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Pituitary diseases: long-term clinical consequences

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Citation

Klaauw, A. A. van der. (2008, December 18). *Pituitary diseases: long-term clinical consequences*. Retrieved from <https://hdl.handle.net/1887/13398>

Version: Corrected Publisher's Version

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Note: To cite this publication please use the final published version (if applicable).

Chapter 10

Recombinant human growth hormone replacement increases CD34+ cells and improves endothelial function in adults with growth hormone deficiency

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European Journal of Endocrinology, 2008

ABSTRACT

Objective

Adult patients with growth hormone deficiency (GHD) are at increased risk for cardiovascular morbidity and mortality. Endothelial function, vascular stiffness and loss of circulating CD34+ cells are considered biomarkers for cardiovascular disease. The aim of this study was to assess vascular structure and function in relation to circulating CD34+ cells in adults with GHD before and during 1 year of recombinant human growth hormone (rhGH) replacement.

Design

1 year intervention with rhGH.

Patients and methods

Vascular function (flow-mediated dilatation (FMD)) and structure (pulse wave velocity (PWV) and analysis) were assessed in 14 adult patients (9 men) with GHD (mean age 57 yrs, range 27-71 year). In addition, the number of CD34+ cells was evaluated using flow cytometric analysis. Study parameters were analyzed at baseline, and after 6 months and 1 year of rhGH replacement.

Results

RhGH replacement increased IGF-I levels from 10.4 ± 4.5 mmol/l at baseline to 18.4 ± 10.1 mmol/l, and 20.5 ± 8.0 mmol/l, at 6 months, and 1 year, resp. ($p=0.001$). FMD increased from $3.5 \pm 1.8\%$ to $6.0 \pm 2.5\%$ and $5.1 \pm 2.5\%$ during 1 yr rhGH replacement, $p=0.008$). There was no beneficial effect on PWV, central pulse pressure, central systolic pressure and augmentation index. The number of CD34+ cells increased from 794.9 ± 798.8 cells/ml to 1270.7 ± 580.1 cells/ml and to 1356.9 ± 759.0 cells/ml ($p=0.010$).

Conclusion

One year of rhGH replacement in adults with GHD improves endothelial function and increases the number of circulating CD34+ cells.

INTRODUCTION

Growth hormone deficiency (GHD) is associated with an increased prevalence of cardiovascular risk factors, such as central obesity, hypertension, dyslipidemia, a decrease in lean body mass and an increase in insulin resistance (1;2). In addition, abnormalities in vascular function and structure have been described in GHD (3-5). Recombinant human growth hormone (rhGH) replacement in GHD is aimed at reversing these abnormalities (6-10).

Since a decade, bone marrow-derived endothelial progenitor cells have been proposed to play an important role in maintenance and repair of the vasculature. Both re-endothelialization and angiogenic capacity have been put forward as mechanisms by which these cells are involved in vascular repair (11). We, and others, have shown that the number of these cells are reduced in patients with type 1 diabetes (12), in patients with other cardiovascular risk factors and with established cardiovascular disease (13;14).

There is, however, a continuing debate on the phenotypic characteristics of endothelial progenitor cells (11;15;16). Many groups perform flow cytometric analysis using CD34, CD133 and/or VEGFR as cell surface markers to characterize the cells. Of these, CD34-positive cells (without VEGFR expression) show a stronger inverse correlation with the presence and number of cardiovascular risk factors than CD34/VEGFR+ cells or cells with other combinations of positive surface markers believed to be endothelial progenitors (17).

Thus, CD34+ cells are a bone-marrow derived biomarker for cardiovascular risk, but there is no information on the effects of rhGH replacement on these cells in adults with GHD. Therefore, the aim of our study was to evaluate the effects of rhGH replacement on the number of circulating CD34+ cells and vascular function and structure in adults with GHD.

