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Cytotoxic T-Lymphocyte Associated Antigen 4 Gene Polymorphisms and Autoimmune Thyroid Disease: A Meta-Analysis

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Context: Cytotoxic T-lymphocyte associated antigen 4 (*CTLA-4*) polymorphisms have been widely examined for their associations with autoimmune thyroid diseases [Graves' disease (GD) and Hashimoto thyroiditis (HT)], but their relative population effect remains unclear.

Objective: The aim was to generate large-scale evidence on whether the *CTLA-4* polymorphisms (*A49G* and *CT60*) and haplotypes thereof increase the susceptibility to GD and/or HT.

Design, Setting, and Participants: Meta-analyses of group-level data were reviewed from 32 (11,019 subjects) and 12 (4,479) published and unpublished studies for the association of the *A49G* polymorphism with GD and HT, respectively (PubMed and HuGeNet search until July 2006). There were 15 (n = 7246) and six (n = 3086) studies available for the *CT60* polymorphism, respectively. Meta-analyses of individual-level data from 10 (4906 subjects) and five (2386) collaborating teams for GD and HT, respectively, were also reviewed.

Main Outcome Measures: Association of gene variants and haplotypes with GD and HT was measured.

Results: Group-level data suggested significant associations with GD and HT for both *A49G* [odds ratios 1.49 ($P = 6 \times 10^{-14}$) and 1.29 ($P = 0.001$) per *G* allele, respectively] and *CT60* [1.45 ($P = 2 \times 10^{-9}$) and 1.64 ($P = 0.003$) per *G* allele, respectively]. Results were consistent between Asian and Caucasian descent subjects. Individual-level data showed that compared with the *AA* haplotype, the risk conferred by the *GG* haplotype was 1.49 (95% confidence interval 1.31,1.70) and 1.36 (95% confidence interval 1.16,1.59) for GD and HT, respectively. Data were consistent with a dose-response effect for the *G* allele of *CT60*.

Conclusion: The CT60 polymorphism of *CTLA-4* maps an important genetic determinant for the risk of both GD and HT across diverse populations. (***J Clin Endocrinol Metab* 92: 3162–3170, 2007**)

AUTOIMMUNE THYROID DISEASES (AITDs) are the most prevalent autoimmune disorders (1), affecting up to 5% of the general population (2). Graves' disease (GD) and Hashimoto thyroiditis (HT), the two most common forms of AITD, and other autoimmune disorders such as type 1 diabetes mellitus and rheumatoid arthritis commonly cluster in the same families, and, thus, they may share some genetic background (3). Several genes have been proposed to be linked and/or associated with AITDs, but the extent of replication of these claims has been variable. The cytotoxic T-lymphocyte associated antigen 4 (*CTLA-4*) gene has been one of the most extensively studied loci in this regard, and considerable genetic and immunological evidence suggests that it may be important for autoimmunity. CTLA-4 is an

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Abbreviations: AF, Attributable fraction; AITD, autoimmune thyroid disease; CI, confidence interval; *CTLA-4*, cytotoxic T-lymphocyte associated antigen 4; GD, Graves' disease; HT, Hashimoto thyroiditis; HWE, Hardy-Weinberg equilibrium; LD, linkage disequilibrium; OR, odds ratio; SNP, single nucleotide polymorphism.

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immune regulatory molecule expressed on the surface of activated T lymphocytes and a key inhibitor of T cell activation (4, 5). *CTLA-4* knockout mice develop a lymphoproliferative disorder resulting in death from autoimmunity within 3–4 wk postpartum (6, 7).

