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Specific down regulation of 3T3-L1 adipocyte differentiation by cell-permeable antisense HIF1 α -oligonucleotide

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Abstract

Hypoxia is a strong modulator of angiogenesis, accelerating adipose tissue expansion, suggesting that hypoxia inducible factor 1 α (HIF1 α) can be a novel target for anti-obesity. We conjugated antisense-HIF1 α -oligonucleotide (ASO) with low molecular weight protamine (LMWP), a cell-penetrating peptide, to enhance its ability to block hypoxic-angiogenesis, thereby eliciting an anti-obesity effect. Nano-sized ASO-LMWP (AS-L) conjugates enhanced cellular uptake of ASO without yielding a cytotoxic effect and protected the ASO against enzymatic attack and chemical reduction. AS-L showed enhanced intra-cellular localization compared to naked ASO and the complex of ASO with lipofectamine during hypoxic-differentiation. Consequently AS-L induced significant down-regulation of leptin and VEGF gene expressions, thereby reducing fat accumulation in the cell.

This proof-of-concept study shows that AS-L produces an inhibitory effect on adipogenesis and angiogenesis during differentiation, indicating LMWP mediated ASO delivery can potentially be a safe and promising treatment for obesity.

Keywords

angiogenesis; antisense oligonucleotide; hypoxia inducible factor 1 α ; low molecular weight protamine; obesity

1. Introduction

The binding of antisense oligonucleotide to its complementary mRNA, in a sequence specific manner, has been utilized for gene silencing and has offered promising therapeutic benefits when used early in the course of disease [1]. FDA approval of antisense agents for clinical use

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[2] demonstrates the possibility of its use in disease management. Indeed, several antisense agents have already reached phase I and II clinical trials, illustrating clinical viability [3].

However, antisense therapy faces a major hurdle in real-time clinical application, primarily due to the poor cellular membrane permeability of these charged macromolecules [4]. Specifically, improvement in the systemic and intracellular delivery of antisense agents is needed [5]. Great effort has been exerted in attempts to overcome this obstacle with the use of viral vectors [6,7], cationic liposome [8-11], polymeric micelles [12-16], peptides [17,18] or cationic polymers [19-21] as carriers to enhance intracellular delivery of antisense oligonucleotide [22]. Although viral vectors such as adenoviruses have shown great promise in achieving effective intracellular delivery of gene compounds, the adverse effects, such as cytotoxicity, immunogenicity and mutagenesis, associated with the use of such a viral system have raised serious safety concerns [23,24].

The current focus of antisense therapeutics is mainly on cancer, autoimmune and cardiovascular diseases, wound healing, and viral infections [1,25]. Only a few results have been reported on the use of antisense therapy for obesity treatment [26]. In addition, the efficiency of adenovirus-mediated transfection of naked DNA into 3T3-L1 cells is still relatively poor [27]. Despite enhanced cellular uptake with adenovirus-poly-L-lysine (PLL) complexes as carriers [27], the immunogenicity concerns still remain.

Obesity, an excess of adipose tissues, caused by hypertrophy and hyperplasia of adipocytes [28], is closely related to a variety of metabolic disorders, including diabetes and cardiovascular diseases such as atherosclerosis. Rising demand for nutrients and oxygen by the growing adipose tissues triggers angiogenesis, to increase the number and/or size of blood vessels [29], and consequently leads to both neovascularization (for adipocyte hyperplasia) and dilation or remodeling of existing capillaries (for adipocyte hypertrophy). Under such physiological changes that occur during the development of obesity, adipocytes become hypertrophic and their size increases up to 140 – 180 μm in diameter [28]. Adipocytes typically have a limited capacity for hypertrophy, partially attributed to the diffusion limit of oxygen, which allows them to reach a size of only about 100 μm [30]. Therefore, it is reasonable to assume that hypertrophic adipocytes would experience a lower oxygen environment; a condition termed hypoxia. It has also been widely suggested that white adipose tissues are poorly oxygenated in individuals with obesity [31,32].

Hypoxia is a major driving force for angiogenesis and can induce increased transcription of both adipogenic and angiogenic factors [31,33-36]. Accumulating reports in recent literature [37,38] support the idea that hypoxic angiogenesis can accelerate adipogenesis through cytokines secreted from adipocytes, and that this process can be inhibited by blocking the upregulated transcription that occurs under hypoxia. It has also been documented that hypoxic adipocytes can secrete various types of cytokines, thereby functioning as a secretory organ. These cytokines, termed adipokines, include leptin, tumor necrosis factor alpha (TNF- α), resistin, and plasminogen activator inhibitor-I (PAI-I). To this regard, several studies have characterized obesity as a chronic inflammatory disease [38]. Inflammation-related-adipokine secretions may trigger the expression of angiogenic factors under hypoxia [36], and local adipose tissue hypoxia can lead to the production and secretion of proangiogenic factors, thereby maintaining adequate blood flow during the development of adipogenesis [39]. Hence, angiogenesis is closely related to adipogenesis in the early stage of white adipose tissue growth.

