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In vivo delivery of cell-permeable antisense hypoxia-inducible factor 1α oligonucleotide to adipose tissue reduces adiposity in obese mice

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ABSTRACT

Ongoing research has gradually recognized and understood the importance of adipose tissue (AT) angiogenesis as a key modulating factor of adipogenesis in the development of obesity. Previously, we carried out the first *in vitro* demonstration of the down-regulation of hypoxic angiogenesis during adipogenesis using cell-permeable chemical conjugates composed of antisense hypoxia-inducible factor 1α (HIF1α) oligonucleotide (ASO) and low-molecular weight protamine (LMWP). To further confirm the *in vivo* feasibility, we administered ASO-LMWP conjugates (AL) to diet-induced obese (DIO) mice by intraperitoneal injection (IP). Results showed that the AL conjugates significantly reduced the body weight, total fat tissue weight, and plasma lipid concentrations in the mice. Moreover, the AL conjugates not only decreased liver weight and hepatic triglyceride concentration but also significantly attenuated subcutaneous adipocyte cell size, which was conversely increased in the AL-untreated high-fat diet (HFD) group. Interestingly, more blood vessels were observed in the HFD group than in the lean group, indicating that blood vessel development could induce growth of the fat mass. This pattern was reversed in the AL-treated groups, which displayed a decrease in blood vessel density compared to the AL-untreated HFD group. This study presents the first *in vivo* evidence, in an obese mouse model, of the feasibility of achieving a biological treatment modality for obesity by blocking the angiogenic transcriptional factor HIF1α, thereby limiting angiogenesis, via the use of an adipose tissue-permeable ASO-LMWP.

1. Introduction

Obesity is reaching epidemic proportions worldwide, and it is currently the most prevalent disease among children and young adults in the United States [1]. The World Health Organization identifies individuals with a body mass index (BMI) of 25 kg/m^2 or greater as overweight, while those with a BMI of 30 kg/m^2 or higher are further classified as obese. It is estimated that over 1.7 billion adults are overweight; more than 300 million are obese. Studies have shown that overweight individuals are more likely to die early, losing an average of 7 years of their lifespan [1]. Obesity is also closely associated with a variety of metabolic disorders, including diabetes, and cardiovascular diseases such as atherosclerosis, hyperlipidemia, and hypertension [2]. This disease burden results in a devastating loss of lives as well as tremendous healthcare costs.

Obesity treatments have thus far focused mainly on lifestyle modifications such as reduction of food intake by influencing the central nervous system (satiety center), or increasing physical activity. While lifestyle change is the ideal solution, this approach has failed to resolve this growing epidemic to date [2]. One of the factors for this failure is the silent nature of this disease, with its insidious effects becoming apparent only when it gives rise to other, more visible health-related problems [1]. The long-term results of these efforts have hitherto been disappointing.

Pharmaco-therapeutic management for obesity is currently limited to 2 drugs: orlistat and sibutramine [3,4]. These therapies, however, are unable to induce substantial weight loss, and are effective only when used in combination with other drugs or strict weight management programs. Additionally, orlistat and sibutramine can cause significant side effects such as gastrointestinal distress and increase of blood

Abbreviations: HIF1α, Hypoxia inducible factor 1α; ASO, Antisense-HIF1α-oligonucleotide; AL, ASO-LMWP conjugates; ALT, Alanine transaminase; AST, Aspartate transaminase; CPP, Cell permeable peptide; DIO, Diet-induced obesity; HFD, High fat diet; MMO, Mismatch-HIF1α-oligonucleotide; ML, MMO-LMWP conjugates; LMWP, Low molecular weight protamine.

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pressure and heart rate [3]. Surgical intervention (e.g., gastric bypass), an option that has recently attracted greater attention in the US, is able to produce weight loss of 25–30%, but is associated with several contraindications, including schizophrenia, depression, and eating disorders, and requires long-term monitoring of patients after surgery [1]. Overall, current practices are unable to manage this growing epidemic, and there is great urgency and need for a novel, safe, and effective therapy.

Obesity, the expansion and growth of white adipose tissue (WAT) caused by hyperplasia and hypertrophy of adipocytes [5], is dependent on the neovascularization and dilation of existing capillaries, respectively [5–7]. Hence, hypertropic adipocytes are typically found to possess low-oxygen microenvironments—hypoxia [8]. Similar to tumor growth, the inhibition of adipose tissue angiogenesis inhibits WAT growth and ultimately, the development of obesity. Indeed, endogenous angiogenesis inhibitors such as angiostatin [9] and endostatin [10] have been reported to reduce the body weight of obese mice [11]. In addition, it has been demonstrated that angiogenesis inhibitors such as TNP-470 and vascular endothelial growth factor receptor 2 (VEGFr2)-specific inhibitors prevent the development of obesity in genetic mouse models raised on high-fat diets (HFD) [12]. It thus appears that specific treatment of adipose tissues is critical to achieving control of obesity. Therefore, pharmacological manipulation of adipose tissue angiogenesis potentially offers a novel therapeutic option for effective treatment of obesity and related metabolic disorders [12].

