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Growth Hormone elicits Specific Effects on Blood Cell Gene Expression levels

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Abstract

The increasing abuse of human growth hormone (HGH) requires the development of innovative methods of detection. Here we present a novel molecular biological technique: DNA microarray technology was utilised for the identification of potential marker genes for the proof of abuse of HGH in athletes. Gene expression levels of distinct blood cell populations were interrogated by whole genome microarrays covering 19.000 human genes, expressed sequence tags (ESTs) respectively. Peripheral blood mononuclear cells were isolated from donated blood and cultivated for the treatment. Cells were treated with recombinant human growth hormone at doping-relevant pharmacological levels of 2 µg/mL and 20 µg/mL, lysed and total RNA was isolated. mRNA was specifically primed and, under the incorporation of fluorescently labelled nucleotides reversely transcribed to cDNA. cDNA from growth hormone stimulated cells was labelled with one fluorescent dye whereas cDNA from un-stimulated cells with another. Both cDNAs were hybridised to microarrays to display patterns of differential gene expression. Specific differential gene expression of genes clearly linked to HGH could be detected allowing the generation of an array targeted at these marker genes.

Introduction

Human growth hormone (HGH) is a peptide hormone secreted by the anterior pituitary gland. The predominant form of HGH comprises 191 amino acids and has a molecular weight of 22 kDa. In the blood stream HGH has a half life of 15 to 20 minutes before being cleared from the circulation by receptor mediated degradation mainly in the kidneys and the liver. HGH molecules bind to two dimerizing receptors, e.g. on the surface of white blood cells (leukocytes). The dimerisation of HGH receptors leads to initiation of intracellular signalling. Genes become activated and are being transcribed into mRNA, the messenger molecule from the DNA to protein synthesis. Many different cellular processes are activated such as the generation of insulin-like growth factor 1 (IGF-1) and its binding proteins. IGF-1 can be considered a marker of HGH action mainly on the liver. Protein synthesis is stimulated and fats are mobilised by a direct lipolytic action.

Therapeutically HGH is employed in the treatment of children of short stature of no known cause. In HGH deficient adults HGH improves the quality of life by exerting effects on body composition, serum lipids and bone and mineral density. Excess levels of HGH such as in the disease acromegaly lead to soft tissue swellings and changes in bony features of the face such as enlargement of the nasal bone and increased spacing of teeth. Patients with acromegaly have increased lean tissues and reduced body fat (Murakami et al. 2004) whereas growth hormone deficient adults have reduced lean body mass and increased central abdominal fat mass.

HGH is a very potent anabolic agent and relatively safe to use. With the advent of recombinant DNA technology, HGH is a readily available product. The first commercially available HGH product by Genentech (Protropin®) was approved by the U.S. Food and Drug Administration (FDA) in 1985. To date, HGH abuse has been difficult if not impossible to detect. It is certain that the prevalence of HGH abuse will increase (Jenkins 2001). There is high demand in methods to detect HGH abuse. We propose to detect the effect of exogenous HGH administration by profiling the gene expression response of leukocytes.

In the last few years microarrays have emerged as useful tools for analyzing large numbers of genes simultaneously. Microarray and microchip technologies can be seen as logical developments of molecular biological hybridisation methods and diagnostic assays on solid surfaces reducing the amount of analytes required. In microarrays the Northern blot principle is reversed so that the labelled moiety is derived from the mRNA sample and the immobilised fractions are the known gene sequences. Microarrays are fabricated by

depositing purified nucleic acid fragments of genes onto solid supports such as microscope glass slides (Watson et al. 1998).

Microarrays have not become a replacement to present techniques but a novel, high-power approach to perform analyses that were previously time-consuming (figure 1). They can contain thousands of genes. This enables global analysis of biological processes. The main areas of application of microarray technology include gene expression analysis, genome analysis and drug discovery.