Human *CTLA-4* gene consists of four exons and three introns (8). Many studies conducted over the last decade have claimed associations of AITDs with an adenine to guanine transition at position 49 of exon 1 (*A49G*) (9), but several other polymorphisms of the same gene have also been evaluated. The results have not always been consistent. A detailed genomic analysis of CTLA-4 in GD, HT, and type 1 diabetes mellitus involving 108 single nucleotide polymorphisms (SNPs) was published in *Nature* in 2003 (3). The *G* allele of the +6230G>A (*CT60*) polymorphism showed very strong association to GD. Other polymorphisms in addition to *CT60*, such as *JO31*, *JO30*, and *JO27_1*, were also highly associated and found to be in strong linkage disequilibrium (LD) (*i.e.* they tend to exist together), making it very difficult to map disease susceptibility to a single SNP. In an attempt to gain a greater understanding of the effects of individual *CTLA-4* variants and address some of the published inconsistencies, we performed a collaborative international meta-analysis. We have included both published and unpublished data from a network of investigators working in the field. Investigators provided individual-level information from their databases, which allowed more detailed haplotype analyses.

Materials and Methods

Identification and eligibility of relevant studies and data

We first identified all published studies that examined the association of any *CTLA-4* gene polymorphism with AITDs. Sources included MEDLINE, EMBASE, and HuGeNet (last search update performed in June 2006). The search strategy was based on combinations of "CTLA4," "CTLA-4," "cytotoxic T-cell lymphocyte associated antigen 4," "CD152," "CD28," "thyroid,*" "Graves,*" and "Hashimoto,*" limited to humans without language restriction. References of retrieved articles were also screened.

Studies were eligible if they had determined the distribution of alleles and/or genotypes for any of these polymorphisms in unrelated cases with one or more types of AITD and in unrelated controls without AITDs. We did not consider family-based studies of pedigrees with several affected cases per family. We also excluded studies that did not discriminate between the various types of AITD because all analyses were to be performed separately for GD and HT.

When the published literature was accumulated, the meta-analysis coordinators (F.K.K. and J.P.A.I.) communicated with the corresponding investigators of all eligible studies, seeking their participation in a collaborative meta-analysis of individual-level data. The prerequisite for participation was that investigators should be able to supply individual-level genotype data on GD and/or HT cases and controls for both *A49G* and *CT60* polymorphisms on their study populations. Participating investigators were also encouraged to provide individual-level data on additional *CTLA-4* SNPs, whenever available.

Databases

For published articles, two investigators (F.K.K. and J.P.A.I.) independently extracted data and reached consensus on potential disagreements. The following information was sought from each report: authors; journal and year of publication; country of origin; selection and characteristics of cases and controls; demographics; "racial" descent of the study population (Asian, Caucasian, African-American, and other); eligible and genotyped cases and controls; and genotype distributions. Whenever a study team included two or more "racial" descent subgroups, these were treated as separate studies in all analyses.

Furthermore, we examined whether matching of cases and controls had been performed, there was specific mention of blinding of the personnel that performed the genotyping to the clinical status of the subjects, the genotyping method had been validated, and genotype frequencies in control groups were in Hardy-Weinberg equilibrium (HWE) according to an exact test.

Individual-level data were sent to the coordinators, and checked for logical errors, inconsistencies, and potential deviations from HWE among controls. Queries were sent back to the primary investigators for clarification and resolution.

Statistical analyses

Group-level data. Analyses of group-level data were performed using all published information, as well as any additional unpublished data retrieved from the investigators. Whenever investigators provided updated information besides the published data, we kept only the updated data to avoid double counting. Primary analyses compared allele frequencies for each polymorphism. We also addressed recessive and dominant models.

The odds ratio (OR) was used as the metric of choice. Heterogeneity across all eligible comparisons was tested using the χ^2-based Cochran's Q statistic (significant for $P < 0.10$) (10) and the I^2 statistics (considered very large for $I^2 \geq 75\%$ and large for values of 50–74%) (11). Data were combined using both fixed effects [Mantel-Haenszel (12)] and random effects [DerSimonian and Laird (13)] models. Unless stated otherwise, random effects estimates are reported. Subgroup analyses estimated ORs per "racial" descent subgroup.

We also performed recursive cumulative meta-analysis to evaluate whether the summary OR for the allele contrast changed as more data accumulate (14, 15). We used the nonparametric τ correlation coefficient (16) to evaluate whether the magnitude of the observed association is related to the variance of each study ("small-study effects").