To inhibit angiogenesis, we propose the use of an RNA-interfering agent, antisense oligonucleotides (ASO), for the biological treatment of obesity. ASO are novel therapeutics designed to bind to a target messenger RNA (mRNA), resulting in degradation of the mRNA and thereby decreasing the production of specific proteins associated with disease progression [13]. Since the introduction of nucleic acid-based therapeutics in the late 1970s, a wide variety of ASO have been developed that display target gene silencing efficacy [13]. The major barrier to achieving effective ASO therapy lies in the poor intracellular delivery efficacy and stability of ASO drugs [14]. Recently, RNA interfering agents (RNAi), siRNA and shRNA, have been introduced as a new class of agents for targeted gene therapy [15]. While RNAi technology continues to advance, ASO nevertheless offer several advantages over RNAi, including 1) flexibility in chemistry and target site design; 2) lower synthesis costs; 3) longer circulation half-life; and 4) relative ease of delivery to the target cells [16]. Furthermore, ASO produced by current state-of-the-art technologies are far more resistant to degradation by nucleases than current siRNA compounds [14]. Indeed, several ASO compounds have been shown to possess improved pharmacokinetic properties and significant activity towards the expression of targeted genes as well as disease progression in preclinical rodent studies and preliminary clinical trials [17,18].

Several delivery methods have been developed to enhance intracellular uptake of ASO, such as the use of viral vectors [19], cationic liposomes [20], polymeric micelles [21], peptides [22], and cationic polymers [23]. Although viral vectors (e.g., adenoviruses) have demonstrated great promise in achieving effective intracellular delivery of gene compounds, the associated adverse effects of cytotoxicity, immunogenicity, and mutagenesis have raised serious safety concerns [24]. Conversely, the use of relatively non-toxic cell-penetrating peptides (CPPs) as drug carriers has displayed several distinctive advantages over other delivery methods without altering the biological properties of the ASO [25]. These benefits are: 1) rapid and efficient intracellular delivery of an ASO with a significant antisense effect; 2) improving ASO stability, resulting in a reduced dose of ASO required for therapeutic efficacy; and 3) reducing ASO-induced side effects [25,26]. In a previous investigation, we reported the development of a cell-permeable ASO–low molecular weight protamine (LMWP) chemical conjugate (AL) by linking hypoxia-inducible factor 1α (HIF1α) (a key regulating transcription factor under hypoxia) ASO with LMWP [27], a nontoxic CPP developed in our laboratory [28]. *In vitro* cell culture studies demonstrated a significantly enhanced intracellular localization of the AL conjugates, which consequently induced marked down-regulation of adipogenic and angiogenic gene expressions, thereby blocking the angiogenesis during adipogenesis of 3T3-L1 cells under hypoxia as well as reducing fat accumulation in these cells [27].

In the current investigation, we further confirmed the feasibility of this novel AL therapy *in vivo* by using a clinically relevant diet-induced obese (DIO) mouse model. The findings from these animal studies further confirmed our hypothesis that blocking HIF1α expression and subsequently reducing angiogenesis and adipogenesis in adipocytes as well as fat mass could potentially be a novel and effective means of clinical treatment of obesity.

2. Materials and methods

2.1. Materials

Salmon protamine and thermolysin were purchased from Sigma (St. Louis, MO). Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and dithiothreitol (DTT) were purchased from Pierce Biotechnology, Inc. (Rockford, IL). Fetal bovine serum (FBS), phosphate-buffered saline (PBS), and 0.25% (w/v) trypsin-EDTA were purchased from Gibco-BRL (Invitrogen, Carlsbad, CA). Human umbilical vein endothelial cells (HUVEC), F-12K media, heparin, and endothelial cell growth factor (ECGS) were purchased from American Type Culture Collection (ATCC) (Manassas, VA). All other chemicals used were reagent-grade commercial products. Phosphorothioate (PS)-modified antisense HIF1α oligonucleotide (ASO) and mismatch HIF1α 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.

2.2. Preparation of LMWP

LMWP was derived by the enzymatic digestion of protamine according to a previously described protocol [29]. In brief, protamine was hydrolyzed with thermolysin at room temperature for 1 h, followed by the 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

To create a reactive sulfhydryl group at the 5′-phosphated end of the oligonucleotides, both ASO and MMO were reacted with 0.25 M EDC solution. Following the addition of 20 μL of 0.1 M imidazole (pH 6.0) and cystamine, the reaction mixture was incubated overnight at 50 °C. Unreacted EDC, imidazole, cystamine, and the reaction byproducts were removed by HPLC using a desalting column eluted with 20 mM sodium phosphate buffer at pH 7.4. Prior to conjugation, LMWP was activated using SPDP according to a previously established protocol [29]. 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) AL conjugate. The chemical conjugates of MMO-LMWP linked with a disulfide bond (ML) were prepared in a similar manner. All conjugates were then mixed with excess LMWP (1:10 molar ratio) to form a protective, ionically stabilized complex around the ASO [30].