METHODS

Patients

Fourteen patients with GHD were included in this prospective, open-label intervention study. GHD was confirmed in all patients by an insulin tolerance test (nadir blood glucose <2.2 mmol/l) with a peak GH concentration <3 µg/l. An additional inclusion criteria was stable hormonal substitution of dysfunctional hormonal axes at least 3 months prior to study start. Exclusion criteria were a hormonally active pituitary tumor, history of cancer, presence of chronic inflammatory disease, diabetes mellitus, and a history of cardiovascular disease. The study protocol was approved by the medical ethics committee of the Leiden University Medical Center, and written informed consent was obtained from all subjects.

Treatment protocol

Patients were treated with recombinant-human growth hormone for 12 months. After initial measurements were obtained, all patients were treated with subcutaneous injections of rhGH (Genotropin® Pharmacia/Pfizer or Zomacton® Ferring, Norditropin® NovoNordisk, or Humatrope® Lilly), given every evening. The initial dose of 0.2 mg/day rhGH was individually adjusted each month in the first half year to achieve physiological serum IGF-I concentrations, within the age-dependent laboratory reference range (IGF-I standard deviation scores (SD scores)). The patients were studied at baseline, and 6 and 12 months after growth hormone replacement. During the study, no antihypertensive or lipid-lowering drugs were prescribed.

Study parameters

Endothelial function

Nitric oxide-dependent flow mediated dilatation (FMD), expressed as percentage diameter change in the brachial artery after reactive hyperemia, was measured non-invasively by ultrasonography using standard procedures in our Vascular Research Unit (18). Measurements were performed at the elbow of the right arm using a vessel wall movement system (Wall Track System, Pie Medical, Maastricht, The Netherlands), which consists of an ultrasound imager with a 10 MHz linear array transducer connected to a data acquisition system and a personal computer. Three measurements were averaged to calculate a baseline diameter of the brachial artery. By inflation of a blood pressure cuff for 5 min at a pressure of 200 mm Hg, ischemia was applied to the forearm distal to the location of the transducer. Ultrasonography continued for 5 min after cuff release with measurements at 30 sec intervals. The widest lumen diameter was taken as a measurement for maximal vasodilatation. Nitroglycerin spray (400µg) was administered to determine endothelium-independent vasodilatation. All measurements were performed by the same technician with patients supine in a quiet temperature controlled environment after at least 15 minutes of rest. All patients were requested to refrain from smoking on the morning of vascular measurements. Control values for FMD were obtained from healthy age-, gender-matched subjects (9 men) with a BMI of 26.6 ± 2.9 kg/m² (age 49.8 ± 12.4 yrs, $p=NS$ compared to patients). Three control subjects smoked.

Pulse wave velocity

Arterial stiffness was assessed non-invasively by aortic PWV using standard procedures in our Vascular Research Unit (19). In short, sequential tonometry was performed at the common carotid artery and the femoral artery using a Sphygmocor device (Sphygmocor, Actor Medical, Sydney, Australia) to record the arterial pulse waveform. Pulse transit time between the two sites was determined by the system software from the average of 10 consecutive heartbeats. The distance between the two recording sites was measured and aortic PWV was calculated as the distance traveled by the pulse wave divided by the transit time (in cm/s). The validation

of this automatic method and its reproducibility have been published previously (20). The measurements were performed twice in each patient and then averaged to obtain the mean aortic PWV, which was used for statistical analysis. The same control subjects as for the FMD were used.

Central pressure and augmentation index

Central pulse pressure was determined by measuring the brachial blood pressure, determining the pulse waveform at the brachial and carotid artery by applanation tonometry using a Millar probe (Millar Instruments, Houston, Texas) and applying the calibration method according to Kelly and Fitchett (21) to determine central systolic blood pressure and central pulse pressure (22). This method assumes that the mean arterial pressure and diastolic blood pressure remain constant from the aorta to the large peripheral arteries which allows central pulse pressure calculation. The same control subjects as for the FMD were used.

