A COMPARATIVE STUDY ON DAILY VERSUS LONG-ACTING GROWTH HORMONE

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Abstract:
Aim of the study is to assess the pathophysiology of the actions of daily growth hormone and long-acting growth hormone in in -vitro models by evaluating JAK2-STAT3, STAT 5, IGF1 and IGFBP signaling pathway. The study also aims to compare the extent of hyperglycemia (as it is the commonest side effect) induced by daily versus long-acting GH.

Articular chondrocytes will be obtained from 12 wk old male Wistar rat, cultured and the expression of GH receptor in chondrocytes will be detected. Then recombinant adenovirus GH(Ad-GH) as well as Ad-LAGH will be transfected to one group of chondrocytes. The expression of collagen type II, matrix metallo proteinase 13 (MMP-13) and signal transducer and activator of transcription 3 (STAT3) in each experimental group will be determined by Western blot.

The protein expression of MMP-13, the protein expression of collagen type II, phosphorylated STAT3 (P-STAT3) will be compared in both the groups (GH and LAGH).

GH can promote the proliferation of chondrocytes and the synthesis of type II collagen, and increase the extracellular matrix, which is achieved by phosphorylated STAT3 protein. The extent of this activation by daily GH and LAGH will be compared.

To clarify the nature of the stimulatory effects of GH on articular cartilage, the time-course of changes in the expression of the proto-oncogene, c-myc and IGF-I mRNAs in cultured articular chondrocytes will be examined. GH induced DNA synthesis of articular chondrocytes which could be neutralized by an antibody raised against IGF-I will also be studied. Furthermore, the modulatory effects of GH on the IGF-I autocrine/paracrine axis, including the production of IGF-I and IGFBPs, and IGF-I binding sites will be examined.

The pathogenesis of hyperglycemia will be tested by assessing the extent of expression of GLUT transporters and enzymes of gluconeogenesis.

Keywords: growth hormone, long acting, signaling pathway

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Introduction

Human growth hormone (GH) is a 22 kDa protein synthesized and secreted in the anterior pituitary gland. Ghrelin and hypothalamic GH-releasing hormone (GHRH) stimulate GH production and release, which is inhibited by somatostatin and negative feedback by insulin-like growth factor (IGF)-I [1]. GH secretion is episodic, having many secretory peaks. The bulk of spontaneous peaks occur during sleep, however age, gender, intercurrent and chronic disorders, and dietary condition all have a role [1, 2].

In childhood and adolescence, GH is essential for linear growth. IGF-I, which is largely generated in the liver, is regulated by GH, and the two together accelerate skeletal growth [1]. GH has a significant metabolic impact throughout life [2]. It improves muscle mass and bone formation, lowers fat mass, and increases total body water through its anabolic, lipolytic, and antinatriuretic actions. GH boosts metabolic activity, resulting in a significant increase in resting energy expenditure and fat oxidation, as well as an increase in protein synthesis [3]. Additionally, peripheral thyroxine (T4) to triiodothyronine (T3) and cortisol to inactive cortisone conversions increase. Furthermore, GH produces hyperinsulinemia by increasing insulin resistance.

Because a new steady state is attained during the first years of replacement therapy, the lipolytic effect, and hence the impact on body composition, is most immediately noticed. Growth retardation, as well as an aberrant body composition, with more body fat than lean body mass, and lower physical capacity and quality of life, are all symptoms of GH insufficiency in children. Treatment with GH in children with GHD has been well established for 20–30 years and has been shown to promote linear development and improve metabolism in various trials [4]. In fact, GH replacement now lasts well beyond adulthood and even senescence.

Growth hormone deficiency (GHD) in children (CGHD) and adults has been proven to benefit from recombinant human growth hormone (rhGH) therapy provided as daily subcutaneous injections (AGHD). Making the accurate diagnosis, delivering the optimum amount of rhGH, and maintaining patient adherence and perseverance with treatment are all important factors in the efficacy of rhGH therapy[5,6]. Device restrictions, annoyance of required dose frequency, lack of apparent benefit, insurance concerns, and expenses are all obstacles to daily rhGH therapy adherence and persistence[7,8].