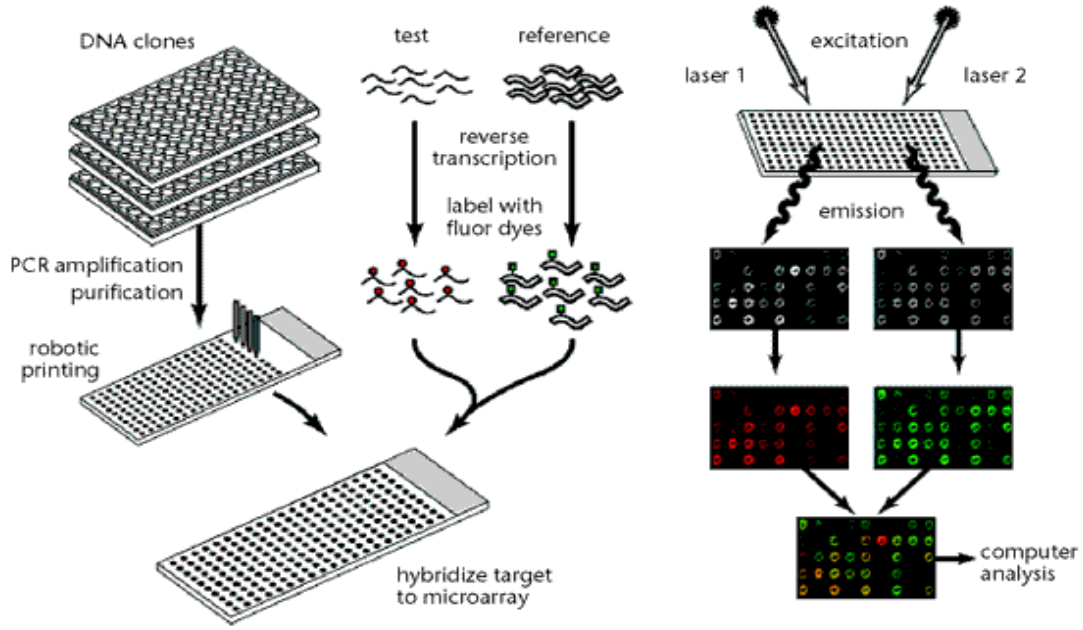

Figure 1. Principle of microarray analysis (Oklahoma Medical Research Foundation 2001). A collection of nucleic acid sequences is immobilised onto a solid support so that each unique sequence forms a feature called a spot. mRNA pools obtained from a test and a reference sample are separately from each other reverse transcribed and fluorescently labelled. The labelled test cDNA and the reference cDNA are co-hybridised on the microarray, which is finally read by a scanner containing two different lasers that emit light suitable for exciting the fluorescent labels. A confocal microscope records the emitted light from each of the spots. These are displayed as for instance red and green spots on the visual display unit and are fed into further computer aided analysis.

In gene expression analysis the composition of mRNA populations is investigated. The transcripts and their expression levels provide information on the state of the cell and the

activity of genes under certain conditions as well as on possible changes in the resulting proteome.

In microarray gene expression studies typically the relative expression levels of specific transcripts in two samples are compared. In the relative expression approach one untreated sample functions as a control and the other one is derived from the cells that were treated with the substance under investigation (HGH). Each sample is labelled with a different fluorescent dye. Equal signal from both samples suggest equal expression in both samples whereas a difference in signal reflects a difference in expression. Leukocytes present an easily extractable target for mRNA isolation and subsequent microarray analysis.

Experimental

Materials - Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of five volunteers by Ficoll gradient centrifugation. Blood samples were taken on two independent days and collected in sodium-heparin vials.
Whole Genome Arrays Human SS-H19k7 were supplied by the University Health Network at Toronto, Canada. 19008 different human expressed sequence tags (ESTs) derived from PCR amplification of EST clones were printed onto Ultra-GAP (Corning, Inc.) glass slides.1 µL HGH stock solution or 2 µg HGH/mL cell suspension simulated HGH dosages potentially used in doping. 10 µL HGH solution or 20 µg HGH/mL cell suspension represented excessive amounts of HGH potentially used in medical treatments.

Methods – Upon treatment with HGH or control treatment with physiological concentrations of NaCl for three hours, cells were lysed in 1 mL TRIzol per 0.5 to 1 x 107 cells. RNA was isolated via the RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The integrity of the RNA was assessed on a 1% agarose gel and on a NanoDrop ND-1000 UV/VIS spectrophotometer device. Upon precipitation with ethanol and 7.5 M ammonium acetate, RNA was reversely transcribed to cDNA and labelled by Superscript II reverse transcriptase (Invitrogen) under the incorporation of CyDye-labelled d-CTP (Amersham Pharmacia Biotech, Piscataway, USA). The cDNA was purified over the CyScribe GFX purification kit (Amersham).

cDNA from treated cells was directly hybridised versus cDNA from untreated cells onto Whole Genome Arrays Human SS-H19k7. Reversely labelled experiments were carried out with samples from the corresponding second sampling days, the biological replicates.

Hybridisation took place in hybridisation chambers (Camlab, UK) in order to prevent drying out during the overnight incubation. Slides were washed with decreasing stringency from 1xSSC, 0.2% SDS wash buffer to 0.1xSSC wash buffer.

Dried slides were scanned in a GenePix 4000A microarray scanner (Axon Instruments, Union City, USA). Images were read out with the GenePixPro 3.0 software (Axon Instruments). Background corrected intensities were normalised by Print tip Lowess normalisation (Yang et al. 2002). Data were analysed by the limma (linear models for microarray data) package based on the programming language (Dettling 2004). Analysis was performed using R version 1.9, Bioconductor version 1.4 and limma version 1.6.5.
Data import, image-plots and quality control were performed according to user instructions.