CD34-positive cells

For enumeration of CD34-positive circulating (CD34+ cells), flow cytometric analysis was performed using a multi-parametric gating strategy based on the International Society of Hematotherapy and Graft Engineering (ISHAGE). This lyse/no wash method uses Trucount tubes (Becton Dickinson, Franklin Lakes, NJ, USA) that contain a defined number of brightly fluorescent microbeads, permitting the acquisition of absolute counts of cells, even at very low numbers. Circulating CD34+ cells are defined as cells with low-expression for CD45, positive for CD34, and located in the lympho-gate on a side- and forward-scatter plot. Within 2h of blood-withdrawal, 50 μ l of EDTA-anticoagulated whole blood was added per Trucount tube (two per subject) by reverse pipetting and directly labeled antibodies were added: CD45-PerCP, CD34-FITC (BD Biosciences, Erembodegem, Belgium). After 30 min incubation on ice and in the dark, cells were fixed using FACS-lysing solution (BD Biosciences) and the samples were measured within 24h using a fluorescence-activated cell sorter (FACS)-Calibur (BD Biosciences). A total of 500.000 CD45+ cells were measured (excluding the beads) and the number of CD34+ cells per microliter blood was calculated. Reference values were obtained from 9 healthy men with a mean age of 61 ± 5 yrs and a BMI of 24.2 ± 1.0 kg/m² obtained in our center.

Biochemical parameters

IGF-I, IGFBP-3, fasting levels of glucose, total cholesterol, HDL cholesterol (HDL), and triglycerides (TG) were measured at baseline, after 6 months, and after 1 year of follow-up. LDL cholesterol concentrations (LDL) were calculated using the Friedewald formula. Patients were requested to fast overnight before blood samples were taken for laboratory measurements of lipid profiles and glucose.

Assays

Serum IGF-I concentrations (ng/ml) were measured using an immunometric technique on an Immulite 2500 system (Diagnostic Products Corporation, Los Angeles, USA). The intra-assay variation was 5.0 and 7.5% at mean plasma levels of 8 and 75 nmol/l, respectively.

IGFBP-3 was measured using an immunometric technique on an IMMULITE Analyzer (Diagnostic Products Corporation, Los Angeles, USA). The lower limit of detection was 0.02 mg/l and inter-assay variation was 4.4 and 4.8% at 0.91 and 8.83 mg/l.

A Hitachi P800 autoanalyzer (Roche, Mannheim, Germany) was used to quantify serum concentrations of glucose, total cholesterol and TG. HDL was measured with a homogenous enzymatic assay (Hitachi 911, Roche, Mannheim, Germany).

Statistics

Statistical analysis was performed using SPSS for Windows, version 14.0 (SPSS Inc. Chicago, Illinois, USA). Results are scored as the mean \pm standard deviation (SD), unless specified otherwise. ANOVA repeated measurements with Sidak correction for multiple comparisons were used. A P-value <0.05 was assumed to represent a significant difference.

RESULTS

Patients

Fourteen patients (9 men) were included in this prospective, open-label intervention study with a mean age of 51 years (range 27-71 years) and a mean BMI of 29.4 ± 3.9 kg/m². GHD was secondary to a non-functioning pituitary macroadenoma in eleven patients, to an enlarged

Table 10/1: Clinical characteristics of the included patients.

		N=14
Age (yrs, mean (range))		50.8 (27-71)
Gender (M/F (n(%)))		9 (64)/ 5 (36)
Etiology of GHD (n(%))	Non-functioning pituitary adenoma	11 (79)
	Other	3 (21)
Treatment of pituitary tumor	Surgery (n(%))	11 (79)
	Radiotherapy (n(%))	4 (29)
Pituitary deficiencies	TSH deficiency (n(%))*	8 (57)
	ACTH deficiency (n(%))*	6 (43)
	LH-FSH deficiency (n(%))*	7 (50)
	ADH deficiency (n(%))	1 (7)
Smoking (n(%))		4 (29)

*TSH deficiency was treated with thyroid hormone substitution in all TSH deficient patients. ACTH deficiency was treated with hydrocortisone substitution in all ACTH deficient patients as was LH-FSH deficiency with either testosterone and estrogen.

pituitary stalk in 2 patients, and was idiopathic in 1 patient. Additional clinical characteristics are detailed in Table 1.