Hypoxia-inducible factor-one alpha (HIF1 α) is a master transcription factor that plays a key role in the adaptive response to low oxygen environments by inducing hypoxic angiogenesis. HIF1 α accumulates during hypoxia and increases the expression of a variety of mRNAs related to erythropoiesis, glycolysis and angiogenesis [36]. Based on this aspect, hypoxia might be a

strong modulator of the angiogenic process associated with adipose tissue expansion, and therefore, in targeting the hypoxia signaling pathway, HIF1 α itself could offer a novel route for treating obesity [36,38,39]. Notably, a recent clinical study [31,33-35] discovered that HIF1 α expression was elevated in the adipose tissues of obese subjects and it was reduced following weight loss. Although the patho-physiological implication of hypoxia and HIF1 α activation in adipose tissue remains to be established, it was nevertheless shown that suppression of HIF1 α could indeed alleviate the conditions needed for obesity [31,33-35].

To this regard, suppression of HIF1 α expression by an antisense HIF1 α oligonucleotide (ASO), designed to contain a specific sequence complementary to the target RNA molecules, can be a promising strategy to control obesity.

We previously reported the development of low molecular weight protamine (LMWP) as a potent yet nontoxic cell-penetrating peptide (CPP) [40,41] or membrane translocation carrier [40,41]. Both *in vitro* and animal investigations demonstrated that, via covalent or electrostatic conjugation, LMWP was able to transduce its attached protein, gene or carrier cargo into various types of cells [40,41]. More recently, it was shown that cell translocation mediated by LMWP does not cause any perturbation or damage to the cell membrane [42]. Therefore, we hypothesized that LMWP could be used as a tool to aid intracellular delivery of ASO to 3T3-L1 cells [43].

In this paper, the feasibility and utility of this ASO delivery strategy for potential treatment of obesity were explored by synthesizing nano-scale conjugates through linkage of ASO to LMWP via a cytosolic self-cleavable disulfide bond. Investigation of the effects of these conjugates on specific down regulation of adipocyte differentiation by controlling the expression of leptin and VEGF genes are reported.

2. Materials and methods

2.1. Materials

Salmon protamine, thermolysin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 3-isobutyl-1-methylxanthine (MIX), insulin, dexamethasone (DEX), DNase I and CoCl₂ were purchased from Sigma (St. Louis, MO). N-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and BCA protein assay reagent were obtained from Pierce (Rockford, IL). Fetal bovine serum (FBS), phosphate-buffered saline (PBS), Trizol reagent, M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase, oligo(dT)₁₅, rTaq DNA polymerase, 0.25% (w/v) trypsin-EDTA, Dulbecco's modified essential medium (DMEM), penicillin, streptomycin, LipofectAMINE 2000 and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Gibco-BRL (Invitrogen, Carlsbad, CA). The 3T3-L1 cell line which is a preadipocytes that can be differentiated with the treatment of adipocyte differentiation inducer, a mixture of insulin, DEX and MIX, was obtained from ATCC (Rockville, MD). The Lab-Tek II chamber coverglass system was obtained from Nunc International Corp., (Naperville, IL). Heparin and desalting columns were obtained from GE Healthcare (Piscataway, NJ). Unless otherwise noted, all chemicals and reagents were of analytical grade and obtained from Sigma (St. Louis, MO).

2.2. Preparation of the LMWP

The LMWP was derived by the enzymatic digestion of protamine according to a previously described protocol [40]. In brief, protamine was reacted with thermolysin at room temperature for 1hr, followed by addition of 50 mM EDTA to quench the reaction. The product was further purified using a heparin affinity column.

2.3. Chemical conjugation of ASO with LMWP

PS modified antisense-HIF1 α -oligonucleotide (ASO) and mismatch-HIF1 α -oligonucleotide (MMO) were synthesized by IDT Corporation (San Diego, CA). For further conjugation with LMWP, these oligonucleotides were phosphorylated at the 5'-end. An individual sequence of these oligonucleotides was specifically designed to consist of 5'-ACA ACG CGG GCA CCG ATT CGC CAT G-3' for ASO, and 5'-GTG ATC CCC TGC TCT TGC CGT-3' for MMO.

To create a reactive sulfhydryl group at the 5'-phosphated end, oligonucleotides were reacted with 0.25 M ethylenediamine/imidazole solution. Following addition of 20 μ l of 0.1 M imidazole (pH 6.0), the reaction mixture was incubated overnight at 50 °C. Unreacted EDC, imidazole, and the reaction by-products were removed by an HPLC desalting column eluted with 20 mM sodium phosphate buffer at pH 7.4. For conjugation, LMWP was activated using SPDP according to a previously established protocol [40]. Activated ASO-SH and LMWP-SPDP were then reacted in PBS buffer (pH 7.4) overnight at room temperature to produce a 1:1 (molar ratio) AS-L conjugate. MM-L was prepared in a similar manner.

