructions, and then purified by NucAwayTM spin column (life technology, Grand Island, NY). The purity of the product was controlled by precipitation of the ODNs with trichloroacetic acid (TCA) (Sigma-Aldrich Co. LLC., St. Louis, MO, USA), and only the batches with precipitation rates >95% were used. Briefly, ice-cold 10% TCA (500 μl) was added to a 1 μl mixture of sample and 50 μl 2.5% bovine serum albumin (BSA, Fisher Scientific, Pittsburgh, PA, USA). The resulting mixture was then vortexed and incubated on ice for 10 min following by centrifugation at 4000g for 5 min. The supernatant was collected and the pellet was dissolved in 3% KOH. The radioactivity of the TCA-precipitated fraction was calculated as % (precipitable fraction) = $(\text{cpm}_{\text{pellet}} \times 100) / (\text{cpm}_{\text{pellet}} + \text{cpm}_{\text{supernatant}})$. The Tfr negative bEND5 cells were generated by transfecting CD71 siRNA with lipofectamine RNAiMAX (Life Technology, Grand Island, NY) according to the manufacturer's protocol. Briefly, Tfr negative and normal bEnd5 cells at 80% confluence were incubated with ^{32}P -labeled ODNs at the concentration of 1 μmol/l (~0.4 μCi) for 40 minutes at 37 °C. After washing the cells three times with phosphate-buffered saline (PBS), the cells were lysed with RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA), and the cell-associated radioactivity was counted by a LSC 6500-multiplepurpose scintillation counter (Beckman Counter, Fullerton, CA). The competition assay for cellular uptake was performed as follows: 20-fold extra unlabeled GS24-NFκB was added to the medium 30 min before adding ^{32}P -labeled ODNs.

Serum stability of GS24-NFκB

GS24-NFκB at a final concentration of 2 μM was incubated in goat serum (life technology, Grand Island, NY, USA) at 37°C for 24 h. An aliquot was withdrawn at appropriate time intervals and treated at 80-100°C for 5 min to quench the digestion reaction. The samples were then electrophoresed on 8% denaturing urea gel to analyze the integrity of the ODNs.

ODN treatments in models *in vitro*: TNF-α model:

The confluent bEND5 cells were incubated with GS24-NFκB or its corresponding controls in serum free medium for 2 hour. Then TNF-α (50 ng/ml) (Shenandoah Biotechnology, Warwick, PA, USA) was added to the medium to stimulate inflammation of bEND5 cells for 4 hours, after which cell samples were harvested.

The OGD/R model

the OGD/R model was used to mimic ischemia/reperfusion *in vitro*. Hypoxia was induced by placing cells in a sealed chamber (BillupsRothenberg, Del Mar, CA) at 37 °C, which has been flushed with 95% N₂ /5% CO₂ gas. The concentration of oxygen in the atmosphere was maintained at 0% oxygen and the PO₂ in the medium was below 25 mmHg. Glycaemia was induced by using RPMI 1640 medium without L-glucose (Hyclone, Logan, UT). After 9 hours OGD, the cells were moved back to normal growth condition for 16 hours. Then the growth medium was changed to serum free medium with the addition of GS24-NFκB or its

corresponding controls for another 8 hours. The cell samples were obtained 24 h after re-oxygenation.