Effects of 1 year rhGH replacement

During rhGH replacement, IGF-I and IGFBP-3 concentrations increased within 6 months after the start of treatment ($p=0.006$ and $p=0.053$, resp., Table 2), and remained unchanged between 6 months and 1-year rhGH replacement ($p=1.0$ and $p=1.0$, respectively). Total, LDL and HDL cholesterol remained unchanged as well as fasting glucose and triglycerides during 1 yr rhGH replacement.

Table 10/2: Vascular endothelial function, PWV and PWA during 1 year rhGH replacement in adult patients with growth hormone deficiency.

	Baseline	6 months rhGH replacement	1 yr rhGH replacement	Overall P-value	Control values*
IGF-I (nmol/l)	10.4 ± 4.5	18.4 ± 10.1	20.5 ± 8.0	0.001	
IGFBP-3 (mg/l)	2.7 ± 1.2	4.0 ± 1.7	4.0 ± 1.1	0.003	
Glucose (mmol/l)	5.1 ± 0.6	5.1 ± 0.7	4.9 ± 0.7	0.062	
Total cholesterol (mmol/l)	5.5 ± 0.1	5.2 ± 1.1	5.2 ± 0.8	0.335	
LDL cholesterol (mmol/l)	3.7 ± 0.8	3.6 ± 0.4	3.5 ± 0.8	0.545	
HDL cholesterol (mmol/l)	1.4 ± 0.4	1.3 ± 0.4	1.4 ± 0.4	0.122	
Triglycerides (mmol/l)	1.6 ± 0.7	1.3 ± 0.6	1.6 ± 0.7	0.064	
FMD (%)	3.5 ± 1.8	6.0 ± 2.5	5.1 ± 2.5	0.008	9.1 ± 4.7
NTG (%)	13.5 ± 5.2	14.9 ± 6.5	13.1 ± 4.8	0.427	18.0 ± 5.9
Aortic PWV (cm/s)	7.9 ± 1.9	7.7 ± 1.9	7.7 ± 2.2	0.777	8.1 ± 1.3
Brachial systolic pressure (mm Hg)	136.2 ± 11.9	131.6 ± 17.2	136.6 ± 15.0	0.302	133.0 ± 7.0
Brachial diastolic pressure (mm Hg)	84.1 ± 8.1	80.7 ± 8.1	81.2 ± 8.1	0.056	83.8 ± 5.6
Brachial pulse pressure (mm Hg)	52.1 ± 11.3	50.9 ± 17.6	55.4 ± 10.2	0.374	49.2 ± 6.9
Central systolic pressure (mm Hg)	135.6 ± 13.5	135.1 ± 22.6	138.1 ± 17.5	0.812	129.8 ± 7.5
Central pulse pressure (mm Hg)	51.8 ± 12.1	54.8 ± 22.3	57.0 ± 13.7	0.569	46.8 ± 8.1
Augmentation index	26.6 ± 10.6	24.9 ± 11.6	25.7 ± 11.4	0.483	32.9 ± 11.9

Data were compared with ANOVA with repeated measurements. *Reference values of age- and gender matched healthy controls. FMD, flow-mediated vasodilatation. NTG, nitroglycerin. PWV, pulse wave velocity.