Although one molecule of CPP is known to be capable of translocating the attached cargo, it would nevertheless be beneficial if the attached ASO could be embedded within a network structure to protect it from proteolytic degradation [44]. For this purpose, conjugates were synthesized by reacting ASO with an excess amount of LMWP to form a protective complex network around ASO.

Unless otherwise stated, ASO labeled with 6-FAM at the 3'-position was used for the cellular internalization study by confocal microscopy.

2.4. Characterization of the AS-L

Size distribution of the AS-L was measured by photon correlation spectroscopy using a submicron particle sizer (Zetasizer, Malvern Instruments Ltd., Worcestershire, UK). The macroscopic morphology of the conjugates of LMWP with ASO or MMO was examined using a CM-100 transmission electron microscope (Philips, Eindhoven, Netherland). Samples (5 μ l) were negative-stained with 5 μ l of 1.0% uranyl acetate solution. Images were digitally recorded with a Hamamatsu ORCA-HR digital camera system equipped with AMT software (Advanced Microscopy Techniques Corp., Danvers, MA).

2.5. DNase I Protection Assay

The DNase I protection assay was carried out by utilizing a slightly modified procedure of a previously established protocol [41]. Briefly, the amine/phosphate ratio (N/P ratio, LMWP/ASO) employed in the preparation of AS-L was controlled at 10:1. DNase I (50 units) was added to the solutions of the test compounds, and the reaction mixtures were incubated for 90 min at 37 °C. During incubation, 50 μ l of each suspension was withdrawn at different time points (0, 10, 30, 60 and 90 min), mixed with 75 μ l of the quenching solution (4 M ammonium acetate, 20 mM EDTA, and 2 mg/ml glycogen), and then placed on ice. ASO was then dissociated from LMWP by addition of 37 μ l of 1.0% SDS to the suspension, and the mixture was heated overnight at 65 °C. ASO was precipitated and extracted after incubation by treating the suspension with phenol/chloroform (1:1, v/v), and then the precipitate was treated with ethanol to extract the ASO. The ASO pellet was then dissolved in 10 μ l TE buffer and subjected to 1% agarose gel electrophoresis. Naked ASO treated with the same procedure was used as the control.

2.6. Release of ASO from the conjugates by glutathione (GSH)

ASO released from the conjugates under reductive conditions was measured using a previously reported method [45]. In brief, the samples were treated with varying concentrations (0, 10, 50

and 100 μ M) of GSH in 25 mM Tris-HCl (pH 7.4). The samples used for the release study were either AS-L, AS+L complex or ASO alone, with 100 nM of ASO in each. After incubation for 1 h or 4 h at 37 °C, sample solutions were subject to 1% agarose gel electrophoresis for detection of the released ASO. The amounts of released ASO were determined using the Image J program (National Institutes of Health, Bethesda, MD).

2.7. Cell Cytotoxicity (MTT) Assay and Proliferation Assay

For both the MTT assay and hemacytometry, we used differentiated cells that were treated by an inducer for 8 days to investigate the effect of the test compounds on the early stage of differentiation. Cell cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) conversion assay [41]. Briefly, after seeding cells in a 96-well plate and incubating varying concentrations (0, 0.5, 1.0, 1.5, 2, 5 and 10 μ g/ml) of ASO for 24 h at 37 °C, 100 μ l aliquots containing MTT (5 mg/ml in complete DMEM) were added followed by an additional 5 h incubation. MTT-containing medium was removed and 200 μ l of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals formed by live cells. Absorbance was measured at 570 nm, and the cell viability (%) was calculated according to the following equation [41]:

$$\text{Cell viability (\%)} = [\text{OD}_{570}(\text{sample})] / [\text{OD}_{570}(\text{control})] \times 100.$$

The number of viable cells after treatment with the test compounds was measured using a hemacytometer. Test groups for both cell MTT and proliferation studies included: 1) ASO alone; 2) MMO alone; 3) ionic mixture of ASO and LMWP; 4) ASO complex with Lipofectamine® (20 μ g/ml, positive control); 5) MM-L; and 6) AS-L. Unless otherwise stated, the test compounds involved in differentiation, transfection and cytotoxicity studies include all the samples mentioned above.

2.8. 3T3-L1 preadipocyte differentiation following treatment with the AS-L

The 3T3-L1 cells were divided into three groups: control group (Con), inducer treated differentiation groups under normoxia (D) and under hypoxia (H). Each group further included several subgroups that were the same as those mentioned in the *Cell cytotoxicity (MTT) assay* method. The 3T3-L1 preadipocytes were seeded into a 6-well plate (3×10^5 cells/well) and differentiated as previously described [46]. In short, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and maintained in a 37 °C incubator with 5% CO₂. After cells reached complete confluency (d0), differentiation was induced by changing the medium to DMEM containing 10% FBS plus 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 10 μ g/ml insulin and 0.25 μ M dexamethasone (DEX). After incubation for 48 h (d2), the medium was replaced with DMEM containing 10% FBS and 1 mg/mL insulin. On day 4 (d4), the medium was replaced with DMEM containing only 10% FBS. This step was repeated every 2 days, until it reached d10. Cell differentiation was then assessed from the morphological changes using the oil red-O staining method [46]. A hypoxia mimetic agent, CoCl₂, was added to the differentiation medium at a concentration of 100 μ M and then CoCl₂ containing medium was changed every 2 days until 10 days.