RT-PCR and Western blotting

The bEND5 cells under different conditions were treated with various doses of ODNs or equal doses of the scramble control ODNs. RNA samples were evaluated by RT-PCR and protein samples were analyzed by western blotting to determine nuclear levels of P-65 and expression of ICAM-1 and/or VCAM-1. β -Actin was used as a protein loading control marker for whole cell lysates and TATA binding protein (TBP) was used as a protein loading control marker for nuclear protein. The following antibodies were used in western blotting procedure: 1: 2,000 (dilution) β -Actin antibody, 1:2000 NF- κ B p65 antibody, 1:1000 phospho-NF- κ B p65 (Ser536) antibody (Cell Signaling Technology, Danvers, MA), 1:1,000 ICAM-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), 1:2,000 VCAM-1 antibody and 1:1,000 TBP antibody (Abcam, Cambridge, MA). mRNA expression was measured using the following forward and reverse qPCR primers: VCAM-1: 5'-AAG ACT GAA GTT GGC TCA CAA-3' and 5'-GGA GTT CGG GCG AAA AAT AG-3'; ICAM-1: 5'-GGG AAT GTC ACC AGG AAT GT-3' and 5'-CAG TAC TGG CAC CAG AAT GA-3'; β -actin: 5'-GGC TGT ATT CCC CTC CAT CG-3' and 5'-CCA GTT GGT AAC AAT GCC ATG T-3'. The mRNA expression of β -actin was used as an internal control. All the primers were purchased from Integrated DNA Technologies Inc.

Nuclear protein extraction

Cells were washed and lysed in ice cold buffer-A containing 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10mM KCl, 0.25% v/v noident P-40, 0.5 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) in deionized water (dH_2O) for 10 min. The supernatant, cytoplasmic, and nuclear pellet fractions were obtained by centrifuging cell samples at 12,000g for 2 min. Nuclear protein was extracted from the pellet with ice cold buffer-B containing 20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.5 mM DTT, 25% v/v Glycerol, and 0.5 mM PMSF in dH_2O for 20 min. Then the nuclear fractions were collected and diluted in buffer-C containing 20 mM HEPES pH 7.9, 50 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, and 0.5 mM PMSF. Finally, the protein concentrations were measured, and western blotting was performed. All the chemicals were purchased from Sigma-Aldrich Inc.

Adhesion assay

U937 cells (ATCC) were incubated with 5 mg/mL BCECF-AM (2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester, Sigma Inc.) for 30 minutes at 37°C, and then the cells were washed and re-suspended in serum free media. For ODN treatments in TNF- α stimulated bEND5 cells, cells were cultured and incubated with ODNs in a 24-well plate for 2 h. Then TNF- α (20ng/ml) was added for 16 h, after which the cells were incubated with BCECF-AM-labeled U937 cells (10^6 cells/well) for 30 minutes at 37°C. For ODN treatments in OGD/R condition, the bEND5 cells were treated as described earlier in the OGD/R model, and then cells were incubated with BCECF-AM-labeled U937 cells (10^6 cells/well) for 30 minutes at 37°C. Non-adhering U937 cells were removed and cells were washed with PBS, and subsequently lysed in 0.1% Triton X-100 in 0.1M Tris-HCl

(pH7.4) (Sigma-Aldrich). Fluorescence (F) was measured with a microplate fluorescence reader (POLAR star OPTIMA, BMG Labtechnologies, Ortenberg, Germany) using an excitation of 492 nm and an emission of 535 nm. The monocyte adhesion was calculated as: Adhesion (%) = $100 \times F_{\text{sample}} / F_{\text{total}}$ (F: fluorescence intensity of 10^6 cells).

Brain distribution of GS24-NF κ B

Male CD1 mice (body weight ~25g, supplied by Charles River Inc, Wilmington, MA, USA) were anaesthetized by injection of Ketamine and Xylazine (Butler Animal Health Supply Inc. Dublin, OH, USA). 32 P-labeled ODNs (13 μ g, 100 μ l, ~2 μ Ci) were intravenously administered through the jugular vein. Blood samples were obtained by puncture of the retroorbital venous plexus at 1 h. Mice were then sacrificed by decapitation and brains were sampled and weighed. Blood was centrifuged at $2000 \times g$ at 4°C for 10min, and the plasma was collected. Brains were cut into pieces and then solubilized in 3% KOH at 50°C for 4h. Radioactivity of all samples was measured on a LSC 6500-multiplepurpose scintillation counter. Measurements of injected solutions were used to determine the injected dose of radioactivity. ODN concentrations in the brains were expressed as percent of injected dose %ID/g. The results were corrected for capillary plasma content (9.3 μ l/g) in the brain.