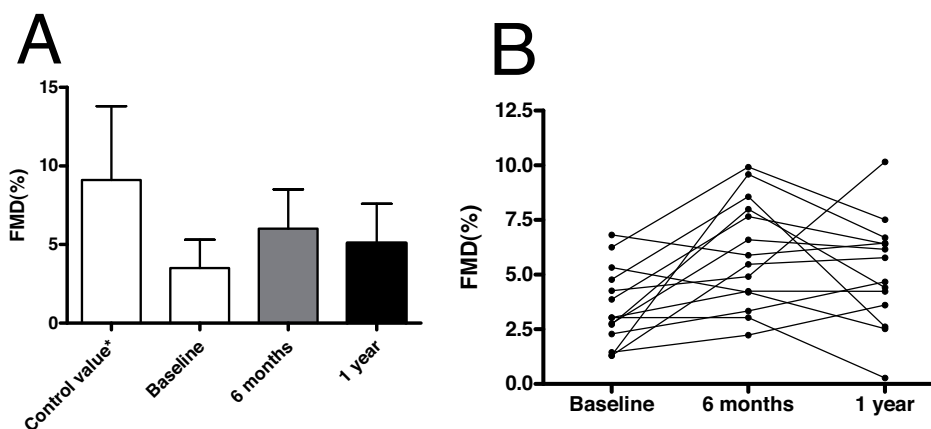


Figure 10/1: Percentage flow-mediated dilatation (FMD) during 1 year rhGH replacement in adult patients with growth hormone deficiency increases significantly, predominantly in the first 6 months of rhGH replacement (overall $P=0.008$). Upper panel (A): The first white bar represents the control value* of FMD in age- and gender-matched healthy subjects obtained in our center. The second white bar represents baseline, grey bar 6 months of rhGH replacement, and black bar 1 year of rhGH replacement. Lower panel (B): Individual FMD during 1 year of rhGH replacement.

Vascular assessment and CD34+ cells during rhGH

FMD increased during 1 yr rhGH replacement ($p=0.008$, Table 2), most markedly during the first half year of rhGH replacement (Figure 1). No change in PWV, brachial systolic and pulse pres-

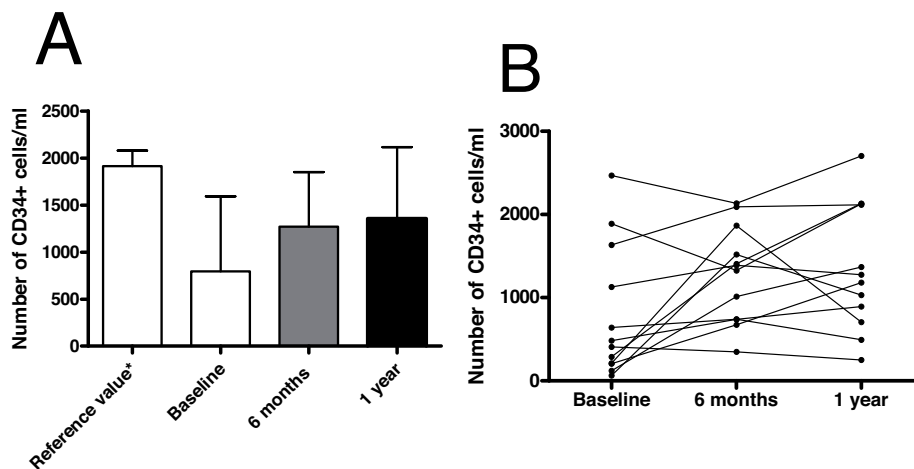


Figure 10/2: Number of circulating CD34+ haematopoietic stem cells during 1 year of rhGH replacement in adult patients with growth hormone deficiency increases (overall $P=0.010$). Upper panel (A): The first white bar represents the reference value* of the number of circulating CD34+ cells in 9 men with a mean age of 61 ± 5 yrs and a BMI of 24.2 ± 1.0 kg/m² obtained in our center. Second white bar represents baseline, grey bar 6 months of rhGH replacement, and black bar 1 year of rhGH replacement. Lower panel (B): Individual concentrations of circulating CD34+ haematopoietic stem cells during 1 year of rhGH replacement.



sure, central pulse pressure, central systolic pressure and augmentation index were observed during GH therapy (Table 2).