2.9. Treatment and Transfection of Cells with Conjugate

Four days after differentiation, cells were treated with the test compounds mentioned in the *Cell cytotoxicity (MTT) assay* method. Test compounds were prepared in 10% serum containing DMEM medium and then incubated with cells at 37 °C for 6 h. Transfection of ASO using lipofectamine was conducted in 6-well plates when cells were at about 50 – 70% confluence. After a 6 h incubation, serum containing medium was added to each well and cells were incubated over night.

The concentrations of all the test compounds were at equivalent concentrations of AS-L (1.5 $\mu\text{g}/\mu\text{l}$ of ASO).

2.10. RT-PCR Analysis

Total RNA was extracted using Trizol solution according to the manufacturer's instructions. First-strand cDNA synthesis was performed with 1 μg of RNA, oligo(dT)₁₅, and M-MLV reverse transcriptase. Semi-quantitative PCR amplification of cDNAs encoding leptin, VEGF and actin were carried out using rTaq DNA polymerase. The primer sequences used for three cDNAs were as follows; VEGF (479 bp) : forward 5'-GAT GTA TCT CTC GCT CTC TC-3', reverse 5'-CTG CTC TAG AGA CAA AGA CG-3'; leptin (597 bp): forward 5'-CCT GTG TCG GTT CCT GTG G-3', reverse 5'-GAA ATG AAT GAT GGA TGT GTG C-3'; Actin (704 bp; used as a control): forward 5'-CCA GAG CAA GAG AGG CAT CC-3', reverse 5'-AGG TCT TTA CGG ATG TCA ACG-3'.

The amplified fragments were analyzed on a 1% agarose gel. The bands on the images were quantified using the Image J software (National Institutes of Health, Bethesda, Maryland).

2.11. Confocal Microscopy

The 6-FAM tagged ASO, at the 3'-position, was used for the chemical conjugation. Both an ionic complex of ASO with LMWP and ASO with lipofectamine were used as positive controls. Cells were exposed to differentiation medium with- (H) or without- (D) hypoxia conditions. The day before transfection, three groups of cells in each 6-well plate were detached at the 4-day mark (d4) after inducer treatment and re-seeded on a 4-well Lab-Tek chamber coverglass slide at 70% confluency per well. Following an approximately 20 hr incubation, cells were treated with the test compounds without serum deprivation. After washing with PBS, cells were subjected to confocal analysis, controlled at 37 °C and 5% CO₂, using an Olympus FluoView 500 Laser Scanning Confocal Microscope.

2.12. Statistical analysis

Statistical analyses of experimental results were performed using the SPSS (Version 11.0) program. All measurements are expressed as mean $\pm$ SD. A statistically significant difference between groups was assessed by using one way-ANOVA, and $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Synthesis and Characterization of the AS-L

The scheme for synthesis of the AS-L conjugates is depicted in Fig. 1. ASO, phosphorylated at the 5'-end, was first reacted with imidazole to form a reactive phosphoroimidazolide, and then cystamine and DTT were added to yield a reactive, ASO thiolation. The degree of thiol substitution in ASO was $68 \pm 13\%$ as measured by Ellman's assay. Thiol-modified ASO was then added to SPDP-activated LMWP to produce the ASO-SS-LMWP conjugate through a sulfhydryl exchange reaction.

The conjugates of ASO and LMWP can be synthesized by site-specific manner of ASO, the conjugates has reacted 1:1 ratio.

A gel retardation study was conducted to confirm the covalent linkage of ASO with LMWP. Conjugates, treated with 0.5 M DTT to break the disulfide bonds, were also analyzed by electrophoresis on a 1% agarose gel. As shown in Fig. 2A, migration of AS-L conjugates after PBS treatment was completely retarded due to charge neutralization (Left lane). In contrast,

following dissociation of the disulfide bond between LMWP and ASO by DTT treatment, only the fast-migrating negatively charged ASO band was observed (Right lane).

Characterization by TEM (Fig. 2B) revealed that the AS-L formed irregular, spherical structures with an average diameter of 189 ± 39 nm (Fig. 2B).

3.2. Stability of the AS-L

We also examined the stability of the AS-L against nuclease degradation. Fifty units of DNase I were added to test samples containing either AS-L or the ionic complexes prepared by mixing ASO with LMWP (AS+L), followed by incubation of the reaction mixture for various time periods (0, 10, 30, 60 and 90 min) at 37 °C. Naked ASO treated with DNase I was used as a control. As shown by the 1% agarose gel electrophoresis results in Fig. 3A, naked ASO and the ionic complexes (AS+L) migrated downward rapidly after incubation with DNase I. In contrast, no migration of the AS-L was observed, indicating that conjugation with LMWP resulted in the protection of ASO against nuclease digestion.