In both adolescents and adults, poor adherence to rhGH medication reduced efficacy, and current studies estimate that only around 30% of patients adhere to daily rhGH therapy (missing just one dosage per week) [9]. Adherence is especially weak among teens, which could explain why children's near-adult height results remain below the mid-parental target height and the population mean [10,11]. Long-acting growth hormone (LAGH) preparations with decreased injection frequency are thought to minimise the difficulty of patient adherence, resulting in longer persistence and maybe better outcomes.

It has been hypothesized that a LAGH analog with a lower injection frequency might help mitigate treatment non-adherence, and thereby potentially improve treatment outcomes. Many pharmaceutical companies have spent a significant amount of money developing LAGH analogs using a several different yet novel technologies to prolong GH action that may allow for weekly [12], bi-weekly [13-15], or monthly administration [16,17]. However, there are theoretical reasons to suspect that LAGH analogs might be inferior to daily rhGH administration. The physiologic secretory pattern of GH occurs in an episodic and pulsatile pattern, with several peaks throughout the day and an increased number in the second half of the night during sleep. Concerns that elevated and non-pulsatile GH exposure may downregulate or desensitize GH receptor signaling were unfounded when a study by Laursen et al. demonstrated that subjects who received continuous subcutaneous infusions of GH over 6 months maintained their serum IGF-I levels within the normal range and did not develop any signs or symptoms of acromegaly [18].

Growth hormone signalling pathway

GH binding to its receptor activates the GH receptor associated JAK2 tyrosine kinase. JAK2 in turn phosphorylates tyrosines within the GH receptor and within itself. These phosphorylated tyrosines can then recruit signaling proteins to GH receptor– JAK2 complexes in the plasma membrane. Proteins recruited to GH receptor-JAK2 complexes and phosphorylated by JAK2 include the transcription factors STATS 1, 3, 5a, and 5b that regulate GH sensitive genes including genes encoding c-Fos and IGF-1; IRS 1 and 2 which recruit PI3K and lead to activation of Akt and other proteins; Shc adapter proteins that initiate the Shc/grb2/SOS/Ras/Raf/MEK pathway leading to activation of Erks 1 and 2; SIRPα1 that recruits a tyrosine phosphatase that appears to be a negative regulator of JAK2 activity; and SH2B1, a scaffold protein that enhances GH-induced changes in the cytoskeleton leading to enhanced motility of cells, including macrophages. These pathways work together, presumably with other signaling proteins,

to lead to a variety of responses to GH, including body growth, regulation of metabolism, and the ever-emerging actions of GH throughout the body.

Current Assessment of Need

Long acting growth hormone analog with a lower injection frequency might help mitigate treatment non-adherence, and thereby potentially improve treatment outcomes.LAGH analogs can be administered weekly , bi-weekly , or monthly administration. However, there are theoretical reasons to suspect that LAGH analogs might be inferior to daily rhGH administration. The physiologic secretory pattern of GH occurs in an episodic and pulsatile pattern, with several peaks throughout the day and an increased number in the second half of the night during sleep. Concerns that elevated and non-pulsatile GH exposure may down-regulate or desensitize GH receptor signaling. Hence it is the need of the hour to investigate the pathophysiology of the actions of both the hormones, their growth stimulating effects as well as their role inducing the hyperglycemia (the commonest side effect of growth hormone administration).

To evaluate and compare the effects and possible mechanisms of daily and long-acting growth hormone analogues on chondrocytes is the main aim of the study.

Objectives:

1. compare the effectiveness of daily recombinant human growth hormone (rhGH) and long-acting growth hormone analogue (LAGH) on chondrocytes in terms of cell proliferation (as assessed by CCK-8 assay), GH signaling pathway, JAK2 STAT3, STAT5, protein expressions of matrix metallo proteinase 13 (MMP-13), phosphorylated signal transducer and activator of transcription 3 (P-STAT 3)
2. Compare DNA synthesis of articular chondrocytes
3. Compare time course changes in the expression proto -oncogene c-myc, IGF-1, IGFBP mRNAs in cultured articular chondrocytes
4. Compare the mRNA and protein expression of GLUT-1 and enzymes of gluconeogenesis in chondrocytes

Project Design and Methods

***i (a). Implementation*:**

Study design

i.Study design: Experimental

Study Center: The study will be carried out in the Molecular Genetics division of Central research Laboratory, KS Hegde Medical Academy, Mangalore, Karnataka, India.