ODN treatments in LPS model *in vivo*

Lipopolysaccharide (LPS, Sigma-Aldrich Inc.) was dissolved in sterile saline, aliquoted, and stored at -20°C until use at which time a single intraperitoneal injection of 1mg/kg LPS was administered. To measure phospho-p65 expression, 15mg/kg GS24-NF κ B or scramble ODNs were administered by intravenous (i.v.) injection 3 h after LPS challenge. Brain samples were obtained 3 h after ODN treatment and tested for detection of phospho-p65 expression. For analysis of VCAM-1 expression, 15mg/kg GS24-NF- κ B or scramble ODNs were administered by i.v. injection 16 h after LPS injection. Brain samples were collected 4 h after ODN treatment.

Brain micro-vessel isolation

Brain samples were homogenized in a tissue grinder with ice cold medium-131 (life technologies, Grand Island, NY) supplemented with 2% FBS, and 1% (v/v) 10,000 IU/ml penicillin/10,000 μ g/ml streptomycin. The homogenates were suspended in 16% dextran (average molecular weight 100,000, Sigma-Aldrich Inc.) and centrifuged at 5000RPM for 20 min. Then the fatty layer of the supernatant was removed, and the vascular pellet was digested with 0.1% collagenase and 10 μ g/ml DNase at 37°C for 1 h with occasional agitation. The digested micro-vessels were filtered through a 70 μ m nylon mesh cell filter and collected with centrifugation at 1000g for 5 min. These samples were subsequently lysed and used for further analysis.

Statistical analysis

Data analysis of multiple groups was performed by ANOVA followed by post-tests (Holm-Sidak test for comparison of experimental groups versus control conditions). The p value of < 0.05 was chosen as the threshold of statistical significance.

Results

Formation of GS24-NF κ B complex and cellular uptake of GS24-NF κ B complex

The design of GS24-NF κ B complex is shown in Fig.2A. NF- κ B-decoy-2 binds to its complementary sequence in the GS24-NF κ B-decoy-1 strand to form GS24-NF κ B DNA complex, which contains two functional structures: a GS24 aptamer and a NF- κ B decoy (Fig.2A). The formation of the DNA complex was confirmed by 8% native-PAGE gel (Fig. 2B). Because the molecular weight of GS24-NF κ B complex is the heaviest among the loaded ODNs, its migration distance is the shortest, as is demonstrated by the signal in lane 5 of Fig.2B.

The cellular uptake of 32 P-labeled GS24-NF κ B complexes was studied in bEND5 cells. The radioactivity value for GS-NF κ B was 42491 ± 11668 CPM in bEND5 cells. While it was 6220 ± 3621 CPM for the scramble ODN complex containing scramble GS-24 sequence, which was only 14% of 32 P-GS24-NF κ B group. The radioactivity value of the 32 P-GS24-NF κ B group was reduced by 99% to 388 ± 49 CPM after adding excess unlabeled GS24-NF κ B (Fig.2C). The specificity of cellular uptake of the GS24-NF κ B was also investigated using TfRs silenced bEND5 cells. As shown in Fig.2D, uptake of GS24-NF κ B by normal bEND5 cells was significantly higher than that by the TfRs knockdown bEND5 cells. The average cellular uptake of GS24-NF κ B was 3.6 times higher than that in the TfR silenced cells (Fig.2D). The serum stability of GS24-NF κ B was also examined using goat serum to determine the stability in the blood stream. The results showed that GS24-NF κ B was stable and visible in goat serum up to 16 h, and the half-life of the GS24-NF κ B in goat serum was about 4 h (Fig.3).