It is expected that the disulfide bond of the AS-L will be severed in the reductive environment of the cytosol, where the ASO exerts its effect. To examine this phenomenon, we treated conjugates with varying concentrations (0, 10, 50 and 100 μ M) of a physiologically relevant reductive reagent, GSH, for 1 h and 4 h, and then examined the release of ASO using gel electrophoresis. As seen in Fig. 3B, after 1h incubation, the extent of ASO released was found to be approximately 20.2% to 29.1%. After a 4 h incubation, nearly 100% of the ASO were detached from LMWP at GSH concentrations of 50 and 100 μ M (4.5 fold and 4.8 fold compared to that of the 10 μ M concentration, respectively). The amounts released after 4h incubation at the concentrations of 50 μ M and 100 μ M were invariably higher than those after 1 h incubation.

3.3 Cytotoxicity and Cell Proliferation Studies

The MTT assay was conducted for two different purposes: 1) to determine the optimum concentration of ASO for subsequent cell treatment; and 2) to assess cell viability during differentiation under hypoxia.

To determine the optimum concentration, non-differentiated control cells were treated with varying concentrations of AS-L containing 0, 0.5, 1.0, 1.5, 2, 5 and 10 μ g/ μ l of ASO. The optimal conjugate concentration was determined to be 1.5 μ g/ μ l. ASO showed significant cell toxicity at concentrations of 2 μ g/ μ l and higher, so 1.5 μ g/ μ l of ASO was determined to be the optimal concentration for AS-L without causing a toxic effect (Fig. 4A).

Although LMWP has been demonstrated to be safe and non-toxic [40], the cytotoxicity of the AS-L still needs to be evaluated, simply because the toxicity of LMWP under adipocyte proliferation and differentiation is not yet known. For this purpose, we examined the effect of the conjugates, containing the optimal concentration of 1.5 μ g/ μ l ASO as determined in Fig. 4A, on both cytotoxicity and cell proliferation by utilizing the MTT assay and a hemacytometer, respectively.

As shown in Fig. 4B, ASO, LMWP and AS-L treated cells yielded statistically indistinguishable cell viability under hypoxia compared to that of control (i.e. treated with MTT buffer). In agreement with reports by other investigators [47], cells treated by ASO chemically conjugated with LMWP displayed a higher viability. On the other hand, cells treated with the ionic complex of ASO with LMWP or MMO-conjugates (MM-L) exhibited a decreased viability to approximately 60% of control. It is known that the effect of MMO on inhibition of cell proliferation is highly sequence-dependent. Hence, after entering the cell cytosol, MMO could bind to non-specific genes in a non-sequence specific manner, therefore decreasing cell viability [48].

Interestingly, cell proliferation was significantly increased by either adipocyte inducer treatment under normoxia or all of the test compounds in the adipocyte differentiation groups under hypoxia (2.2- or 5.7-fold increase compared to that of the control, respectively, $P < 0.05$) (Fig. 4C). However, when comparing with the PBS treated group under hypoxia, significant inhibition (20%; $p < 0.05$) on cell proliferation was observed only in the AS-L treatment group, as the change induced by the other test compound (e.g. AS+L) was not statistically significant.

3.4. Cellular Uptake of the AS-L

Cellular uptake and intracellular localization of the AS-L were carried out with 3T3-L1 cells and analyzed using confocal microscopy (Fig. 5). Both the unmodified ASO and MMO alone were unable to internalize into cells, as shown, in the control (a, d) or differentiation groups, either under normoxia (b, e) or hypoxia (c, f). While the ionic complex of ASO with LMWP could transverse into the cell cytosol in the control (g), it could not do so in the differentiation groups under either normoxia (h) or hypoxia (i). With the aid of lipofectamine, ASO was able to reach the cell cytosol in the control (j) and the differentiation group under normoxia (k). However, lipofectamine could not facilitate the cell translocation of ASO under hypoxia (l). In contrast, chemical conjugation of ASO with LMWP displayed a significant enhancement in cellular uptake under all of the conditions studied, including the control group (p) and differentiation groups under both normoxia (q) and hypoxia (r). In addition, the cell penetration effect was also seen with chemical conjugation of MMO with LMWP in control group (m) and differentiation group under both normoxia (n) and hypoxia (o).

3.5. Adipogenic- and Angiogenic-Gene Expression

The leptin and VEGF genes, expressed during adipogenic and hypoxia-related angiogenic processes, respectively, were examined during adipocyte differentiation under both normoxia (D) and hypoxia (H) (Fig. 6A). As seen, the leptin gene expression was significantly increased by 1.5-fold or 2.7-fold compared to the control group during differentiation under normoxia or hypoxia, respectively. Expression of the VEGF gene followed a pattern similar to that of the leptin gene as a 2.6-fold and 3.4-fold increase over the control group was observed under normoxic and hypoxic conditions, respectively. Based on these results, we concluded that the angiogenic process could be one of the key factors in regulating adipogenesis during adipocyte differentiation.