Results

Growth hormone treatment of the freshly isolated PBMCs demonstrated anabolic effects mainly by diversion of energy to protein synthesis. More genes were down-regulated than up-regulated. Effects were in-line with known symptoms of growth hormone over-dosage such as increased lean tissue mass and fluid retention together with reduced body fat. Growth hormone administration led to increased cell maintenance and anti-apoptotic activity. Cell proliferation, C21-steroid hormone biosynthesis, insulin receptor signalling pathways and protein amino acid phosphorylation and glycosylation were activated. Genes were clustered with the Gene Ontology Tree Machine basing on the GO project (Harris et al. 2004) (figure 2). The results of this study are in accordance with published data; furthermore new growth hormone sensitive genes were discovered by this novel approach.

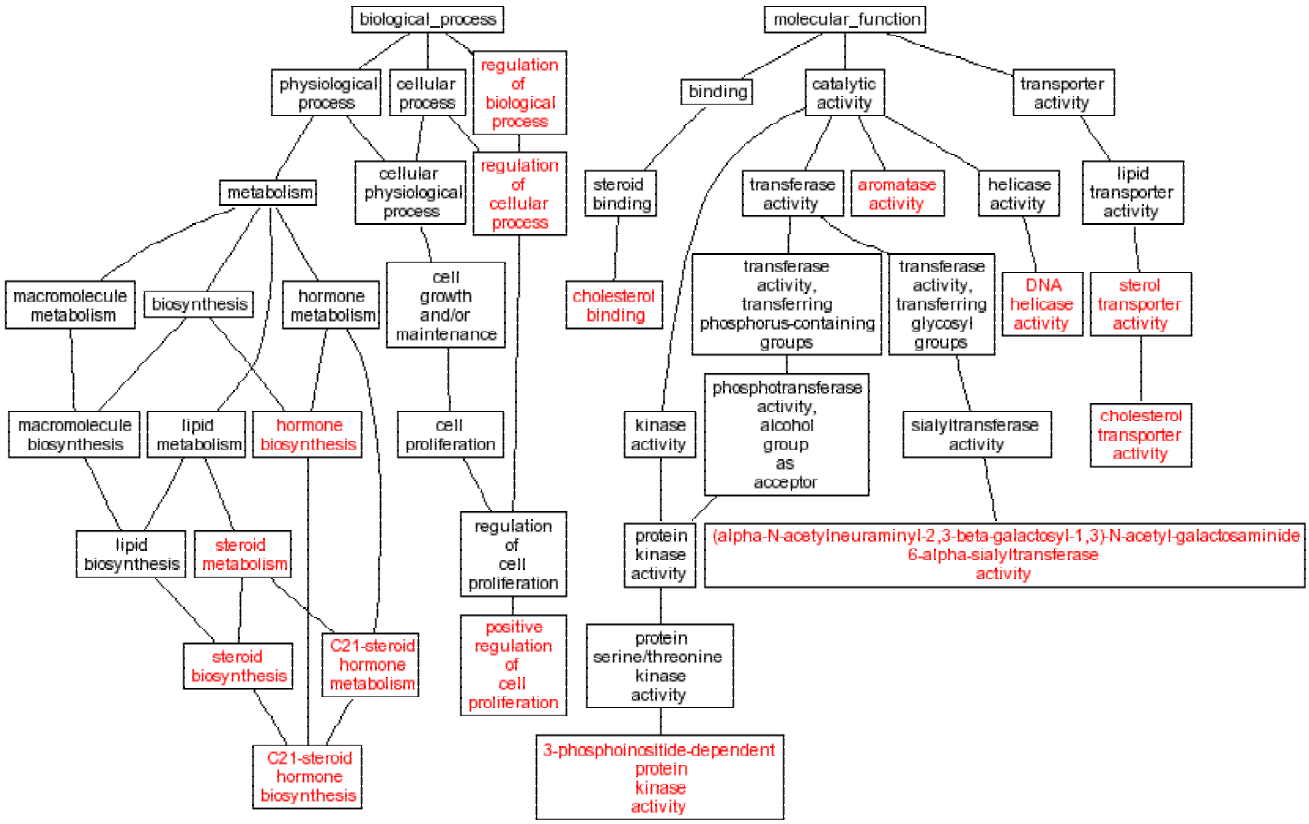

Figure 2. Directed acyclic graph of the most strongly regulated gene groups from the 500 most strongly differentially expressed genes of PBMCs treated with a doping-relevant pharmacological concentration of 2 µg HGH/mL cell suspension for 180 min.

Conclusions

It is now clear that HGH does alter gene expression in PBMCs in a specific way. Anabolic effects were reflected in the gene expression patterns. Not only could known effects of HGH be explained at a molecular level, but also new and sometimes even uncharacterised HGH-responsive genes could be identified in this approach. There is the potential of the design of a selective growth hormone chip upon validation of the detected HGH-marker gene candidates. Whether the concept of detection of growth hormone abuse by means of gene expression also proves to be applicable to an in vivo situation still has to be thoroughly investigated.

Acknowledgements

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