Anti-inflammatory effects of GS24-NF κ B complex in murine brain-derived endothelial cells induced by TNF- α

TNF- α treatment was used to induce an inflammatory response in bEND5 cells. TNF- α significantly enhanced VCAM-1, and ICAM-1 levels in bEND5 cells and also enhanced levels of p65 in the nucleus. TNF- α stimulation increased nuclear p65 protein level to 204.8% of control in bEND5 cells, while 2 μ M of GS24-NF κ B complex mitigated the TNF- α -induced up-regulation of nuclear p65 by 40% (Fig.4A). VCAM-1 molecules were expressed at very low level in untreated bEND5 cells however, expression of VCAM-1 was remarkably increased by TNF- α stimulation. Remarkably, treatment of TNF- α stimulated cells with 2 μ M GS24-NF κ B reduced the level of VCAM-1 by 32.6% (Fig.4B). Scramble complexes or NF- κ B decoy alone did not have any effect on the levels of VCAM-1 and nuclear p65. Similar results were also seen for the other inflammatory marker, ICAM-1 (Fig. 4C). The mRNA levels of ICAM-1 and VCAM-1 were also detected by RT-PCR. As indicated in Fig.5, TNF- α stimulation increased mRNA levels of ICAM-1 and VCAM-1 about 30 fold and 18 fold, respectively, compared to untreated cells. The mRNA levels of ICAM-1 and VCAM-1 were reduced by 42% (Fig.5A) and 50% (Fig.5B) respectively in the 2 μ M GS24-NF κ B treatment group compared to the TNF- α stimulation group.

Because increased levels of ICAM-1/VCAM-1 lead to an increase in monocyte adhesion to endothelial cells, we performed monocyte adhesion assays with U-937 cells to examine the

effects of GS24-NF κ B at the functional level (Fischer et al., 2005). As shown in Fig.6A, when endothelial cells were stimulated by TNF- α , the percentage of U-937 cells adhering to endothelial cells increased to 11.3% compared to that in non-activated endothelial cells which had 6.1% adherent monocytes. GS24-NF κ B treatment at concentrations of 2 and 4 μ M reduced the percentage of adherent U-937 cells to 7.7% and 7.5% respectively. While the same doses of scramble GS24-NF κ B complex (with scramble GS24) did not result in significant decrease in percentage of U-937 cell adhesion at 11.5% and 10.5% respectively (Fig.6A&B).

Anti-inflammatory effects of GS24-NF κ B complex in murine brain-derived endothelial cells induced by OGD/R

Nuclear p65 levels in OGD/R stimulated bEND5 cells was increased to about 195.8% of non-stimulated control cells, and scramble GS24-NF κ B did not have any effect on the level of nuclear p65 in stimulated cells. Treatment of 4 μ M and 8 μ M of GS24-NF κ B complex significantly mitigated the OGD/R-induced up-regulation of nuclear p65. The levels of nuclear p-65 were back to the normal level, which were 136.9 %, 129% and 98.9% of control in 2 μ M, 4 μ M and 8 μ M GS24-NF κ B treatment groups respectively (Fig.7A). GS24-NF κ B treatment of OGD/R stimulated bEND5 cells had a similar effect on the expression of another inflammatory cytokine, VCAM-1 (Fig.7B). 2 μ M, 4 μ M, or 8 μ M GS24-NF κ B treatment reduced VCAM-1 expression to 72%, 67%, and 54% of the OGD/R-alone cells respectively. Scramble controls did not significantly reduce VCAM-1 expression (Fig.7B).

To determine if the inhibitory effects of GS24-NF κ B on nuclear p-65 and its down-stream adhesion molecules were sufficient to have effect on functional level we performed an adhesion assay. As shown in Fig.8A, the percentage of U-937 cells adhering to endothelial cells in OGD/R-stimulated bEND5 cells increased to 9.9% compared to the medium control bEND5 cells (5.2%). 2 μ M, 4 μ M, or 8 μ M GS24-NF κ B treatment reduced the percentage of adherent U-937 cells in OGD/R-stimulated cells to 5.9%, 6.7%, and 3.9% respectively. The same doses of scramble GS24-NF κ B complex did not have effect on percentage of U-937 cell adhesion (Fig.8A, B).