We further examined the effect of the AS-L on both angiogenesis and adipogenesis under differentiation. As shown in Fig. 6B, up-regulated leptin gene expression was significantly reduced by treatment with the AS-L to the level of 32% or 61% of the PBS-treated group under normoxia (D) or hypoxia (H), respectively. It can be seen that ASO delivered by using lipofectamine or the MM-L was also able to cause decreased leptin expression. However, AS-L displayed a stronger inhibitory effect on leptin gene expression, by about 2.0-fold under normoxia and 1.5-fold under hypoxia, when compared to lipofectamine or MM-L.

VEGF expression showed a pattern similar to that of leptin expression. Up-regulated VEGF expression was significantly reduced by the AS-L treatment in both the normoxic and hypoxic differentiation groups (Fig. 6C). In comparison to the leptin gene expression, only the AS-L showed a statistically significant inhibitory effect under normoxia or hypoxia (20% or 56%, respectively, when compared to the PBS group).

3.6. Oil red-O Staining Method for Measurement of Fat Accumulation

The oil red-O staining method was employed to assess fat cell proliferation and the anti-angiogenic effect of test compounds during 3T3-L1 adipocyte differentiation. As shown in Fig. 7A, during adipogenesis under hypoxic condition there was significant cell proliferation as well as fat accumulation, as evident by the increase in the oil red-O stain color. Fig. 7B revealed

that the absorbance at 500 nm, reflecting fat accumulation in the differentiation groups under normoxia (D) or hypoxia (H) was increased by 1.9- and 4.8-fold, respectively, when compared to the control group (Con). Fig. 7C further demonstrated that treatment by AS-L yielded a significant inhibition on fat accumulation in the cell cytosol under normoxic differentiation, as its OD 500 nm decreased to 48% of that seen in the PBS control group ($p < 0.05$). In comparison, cells treated with ASO or the ionic complex displayed only a 10% or 21% decrease, respectively, compared to the PBS control group. Similarly under the hypoxic condition, during which the other test compounds did not inhibit fat accumulation, AS-L treatment blocked 32% of fat accumulation when compared to the PBS control ($p < 0.01$) (Fig. 7D).

4. Discussion

We designed an antisense-HIF1 α oligonucleotide (ASO) and used it for synthesis of chemical conjugates with LMWP. Although antisense gene therapy for HIF1 α has been widely used for cancer therapy for several decades [49,50], herein we provide the first piece of evidence for obesity control by delivering ASO to 3T3-L1 preadipocytes using a protein transduction domain peptide, LMWP.

Research has attempted to overcome the exceedingly low transfection efficiency of naked ASO, or DNA, to pre-adipocyte cell lines [51] by utilizing cationic polymers like polyethylenimine (PEI), adenoviruses [52,53] or lipofectamine to assist the transduction systems [52]. However, adenoviral delivery is dependent on the cellular receptor CAR, which is expressed at a very low level in 3T3-L1 cells [27]. In addition, high MOI (Multiplicity of Infection) of adenovirus is needed to improve the efficiency.

In contrary, the use of a cell penetrating peptide (CPP) as the drug carrier possesses several distinctive advantage such as: 1) rapid and efficient intracellular delivery of ASO; 2) achieving a significant antisense effect [54]; 3) improving the ASO stability; 4) reducing the dose of ASO required for therapeutic efficacy; and 5) alleviating ASO-induced cytotoxic side effects [55]. Additionally, it has been shown that chemical conjugation with a CPP does not interfere with the biological properties of the ASO.

In this investigation, LMWP was selected as the ASO carrier because, aside from its proven cell penetrating capability [27], LMWP was demonstrated to be non-toxic in animals [56] as well as devoid of antigenic [57] or mutagenic [58] properties.

The AS-L was synthesized, via the formation of a disulfide bond, by utilizing a well-established SPDP coupling protocol [27]. TEM revealed that the synthesized conjugates were of a nano-sized (189 ± 39 nm) structure within the size range (100 – 200 nm) favorable for cellular drugs delivery [59].

As expected, conjugation of ASO with LMWP did not induce any measurable cytotoxic effect, and the effect of the conjugate was similar to ASO alone whereby the cell population was significantly increased under normoxia and even more under hypoxia.

The cytotoxicity of AS-L is based on the range of its concentration, and we observed cell toxicity at a concentration > 1.5 $\mu\text{g}/\mu\text{l}$. This concentration as an ASO is higher concentration (193 μM) compared to that of ASO alone used for the other cell cytotoxicity, which is at the maximum 0.25 – 20 μM range [60,61]. However, no measurable cytotoxic effect was seen using AS-L at the concentration range used in our reported study. An ASO dose of 6 mg/kg was reported for a anti-cancer clinical study [62]. Based on a rough calculation, the drug plasma concentration is 0.15 $\mu\text{g}/\mu\text{l}$. As a rule of thumb, we used an experimental concentration of 15 $\mu\text{g}/\mu\text{l}$, i.e. 10-fold clinical dose.