The number of circulating CD34+ cells increased from 794.9 ± 798.8 cells/ml to 1270.7 ± 580.1 cells/ml and, 1356.9 ± 759.0 cells/ml, at 6 and 12 months, respectively, after treatment ($p=0.010$, Figure 2). The reference values of the number of circulating CD34+ cells in 9 men with a mean age of 61 ± 5 yrs and a BMI of 24.2 ± 1.0 kg/m² obtained in our center was 1913.6 ± 1640.2 cells/ml. The number of erythrocytes, lymphocytes, and leukocytes (CD45 positive cells) remained unchanged.

There were no correlations between the change in FMD and the change in number of CD34+ cells ($R=0.217$, $p=0.499$), or between the change in IGF-I and the change in FMD ($R=0.080$, $p=0.785$) and the change in CD34+ cells ($R=0.425$, $p=0.169$). Smoking habits and gender were not related to either change in FMD or change in number of CD34+ cells. Age was correlated with change in CD34+ cells after 1 months ($r^2=0.367$, $p=0.04$, Figure 3).

DISCUSSION

The novel finding in this study is the beneficial effect of treatment with rhGH both on the number of circulating CD34+ cells and on endothelial function, which was manifest within 6 months after the start of treatment and maintained 6 months thereafter.

Hypopituitarism in general is associated with increased mortality, predominantly due to cardiovascular diseases (23), which has been ascribed to untreated GHD (24). These observations in patients with GHD are related to an increased prevalence of cardiovascular risk factors, such as hypertension, dyslipidemia, and alterations in body composition (2). Indeed, intima-media thickness (IMT) is increased in patients with GHD compared to control subjects (25). However,

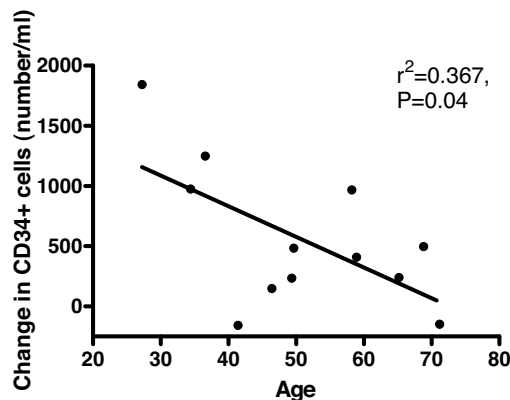


Figure 10/3: Correlation between change number of circulating CD34+ haematopoietic stem cells during 1 year of rhGH replacement in adult patients with growth hormone deficiency and age.



the effect of rhGH replacement on cardiovascular health is still subject to debate. IMT decreased during rhGH replacement in one study (25), whereas others reported that intima-media thickness was not different compared to controls (3;26) and remained unchanged during rhGH replacement (6).

In our study, the number of circulating CD34+ cells in adults with GHD increased within 6 months of rhGH replacement and remained stable thereafter. These results are in line with the very recently observed potential of rhGH treatment to increase the number of circulating endothelial progenitor cells (classified as CD133/VEGFR2 cells and colony-forming units) in healthy volunteers (27). In addition, the potential of rhGH to positively influence hematopoiesis has previously been shown in another clinical setting, i.e. harvesting of CD34+ cells destined for autologous hematopoietic stem cell transplantation in patients with relapsed or refractory hematologic malignancies (28).

Endothelial function was measured in our study before and during rhGH replacement by assessing flow-mediated vasodilatation (FMD). Indeed, at baseline FMD was decreased compared to reference values obtained in our vascular unit. The observed improved FMD after rhGH replacement was also observed within 6 months and continued until the end of the study. These data are in agreement with earlier other reports assessing the effects of rhGH replacement on endothelial function (6;25;29). A putative mechanism by which rhGH replacement improves vascular function is IGF-I mediated stimulation of nitric oxide synthesis in endothelial cells (30;31).