Brain uptake and the effect of GS24-NF κ B on cerebral vascular inflammation *in vivo*

Following intravenous injection of 32 P-labeled GS24-NF κ B or scramble ODNs, whole brain samples were found to have significantly higher uptake of GS24-NF κ B than that of the scramble ODN complexes. As shown in Fig.9, brain accumulation of GS24-NF κ B was $0.39 \pm 0.05\%$ of injection dose/gram of tissue (ID/g), while it was only $0.23 \pm 0.01\%$ of ID/g for the scramble ODNs. Furthermore, we used a LPS-induced inflammatory model *in vivo* to evaluate the effect of GS24-NF κ B on cerebral vascular inflammation. LPS, a component of the Gram-negative bacteria cell wall, is a potent inducer of inflammation and now commonly used to produce inflammation. Since brain endothelial cells were the main targets in this study, the brain micro-vessel fraction was isolated and used to analyze the effects of GS24-NF κ B by measuring the levels of phospho-p65 and VCAM-1. LPS administration significantly increased the level of phospho-p65 and VCAM-1. However, GS24-NF κ B reduced the level of phospho-p65 ($141 \pm 26\%$ of PBS control) by 30% compared to that of

scramble ODN ($201 \pm 34\%$ of PBS control) (Fig.10A). In addition, VCAM-1 expression in the scramble control group was $218 \pm 39\%$ of PBS control, while the level was significantly decreased by 35% ($142 \pm 29\%$ of PBS control) with systemic administration of GS24-NF κ B (Fig.10B).

Discussion

There are a number of receptor-mediated transport systems existing on the endothelial cells of the BBB, which are excellent targets for brain drug delivery. Among these receptor transport systems, TfRs are the most studied receptors for brain drug delivery. TfRs are highly expressed on the microvascular endothelial cells of the BBB, glia, and neurons in the CNS (Moos, 1996). The bEND5 cells used in this study also have high expression of TfR (Bhattacharya et al., 2008). Furthermore, expression of TfRs is up-regulated in some brain diseases like ischemic stroke (Omori et al., 2003). Due to the high expression of TfRs at the BBB, targeting them for drug delivery gives us greater specificity for delivery to the brain. The TfRs at the BBB have been utilized to deliver neurotrophic factors (Zhang and Pardridge, 2001), peptide hormones (Bickel et al., 1993), and immunoliposomes plasmids (Zhang et al., 2003) into the brain. However, none of these approaches have translated to clinical therapy because of a poor pharmacokinetic profile due to the large size and protein content of these molecules. In the present study, we explored an aptamer-directed DNA complex as an alternative approach for brain drug delivery. An ODN-based brain drug delivery system was developed, which consists of an NF- κ B decoy and a GS24 aptamer (Fig. 2A) for targeted delivery of NF- κ B decoy into brain endothelial cells. GS24 is a DNA aptamer (Fig. 2A) that can specifically bind to the extracellular domain of mouse TfR (TfR-ECD) for cellular uptake. It was discovered and produced by systematic evolution of ligands by exponential enrichment (SELEX) (Chen et al., 2008). The central goal of the present study is to target endothelial NF- κ B using GS24-NF κ B to inhibit vascular inflammation. As shown in Fig.2B, a completely DNA-based complex was synthesized (lane 5). Uptake of GS24-NF κ B was investigated both *in vitro* and *in vivo* using brain-derived endothelial cells and mouse models respectively. Compared with the scramble DNA complex, cellular uptake of 32P-GS24-NF κ B was significantly higher, and this uptake could be inhibited by adding excess unlabeled GS24-NF κ B (Fig.2C). Moreover, cellular uptake of GS24-NF κ B was reduced when TfR expression in bEND5 cells was knocked down (Fig.2D). This data indicates that delivery of GS24-NF κ B to cells via TfR is specific. Furthermore, the stability of GS24-NF κ B was stable and detectable in goat serum for up to 16 h (Fig. 3).