Core Project Components:

- Study of pathogenesis of daily GH and long-acting growth hormone in terms of signal transduction and genes responsible for hyperglycemia
- Techniques used will be RT PCR for mRNA, protein expression by Western blot,cell proliferation by CCK 8 assay.

Articular chondrocytes will be cultured and the expression of GH receptor in chondrocytes will be detected.GH receptor expressed in chondrocytes, and this provided a basis for further study of the role of GH in chondrocytes. Cell proliferation of the Ad-GH group will be compared to Ad-LAGH group by CCK-8 assay.

Then recombinant adenovirus GH(Ad-GH) as well as Ad-LAGH will be transfected to one group of chondrocytes. The expression of collagen type II, matrix metallo proteinase 13 (MMP-13) and signal transducer and activator of transcription 3 (STAT3) in each experimental group will be determined by Western blot.

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To clarify the nature of the stimulatory effects of GH on articular cartilage, the time-course of changes in the expression of the proto-oncogene, c-myc and IGF-I, IGFBP mRNAs in cultured articular chondrocytes will be examined. GH induced DNA synthesis of articular chondrocytes which could be neutralized by an antibody raised against IGF-I will also be studied. Furthermore, the modulatory effects of GH on the IGF-I autocrine/paracrine axis, including the production of IGF-I and IGFBPs, and IGF-I binding sites will be examined.

ii. Project implementation plan

Thirty-two male wistar rats (200 g; 12 weeks old) will be obtained. The maintenance and care of the experimental rats will be in accordance with international relevant laws and regulations. The animals will be maintained at the laboratory Animal Center for 1 week before the experiment.

They will be housed at humidity 50% ± 5%, room temperature 23°C ± 1°C with a 12-hour light cycle and will be given food and water.

The rats will be randomly divided into daily GH and LAGH group. The rats will be anesthetized (intraperitoneal injection of 10% chloral hydrate 4 mL/kg). A para-patellar skin incision will be made on the medial side of the left knee joint, and thereafter on the medial side of the patellar tendon. Then the patella will be dislocated laterally to provide access to the joint space. The anterior cruciate ligament will be cut by ophthalmic scissors. A positive anterior drawer test will be used to validate complete transection of the ligament. The joint will then be irrigated with 0.9% sodium chloride injection. The patellar ligament and skin will be sutured. The same surgical procedure without cutting the anterior cruciate ligament will be carried out in the control group. Each animal will be given an injection of penicillin 100,000 U daily for 3 days after surgery.

Cell culture

The rats will be sacrificed and the limbs will be sterilized with alcohol. The thigh bone will be separated with aseptic scissors. Fascia, muscle and connective tissue will be removed as much as possible. Cartilage tissue will be removed from the joints and cut into 1 mm3 pieces. The tissue will be washed for two times with phosphate buffer saline and 0.25% pancreatin will be added. Tissue will be digested for 15 to 20 minutes in a 37°C rocking bed, followed by addition of 0.2% collagenase for 30 to 60 minutes. Culture medium will be added to terminate digestion. The digested solution will be filtered through 200-μm nylon mesh and centrifuged at 12,000 ×g for 5 minutes. The cells will be cultured with DMEM in an atmosphere of 5% CO2 at 37°C.

Isolated chondrocytes will be obtained by enzymatic digestion in a spinner bottle with 0.25% trypsin and 0.01 Methyl enediaminetetra acetic acid (EDTA) in phosphate buffered saline (PBS) for at 37°C, followed by 0.2% collagenase in a mixture of I:IF-12/DMEM supplemented with 10% FBS for 4 h. Digested cells will be seeded at 7 × 104 cells/cm 2 in 175-cm 2 flasks in the:medium supplemented with I0% FBS, penicillin (I00 IU/ml) and streptomycin (100#g/ml) in a 5% CO 2 environment, and fed every 3 days. Five days later, upon reaching confluence, cells will be passaged with trypsin/EDTA and re-seeded in a 9.6-cm diameter culture dish, 6-well, 24-well, or 96-well plastic tissue culture plate. Only first subcultures will be used in the present study.