Since we intended to use circulating bone marrow-derived cells as biomarkers for cardiovascular health, we focused on CD34+ cells only, which are closely linked to cardiovascular risk, even more closely than CD34+/VEGFR+ cells (17). The potential mechanisms, responsible for the increase in the number of CD34+ cells in our study, are not clear. Improvement in endothelial function is associated with increased nitric oxide bio-availability, in particular in the bone marrow (32), which is associated with increased mobilization of CD34+ cells. Indeed, growth hormone treatment was found to induce markers of nitric oxide bio-availability in health volunteers (27). In addition, CD34+ cells express both GH and IGF-I receptors (33) as is the case for several other cell types that could be involved. Indeed, studies in rodents and on fetal bone marrow demonstrate direct effects of GH and IGF-I on hematopoiesis (33;34). It is likely that complex interactions between circulating IGF-I, IGFBP-3 and their effects on nitric-oxide bioavailability result in the increase in CD34+ cells in our study. Indeed, a recent study reported that IGFBP-3 also promotes migration, tube formation of CD34+ cells and differentiation of these cells into endothelial cells, leading to increased vessel stabilization and quicker blood vessel development (35) illustrating the complexity of potential mechanisms involved in rhGH effects.

In addition, we also determined several measures of arterial stiffness before and during one year of rhGH replacement. Pulse wave velocity, as a direct measure of arterial stiffness, did not change during the study. This is in contrast with an earlier report by McCallum et al.



(36). In that study PWV decreased from 8.1 to 6.7 m/s during 6 months of rhGH replacement in 16 patients with GHD (36). In our study no change in PWV was found after 6 months or 1 year of rhGH replacement. The discrepancies between the two studies might be related to a more disadvantageous cardiovascular risk profile in our patients group, since they were older (average 7 years), included more men (64% versus 37%) and had a higher BMI (29.4 vs. 27.8 kg/m²). The small improvement in central systolic blood pressure and augmentation index in a previous report (9) was not observed in our study possibly due the differences in patient groups or the limited number of patients studied.

Although we did not find a statistically significant decrease in lipid concentrations in our limited number of patients, LDL cholesterol decreased by 0.2 mmol/l and total cholesterol by 0.3 mmol/l. In a metaanalysis of short-term trials (treatment up to 18 months) with rhGH replacement in GHD (7), the weighted mean differences for LDL and total cholesterol were -0.53 mmol/l and -0.34 mmol/l respectively. Thus, the changes in lipid concentrations in our study move in a similar direction to the changes noted in rhGH replacement in general.

The major study limitation is the low number of patients that were included due to the fact that GHD is a rare disease and that our study design excluded subject with a history of cardiovascular disease, which could influence our measurements. In addition, the beneficial effects of rhGH replacement have been widely accepted which limits the possibilities to study the natural course of this disease with respect to CD34+ cells. Nonetheless, the differences in CD34+ cell numbers found in this group with a wide age range are relatively major which supports a role of GH in the regulation of this cell type. Thus, this study provides new data into the relationship of circulating endothelial progenitor cells and growth hormone which can be used as a basis for additional larger studies. Interestingly, the change in CD34+ cells showed an inverse relationship with advancing age indicating that the effect of rhGH on CD34+ cells is age-dependent.

In conclusion, one year of rhGH replacement in adult patients with GHD improved endothelial function and increased the number of CD34+ cells. Since these outcome parameters are strong biomarkers for cardiovascular disease risk, our data indicate that growth hormone replacement in adults with GHD may have beneficial effects on the vasculature.

ACKNOWLEDGEMENTS

We thank Jos op 't Roodt for skilled performance of vascular function tests and Jacques Duijs for expert technical assistance.

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