We used two *in vitro* inflammatory conditions, TNF- α stimulation and OGD/R, which induce inflammation in endothelial cells to examine the anti-inflammatory capability of GS24-NF κ B. Our results indicate that, in the absence of a transfection reagent, GS24-NF κ B inhibited the levels of ICAM-1, VCAM-1, and nuclear p65 in bEND5 cells under inflammatory conditions (TNF- α stimulation) (Fig.4, Fig.5). Furthermore, GS24-NF κ B inhibited monocyte adhesion to TNF- α stimulated bEND5 cells (Fig.6). Similar results were observed in the OGD/R stimulated cells as shown in Fig.7 and Fig.8. This finding is significant because it demonstrates that GS24-NF κ B could potentially be used as a therapy for ischemic stroke or other neuro-inflammatory diseases. In order to demonstrate the feasibility of using GS24-NF κ B for vascular inflammation therapy, we performed an *in vivo*

analysis. Brain accumulation of GS24-NF κ B, and its effect on the NF- κ B signaling pathway in cerebral vasculature were evaluated in the mouse. Brain accumulation of GS24-NF κ B was significantly higher than that of scramble DNA complex (Fig.9). Furthermore, GS24-NF κ B significantly inhibited LPS-induced up-regulation of phosphorylation of p65 and expression of VCAM-1 (Fig. 10). The reduced level of p65 in the nucleus in response to GS24-NF κ B treatment is consistent with a previous study (D'Acquisto et al., 2001). Previous studies have shown that NF- κ B decoy resides both in the cytoplasm and nucleus of cells after entering the cell, however, the majority of NF- κ B decoy is located in cytoplasm (Griesenbach et al., 2000, Griesenbach et al., 2002, Liu et al., 2010).

Based on this study and others, we hypothesize that the reduced nuclear level of p65 observed in our study may be due to: (1) NF- κ B decoy may interact with NF- κ B dimers in the cytoplasm causing increased degradation. (2) NF- κ B-decoy/NF- κ B-dimer complex may reduce its nuclear translocation due to an increase in size. As illustrated in Fig.1, the mechanism of GS24-NF κ B activity proposed in our study is that after GS24-NF κ B enters the cell by an interaction between GS24 and TfR-ECD, the NF- κ B decoy portion of the GS24-NF κ B will bind to the "cis"- binding sequence of free NF- κ B units. The formation of this new ODN-NF- κ B complex will prevent the cis-trans interaction between the NF- κ B unit and the promoter region of the target gene (Morishita et al., 1998) and/or prevent NF- κ B nuclear translocation, both of which lead to inhibition of NF- κ B activity. Phosphorylation of p65 is an indicator of NF- κ B activity. To obtain a maximal NF- κ B response, phosphorylation serves to regulate NF- κ B activity (Cheng et al., 2005). Upon stimulation, NF- κ B translocates to the nucleus and is phosphorylated (Ser536). Phosphorylated p65 (Ser536) facilitates the recruitment of transcriptional coactivators (Perkins, 2006). Our results indicate that the phosphorylation of p65 decreased with NF- κ B decoy treatment. This observation may be due to the reduction in the overall level of nuclear p65. While phospho-p65 levels and VCAM-1 expression were significantly reduced in brain endothelial cells *in vitro*, the remaining question is whether the treatment will reduce inflammation at the endothelium *in vivo*. Further study should be performed to investigate the effect of GS24-NF κ B on the adhesion and trans-endothelial migration of neutrophils, and vascular inflammation *in vivo*.

As described earlier, NF- κ B is formed by homo- and heterodimers made up by pair of the five different subunits (Gilmore, 2006), and associates with I κ B proteins in the cytosol in its inactive form (Jacobs and Harrison, 1998, Schwaninger et al., 2006). Different cell types may have different combinations of NF- κ B subunits, indicating its involvement in a variety of functions. Dimers of NF- κ B subunits bind to a set of specific DNA elements in the enhancers/promoters of target genes, which may lead to change in the target gene expression. Although there are many combinations of NF- κ B, the p50/p65 dimer is the most abundant one, found in almost all cell types (Oeckinghaus and Ghosh, 2009). Data from cultured endothelial cells indicated that p50 and p65 are the predominant species found in the nucleus upon cytokine activation (Collins et al., 1995, Poher, 2002). Since all NF- κ B proteins share a highly conserved Rel homology domain (DNA-binding/dimerization domain), NF- κ B decoy is supposed not specific to certain type of NF- κ B units combination (Gilmore, 2006). We propose that the binding of NF- κ B decoy to free NF- κ B dimers

induced by an inflammatory stimulus leads to NF- κ B inhibition in brain endothelial cells, and subsequently blocks the activation of target genes involved in vascular inflammation.