The cell growth was prompted by the adipocyte inducer treatment (normoxia, Fig. 4C-dashed bar) and accelerated under hypoxia (Fig. 4C-white bar). Namely, the cell number of differentiated cells under hypoxia was already significantly increased compared to those of under non-differentiated status (Fig. 4C-black bar) and under normoxia status (Fig. 4C-dashed bar). To evaluate the effect of AS-L on cell proliferation under hypoxia, we checked the cell number after AS-L treatment under hypoxia and compared to that of PBS treatment under hypoxia. Once the cell number is increased, it is hard to be reduced. Furthermore, since the AS-L did not show significant cytotoxic effect (Fig. 4-B), the reduced cell number by the AS-L treatment compared to PBS treatment can be explained that AS-L has inhibition effect on cell proliferation during hypoxic differentiation.

Since the AS-L did not show significant cytotoxic effect (Fig. 4B), reduced cell number by the AS-L treatment compared to PBS treatment can be explained that AS-L has inhibition effect of cell proliferation during hypoxic differentiation.

An important factor for delivery of ASO into the cells is the maintenance of its biological activity and stability. Protection of ASO from degradation by DNase I for up to 90 min was seen for the conjugate, implicating that LMWP protects ASO from DNase I attack. Although the mechanism of such protection is not yet understood, this behavior is certainly of great importance concerning any possible future *in vivo* application. In comparison with chemical conjugates, ASO was detached from the ionic complex when incubated with DNase I. The phosphorothioate (PS) modified ASO, which replaces the oxygen atom in the phosphate backbone with a sulfur moiety, has considerable stability and resistance to extracellular and intracellular nucleases [63], thus when ASO was detached from the ionic complex it migrated downward on the gel similar to the control and there was no degradation.

For the ASO to be therapeutically effective, it must be fully released from the AS-L once inside the cells. To achieve this purpose, LMWP was attached to ASO via a disulfide bond that is self-degradable in the cytosol [45] by the elevated levels of GSH and reductive activity [22]. It should be noted that concentrations of GSH in circulation are in the micromolar range, whereas in the cytosol it is approximately three orders of magnitude higher and in the millimolar range [64]. Findings in Fig. 3B show that the amounts of ASO released from AS-L after 4 h of incubation at GSH concentrations of 50 μ M and 100 μ M were significantly higher than those seen with 10 μ M GSH, as well as after only 1 h of incubation. Based on these results, it was concluded that the AS-L would maintain its stability in the circulation for at least 90 min against reduction by plasma levels of GSH and against DNase I degradation.

Confocal results (Fig. 5) revealed that the AS-L could be delivered into the cytosol of differentiated adipocytes, apparently through a LMWP-mediated cell-internalization mechanism. In addition, fat accumulation and leptin expression in the cells were remarkably decreased by treatment with the AS-L under hypoxia (Fig. 6), primarily through inhibition of the angiogenic phenomena of the angiogenic transcription factor, HIF1 α . In contrast, all other test compounds were not translocated into the cells, except for the MM-L which displayed a similar cell uptake pattern to that of AS-L (Fig. 5). Nevertheless, treatment with the MM-L neither blocked adipogenesis nor angiogenesis (Fig. 6 and 7), suggesting that suppression of the VEGF and leptin genes was specifically due to down regulation by the LMWP-mediated intracellular delivery of ASO.

In summary, AS-L displayed markedly enhanced resistance against cellular nucleases and yet still retained a high binding affinity towards targeted mRNA, thereby yielding the desired transcriptional inhibition. Results presented here demonstrate that LMWP possesses a high efficiency for intracellular localization compared to that of the cationic liposome, lipofectamine. It was specifically unique that AS-L could internalize into the highly

proliferated cell population, improving on the poor cellular membrane permeability of the charged ASO and thus overcoming the primary hurdle for this drug in the treatment of the pathophysiological condition of obesity.

ASO therapy for obesity treatment was investigated in the present study. Based on the concept of Ingber *et al* [65] in linking new blood vessel formation under hypoxia with obesity, we hypothesized that adipogenesis was closely related to angiogenesis, as observed by up-regulated VEGF mRNA gene expression during adipocyte differentiation. Our results provided strong evidence to support this hypothesis, because with inhibition of the HIF1 α angiogenesis-related gene, we were able to control hypoxic angiogenesis. It should be emphatically pointed out that although ASO gene therapy for HIF1 α has been widely used for cancer therapy for several decades [49,50], our data provides the first piece of evidence of obesity control by delivering ASO to 3T3-L1 preadipocytes using the cell-penetrating peptide LMWP.

Overall, this delivery strategy offers several unparalleled advantages. First, highly proliferated cells such as 3T3-L1 are in essential need of an efficient delivery method that carries no adverse effects during their differentiation. In addition, chemical modification of ASO with LMWP could improve its stability, thereby significantly reducing the dose of ASO required for attaining the highest efficacy and lowest non-specific cytotoxicity.

To this regard, LMWP-mediated ASO delivery is a promising strategy for adipogenesis inhibition by silencing the related genes and preventing hypoxic angiogenesis, and is potential in obesity control.

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Fig. 1.
Scheme for synthesis of ASO and LMWP conjugates.

