Anti-POMC siRNA reduces ACTH secretion in a cell culture model of Cushing’s disease
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Background
Cushing’s disease is due to a pituitary corticotroph adenoma autonomously-secreting excessive amounts of adrenocorticotropic hormone (ACTH) and is the most common cause of ACTH-dependent, endogenous Cushing’s syndrome. Increased ACTH secretion stimulates excess cortisol production and release into the circulation. Long-term sequelae of chronic exposure to hypercortisolemia include physical, metabolic and psychological co-morbidities and increased standard mortality rate. Transphenoidal resection of the adenoma is currently the only curative treatment for Cushing’s disease and remains the first-line treatment, unless contraindicated. However, there are long-term side effects and long-term recurrence is high, even amongst patients who achieve initial remission? There is a lack of suitable medical alternatives.

RNA-interference is a naturally-occurring regulatory process that causes post-transcriptional gene silencing by neutralising targeted mRNA molecules before they can be translated into protein. This can be utilised to knock-down the expression of specific genes using double-stranded small interfering RNAs (siRNA).

Proopiomelanocortin (POMC) is the propeptide precursor of ACTH. It is cleaved in the anterior pituitary gland to form ACTH, and is coded for by the POMC gene.

Hypothesis
We hypothesise that anti-POMC siRNA could be a novel effective medical treatment for Cushing’s disease; post-transcriptional silencing of POMC expression could ultimately lower the concentration of circulating ACTH, leading to decreased stimulation of adrenal steroidogenesis.

Aim
To investigate if siRNAs directed against the POMC gene can cause a reduction in the levels of ACTH secreted by an in vitro cell culture model (AT-20 cells) of Cushing’s disease.

Methods
Cell culture model – AT-20 (a neuroendocrine cell line of immortalised murine anterior pituitary corticotrope tumor) cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with Glutamax™, 10% foetal calf serum and 1% penicillin/streptomycin.

siRNA molecules - three siRNAs referred to as siRNA-1, -2 and -3 (Ambion), were designed to target different exonic regions within the POMC mRNA (Figure 1). Designed to have homology to both mouse and human sequences. Control siRNAs were ON-TARGETplus™ non-targeting siRNA (Dharmacon), designed to have no known target, to distinguish the sequence-specific silencing action of anti-POMC siRNA on ACTH level from any non-specific effects, and “Mismatch” and “Scrambled” siRNA-3 molecules custom-designed to investigate sequence specificity.

siRNA transfections - AT-20 cells were plated in 2 ml of antibiotic-free medium into 6-well plates, at 2×10⁵ cells per well. Cells were incubated for 24 hours at 37°C in a 5% CO₂ humidified incubator. After 24 hours, the medium was removed and replaced with 1 ml of fresh antibiotic-free medium and 0.5 ml of OptiMEM per well. AT-20 cells were transfected with each POMC siRNA at 30 nM using Lipofectamine™-2000 (Invitrogen) as the transfection reagent (TR). The cells were incubated for 24 hours post-transfection and the culture medium collected for ACTH assay. Transfection experiments were repeated independently 3 times.

Control treatments – These were: 1) a no-transfection-reagent control, to assess the efficiency of siRNA taken up by the cells in the absence of transfection reagent; 2) a minus-siRNA control with transfection reagent only, which allowed any toxic effects due to the Lipofectamine to be accounted for; and 3) an untreated control, with no siRNA or transfection reagent added, to measure baseline ACTH secretion by the AT-20 cells.

ACTH measurement - An Immulite 2000® ACTH immunoassay (Siemens Healthcare) was used to detect the presence of ACTH in the AT-20 cell culture supernatants. The concentration of ACTH was measured in pg/ml.

Cell viability – Trypan Blue staining was used to estimate viable cell numbers.

Results

Figure 2: Effect of anti-POMC siRNAs on secretion of ACTH from AT-20 cells. The graph shows the mean (±SE) results of three independent siRNA transfection experiments on ACTH levels. Culture media was removed from wells containing AT-20 cells 24 hours after transfection. ACTH levels in the medium were analysed by immunoassay. Controls such as transfection reagent only and no transfection were included. No treatment control provided the baseline ACTH levels secreted into culture media by the AT-20 cells. All 3 siRNA molecules successfully reduced ACTH levels when transfected with transfection reagent, compared to naked siRNA transfection, and siRNA-3 produced the greatest overall decrease in ACTH.

Figure 3: ACTH concentration and total number of viable AT-20 cells following transfection with anti-POMC siRNA-3. The graph displays the mean (±SD) results of three independent experiments using 30 nM of anti-POMC siRNA-3 to establish whether the reduction in ACTH concentration secreted by the AT-20 cells was due to transfection with siRNA or due to cytotoxic effects of the Lipofectamine transfection reagent. Cell viability remained similar to untreated cells when Lipofectamine was included in experiments to transfect AT-20 cells.

Figure 4: Effect of control siRNAs on ACTH secretion by AT-20 cells. siRNA-3, mismatch and scrambled siRNAs and a commercial non-targeting siRNA were transfected into AT-20 cells with Lipofectamine™-2000 reagent (TR) or naked and ACTH concentration was measured. Transfections with control siRNAs had no effect on ACTH secretion.

Figure 5: Dose-response effect of varying the concentration of anti-POMC siRNA-3 on ACTH secretion by the AT-20 cells. AT-20 cells were transfected with siRNA-3 molecules at 30, 10 and 1 nM. Controls were untreated cells and cells that had been treated with Lipofectamine™-2000 (TR) only. Results display the mean ACTH concentration ± SD of 4 replicates from one experiment. Increasing the concentration of siRNA suppressed the ACTH secretion in a dose-dependent manner.

Conclusions
• It is possible to reduce ACTH secretion using anti-POMC siRNAs.
• The reduction in ACTH secretion was not solely due to transfection reagent-mediated cell death.
• The decrease in ACTH is due to the specific silencing effects of anti-POMC siRNAs and not to non-specific silencing by double-stranded siRNA.
• Further work will investigate the effects of siRNA POMC mRNA using in vivo experiments in a mouse model.

References