It is important to note that certain NF- κ B activities may be needed for maintaining BBB integrity and function (Ridder et al.), and normal NF- κ B activity in the brain vascular endothelial cells is important. Excessive suppression of NF- κ B activity may have an adverse effect on the normal functions of BBB. However, over-activation of NF- κ B in response to inflammatory conditions upregulates inflammatory molecules, exacerbating inflammation and further damaging the BBB. GS24-NF κ B was able to inhibit the hyper-activated NF- κ B in brain endothelial cells under inflammatory conditions, but did not completely block its activity. Our data showed that GS24-NF κ B reduced the inflammation-induced up-regulation of nuclear p65 and its downstream cytokines, returning levels to basal conditions without completely blocking expression.

Another concern is that NF- κ B can be neuroprotective, and preventing its activity could be adverse to neuronal survival (Yu et al., 2000, Dutta et al., 2006, Fan et al., 2008). Our study utilizes an ODN delivery system that selectively targets TfRs, which are highly expressed on brain endothelial cells, and thus, may alleviate this issue. TfR can mediate transport via endocytosis or transcytosis, and is then recycled back to cell membrane (Bien-Ly et al., Dufes et al., Orita et al., 1990), with endocytosis being the most commonly occurring process. Many studies have provided evidence that substances delivered by anti-TfR ligands were primarily trapped in brain endothelial cells (Moos and Morgan, 2001, Paris-Robidas et al., 2011). Therefore, transcytosis of GS24-NF κ B should be limited, reducing the likelihood of GS24-NF κ B inhibiting NF- κ B activity in the rest of the brain. Therefore, we hypothesize that the majority of GS24-NF κ B will be sequestered at the BBB level after i.v. administration. Further experiments should be performed to study the effect of GS24-NF κ B on the normal NF- κ B activity in brain endothelial cells, and on neuronal survival, as well as to study the distribution of GS24-NF κ B in the brain beyond the BBB. Lastly, because there are other types of TfR expressing cells in the body, e.g. hepatocytes, red blood cells, and white blood cells etc., GS24-NF κ B can also be taken up by these cells. Although those TfR expressing cells may reduce the efficacy of brain delivery, we anticipate that the overall outcome will be anti-inflammatory, because inhibition of NF- κ B activity in white blood cells will also suppress inflammation. However, because GS24-NF κ B may causes adverse effects on hepatocytes or other TfR expressing cells, further studies should be performed to investigate these potential toxic effects of GS24-NF κ B.

In conclusion, the present study has synthesized an ODN based complex, GS24-NF κ B, and delivered it into brain endothelial cells to inhibit NF- κ B activity. The GS24-NF κ B may give more specificity and efficacy in NF- κ B inhibition by targeting a disease relevant cell population, the brain endothelial cells. Therefore, the refined approach used in the present study will only inhibit deleterious NF- κ B in cerebral vascular endothelium but leave beneficial NF- κ B activity unaffected. This study provides proof of concept for brain-targeted drug delivery and modulating a particular cell population relevant to the disease, the brain endothelial cells. The study explores the cerebral endothelial cell as a novel site of action for brain drug delivery and neuro-vascular inflammation therapy.

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Highlights

- A novel DNA-based complex was synthesized for inhibition vascular inflammation.
- The GS-24 is the moiety to transport DNA complex into endothelial cell.
- The DNA complex could be potentially used for inhibiting inflammation at the BBB.

Figure 1.
NF-κB activation and the mechanism of GS24-NFκB inhibition on NF-κB activity.

Figure 2.