Fig. 2. Characterization of ASO and LMWP conjugates. (A) Migration profiles of conjugates before (left lane) and after (right lane) treatment with 0.5 M DTT for 15 min. (B) Morphology and diameter of conjugates measured by TEM analysis. White arrows indicate randomly selected particles used for conjugate size calculation. Bar size indicates 200 nm. (AS-L: Chemical conjugate of ASO with LMWP, ASO: AS-HIF1 α -oligonucleotide)

Fig. 3. Stability of conjugate using DNase I and GSH. (A) Gel retardation assay with DNase I. Chemical conjugates and ionic complex samples were treated with DNase I (10 units) for varying times (0, 10, 30, 60 and 90 min). All the samples were analyzed by 1% agarose gel electrophoresis for 20 min at 100V. (B) Release of ASO from the conjugate with LMWP after addition of varying concentrations of GSH (0, 10, 50 and 100 μM) for 1 h and 4 h incubations at 37 °C. After incubation, samples were used for 1% agarose gel electrophoresis to detect the released ASO. The band densities of all samples were measured using the Image J program. Un-conjugated naked ASO was used as a standard to measure the concentration. (ASO: AS-HIF1α-oligonucleotide, AS+L: Ionic complex of ASO with LMWP, AS-L: Chemical conjugate of ASO with LMWP)

Fig. 4.

Effect of conjugate on cytotoxicity and cell proliferation. (A) The MTT assay with varying concentrations of AS-L using non-differentiated 3T3-L1 cells. (B) After seeding the cells, they were treated with test compounds mentioned below for 24 h. After five hours of additional incubation with MTT, the optical density (OD) at 620 nm was measured. (C) Cell proliferation assay was conducted using a hemacytometer. Black bar: Non-differentiated control group, Dashed bar: Differentiation group under normoxia, White bar: Differentiation group under hypoxia. (ASO: AS-HIF1 α -oligonucleotide, L: LMWP, AS+L: Ionic complex of ASO with LMWP, MM-L: mismatch oligonucleotide conjugate with LMWP, AS-L: ASO conjugate with LMWP)

Fig. 5.

Intracellular localization of conjugates. Three groups of cells in 6-well plates were detached 4 days after differentiation inducer treatment and re-plated in a 4-well Lab-Tek Chambered Coverglass (Nunc) at 70% cell population/well the day before transfection. Cells were treated with 6-FAM-tagged AS-L and its comparative amount of ASO and ionic complex for 24 h. After treatment, the cells underwent microscopy analysis using an Olympus FluoView 500 Laser Scanning Confocal Microscope (water immersion $\times 60$) under temperature and CO_2 control (37°C and $5\% \text{CO}_2$). (Con: Control group, D: Differentiation group under normoxia, H: Differentiation group under hypoxia, ASO: AS-HIF1 α -oligonucleotide, MMO: Mismatch-

HIF1 α -oligonucleotide, AS+L: Ionic complex of ASO with LMWP, MM-L: mismatch oligonucleotide conjugate with LMWP, AS-L: ASO conjugate with LMWP)

Fig. 6.

Inhibitory effect of AS-L on angiogenesis and adipogenesis. (A) After complete differentiation by a 14-day treatment with an adipogenic inducer, all cells in each group, control group and differentiation groups both under normoxia and hypoxia, were harvested and total RNA was extracted for RT-PCR. Four days after the differentiation period both under normoxia and hypoxia, cells were treated with test compounds as mentioned below. Leptin (B) and VEGF (C) mRNA, adipogenesis and angiogenesis markers, respectively, expressions were detected using the RT-PCR method. The band densities were measured using the Image J program. Black bar: Non-differentiated control group, Dashed bar: Differentiation group under normoxia, White bar: Differentiation group under hypoxia. (PBS: Phosphate buffered saline; Lipo.: ASO complex with Lipofectamine; MM-L: mismatch oligonucleotide conjugate with LMWP; AS-L: ASO conjugate with LMWP; *: $p < 0.05$, **: $p < 0.01$ compared to PBS treatment in Control group; #: $p < 0.05$ compared to PBS treatment in differentiation group either under normoxia or hypoxia)

Fig. 7.

Inhibition of fat accumulation by AS-L. The blocking of fat accumulation by the test compounds was assessed by morphological changes and the oil red-O staining method. (A) Morphological changes and oil red-O stained differentiated cells. (B) Fat accumulation in differentiated cells of all experimental groups (Con, D and H group) was quantified by measuring the absorbance at 500 nm of fat extracted from the cell cytosol with isopropyl alcohol. Fat accumulation after treatment with test compounds in differentiation groups either under normoxia (C) or hypoxia (D) was measured by the absorbance at 500 nm. (Con: Control group, D: Differentiation group under normoxia, H: Differentiation group under hypoxia, PBS: Phosphate buffered saline-treated control group, MM-L: mismatch oligonucleotide conjugate with LMWP, AS-L: ASO conjugate with LMWP, ASO: naked ASO, AS+L: ASO and LMWP ionic complex, *: $p < 0.05$)