Expression of GH receptor in chondrocytes will be assayed by ELISA.

5-BROMO-2t-DEOXYUR~DINE INCORPORATION

Using 96-well dishes, DNA synthesis will be measured with an immuno cytochemical cell proliferation assay kit. Chondrocytes subplated in 96-well plates at a density of 1.5 × 104 cells/well will be cultured to subconfluence for 24 h. They will then be incubated in serum-free F-12/DMEM for a further 24 h and exposed to varying concentrations of GH (0.001-1 gg/ml) in serum-free medium containing 0.3% bovine serum albumin (BSA) or in the latter medium. In some cases, monoclonal anti-IGF-I antiserum will be added 5 min prior to GH administration to assess the role of IGF-I in GH-induced DNA synthesis. This antibody will be demonstrated previously to inhibit the activity of IGF-I at 1000-times dilution in Balb/c 3T3 cells [19]. After 21 h, 5-bromo-2'-deoxyuridine (BrdU), a thymidine analogue, will be incorporated for 3 h and subsequently localized using a specific monoclonal antibody. Bound antibody will be detected using a peroxidase-conjugated antibody against mouse IgG and peroxidase substrate. Reaction products, proportional to the amount of BrdU incorporation, will be measured spectrophotometrically (optical density 405 nm) using an ImmunoReader.

IGF-I RELEASE ASSAY

The concentration of rat IGF-I and IGF binding protein in the medium will be measured by ELISA. Subconfluent chondrocytes plated in 6-well dishes will be washed twice with PBS and cultured for an additional 3 days with GH (0.1 and 1 microgram /ml in serum free F-12/DMEM.Conditioned medium samples will be prepared for electrophoresis and ligand blotting analysis will be performed as per Hossenlopp et al for IGF1 and IGFBP [20].

RNA will be isolated from whole blood and expression of RNA will be quantified by RT-PCR. To determine whether GH stimulated DNA synthesis directly, the time-course changes in mRNA expression of IGF-I and the proto-oncogene c-myc will be investigated. Induction of IGF-I mRNA, maximum level as well as the expression and peak of c-myc mRNA after GH and LAGH treatment will be assessed.

Furthermore, administration of cycloheximide, an inhibitor of protein synthesis, will be checked for the induction of IGF-I and c-myc mRNAs. Western ligand blot analysis of IGF-binding proteins will be

carried out.

Ethics Review

Animal Ethical committee and institutional biosafety committee approval will be sought before starting the study.

Statistical analysis

Statistical analysis will be done using SPSS version 23. Two-tailed t-tests/Mann Whitney U tests will be used to assess the significance of differences in the expression of mRNA and protein expression between the two groups. Association of the daily versus LAGH with the gene expressions of signaling pathway was analyzed by chi-square test. The sensitivity, specificity of the expression of IGF1,IGFBP,MMP-13,collagen II, c-myc, JAK 2-STAT 3,STAT 5 in inducing growth will be compared by constructing ROC.The level of confidence was set at the 0.05 significance level.

Endpoint Measures

Expressions of IGF-1, IGFBP, JAK2-STAT3, STAT5
Expressions of collagen type II, matrix metallo proteinase 13 (MMP-13)

Expressions of GLUT and enzymes of gluconeogenesis

Expected outcome

The study may able to evaluate the pathophysiology of daily GH compared to long-acting GH in terms of their signal transduction pathway, expression of several proteins involved in the chondrocyte proliferation and expression of GLUT and enzymes of gluconeogenesis so as to assess the pathogenesis of hyperglycemia induced by both the analogues of growth hormone.

The study of the signaling pathway may suggest the importance of daily GH versus long-acting analogue of GH in modulating growth. As hyperglycemia is the commonest side effect of GH administration, the evaluation of the expression of glucose transporters induced by both the type of growth hormones may throw light on the basis and extent of this side effect.

Conclusion

The project will benefit the whole population suffering from GH deficiency, as it intends to establish the pathogenesis of daily versus long-acting growth hormone action in terms of signal transduction as well as hyperglycemia as side effect.

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