A. Design and predicted structure of GS24-NFκB complex by M-fold. B. Formation of GS24-NFκB complex: the ODNs were electrophoresed in 8% native-PAGE gel: NF-κB-decoy-1 strand (lane 1), NF-κB-decoy-2 strand (Strand-2 in lane 2), NF-κB decoy (lane 3), GS24-NFκB-decoy-1 (Strand-1 in lane 4), GS24-NFκB complex (lane 5). C. Quantitative analysis of cellular uptake of GS24-NFκB in bEND5 cells. Competition assay was performed by adding 20 times additional cold GS24-NFκB (unlabeled) into the media containing hot GS24-NFκB (labeled with ^{32}P). $n=6\sim8$, Mean $\pm$ SD, ** indicates $p<0.05$. D. The cellular uptake of GS24-NFκB by bEND5 cells Vs TfR-silenced bEND5 cells. Mean $\pm$ SD, $n=3$, $p<0.05$.

Figure 3. Stability of GS24-NFκB in goat serum. The first two lanes are strand-1 or strand-2 of GS24-NFκB in H₂O respectively.

Figure 4.

A. Effect of GS24-NFκB on nuclear P65 level in bEND5 cells subjected to TNF-α stimulation *in vitro*. Protein levels for p65 are expressed as percentage of media control (=100). Media control is normal cell culture media. B. Effect of GS24-NFκB on VCAM-1 level in bEND5 cells subjected to TNF-α stimulation *in vitro*. The protein levels for VCAM-1 are expressed as percentage of TNFα stimulation (=100). C. Effect of GS24-NFκB on ICAM-1 level in bEND5 cells subjected to TNF-α stimulation *in vitro*. The protein levels for ICAM-1 are expressed as percentage of media control (=100). Mean±SD, n=5-8, ** indicates p<0.05.

Figure 5.

A. Effect of GS24-NF κ B on mRNA level of ICAM-1 level in bEND5 cells subjected to TNF- α stimulation *in vitro*. Mean $\pm$ SD, n=4, ** indicates p<0.05. B. Effect of GS24-NF κ B on mRNA level of VCAM-1 level in bEND5 cells subjected to TNF- α stimulation *in vitro*. Mean $\pm$ SD, n=3, ** indicates p<0.05.

Figure 6.

A. Effect of GS24-NFκB on adhesion of U937 monocytes to bEND5 cells subjected to TNF-α stimulation. Mean±SD, n=3, ** indicates p<0.05. B. Representative images of cell adhesion. Image-A: medium control condition; Image-B: TNF-α condition; Image-C: scramble control 4μM condition; Image-D: GS24-NFκB 2μM condition; Image-E: GS24-NFκB 4μM condition.

Figure 7.

A. Effect of GS24-NFκB on nuclear P65 level in bEND5 cells subjected to OGD/R. B. Effect of GS24-NFκB on VCAM-1 level in bEND5 cells subjected to OGD/R. Mean±SD, n=3, ** indicates $p<0.05$.

Figure 8.

A. Effect of GS24-NFκB on adhesion of U937 monocytes to bEND5 cells subjected to OGD/R. B. Representative images of cell adhesion. Mean±SD, n=3, ** indicates $p<0.05$.

Image-A: Medium control; Image-B: OGD/R; Image-C: scramble control (4μM); Image-D: GS24-NFκB 2μM; Image-E: GS24-NFκB 4μM; Image-F: GS24-NFκB 8μM.

Figure 9.

Brain uptake of GS24-NFκB in mouse brain. Brain accumulation of GS24-NFκB is expressed as % of injection dose (ID/g). Mean±SD, n=3, ** indicates p<0.05.

Figure 10.

A. GS24-NFκB by i.v. injection decreased nuclear p-P65 level in brain endothelial cells of mouse subjected to LPS injection. B. GS24-NFκB by i.v. injection decreased VCAM-1 level in brain endothelial cells of mouse subjected to LPS injection. Mean±SD, n=6, ** indicates $p<0.05$