Individual-level data. Analyses of individual-level data focused on the *A49G* and *CT60* polymorphisms for which meaningful amounts of data were available to be examined as haplotypes across a large number of studies. First, we examined whether analyses using group-level data from the studies with individual-level information gave similar results for each polymorphism as those obtained from the group-level analyses, including all studies. Then, the main analyses of individual-level data used haplotypes.

Haplotype reconstruction of the *A49G* and *CT60* polymorphisms was performed using the population genotypic data separately for each case group (GD or HT) and controls of each participating team. The possible haplotypes are *GG*, *GA*, *AG*, and *AA* (the first allele corresponds to the *A49G* and the second one to the *CT60* polymorphism, respectively). Haplotypes were inferred performing 100 iterations and using 100 individuals (randomly chosen) in each input file; 96.2% of the haplotypes were inferred with a probability exceeding 90%. Presented analyses used the most likely inferred haplotype for each subject. Analyses weighting each haplotype by its probability of inference yielded very similar results (data not shown).

Primary analyses used logistic regression to calculate in each study the OR per haplotype copy using the *AA* haplotype as reference. We then combined the natural logarithms of the ORs for each haplotype using an inverse variance random effects model. Between-study heterogeneity was measured with the Q and I^2 statistics. Secondary analyses considered for each haplotype two variables instead of one (having one or two copies).

Individual-level data were used to calculate separately for each "racial" descent the attributable fraction (AF) (the complete list of data can be found in supplemental Appendix 1, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

Analyses were conducted in Intercooled Stata 8.2 (Stata Corp., College Park, TX) using the meta and the metan module. Haplotype reconstruction was performed in PHASE 2.1 (17, 18) using the -T option. *P* values were two-tailed.

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Results

Eligible studies

The electronic search yielded 157 articles. Of those, 114 were excluded (Fig. A1, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). A total of 43 articles examining the relation of AITD and the *CTLA-4* polymorphisms were eligible (3, 9, 19–59) (the complete list of articles can be found in supplemental Table A1, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). There were 27 and nine studies that contained data on the *A49G* polymorphism and GD (3, 9, 21, 23, 25–27, 29–33, 36–39, 42–44, 46, 47, 49–53, 57) and HT (3, 26, 30, 31, 35, 41, 48, 58, 59), respectively. One of the eligible articles included subjects from two different racial descent groups (37). Therefore, a total of 28 comparisons of published studies were considered for the *A49G* polymorphism and GD. Seven and three studies reported data for the *CT60* polymorphisms and GD (3, 43, 48, 50, 52–54) and HT (3, 48, 54), respectively. There was considerable diversity of ethnic groups and eligibility criteria (the complete list of results can be found in supplemental Table A1, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). For controls, varying details were presented regarding the extent of testing that had been performed to exclude controls with disturbed thyroid function. There were 19 studies (3, 9, 19–22, 25, 29, 31–33, 35, 36, 38, 41, 46–48, 50–53, 55–57, 59, 60) that excluded subjects with a family history of AITD and/or other autoimmune disorders from controls.

Seven studies matched for age (9, 38, 39, 43, 44, 48, 50), six for gender (9, 38, 43, 44, 48, 50), four for geographic region (32, 35, 42, 52), one for age, gender, and geographic region (50), one for ethnical descent, age, and gender (9), and five for ethnical descent (3, 19, 42, 52, 59). PCR methods were used for genotyping. No articles mentioned explicit blinding of the personnel that performed the genotyping. In three studies (two for *A49G* polymorphism and one for *CT60*), the distribution of genotypes in the control group deviated significantly from HWE (36, 38, 41).

There were 10 teams of investigators that provided individual-level genotyping data for both *A49G* and *CT60* polymorphisms, all teams provided data for GD and five of them provided data for HT, as well. Four teams were from Europe and six from Asia. Nine teams had already published data on *A49G* polymorphism (3, 26, 28, 29, 37, 38, 43, 44, 47), but only two on *CT60* polymorphism (3, 48). Three teams provided genotyping data from different cohorts than those previously published (29, 38, 48). Seven teams clarified that they had used blinding of personnel in genotyping.

Data on other *CTLA-4* polymorphisms were more limited (the complete list of data can be found in supplemental Tables A17–A23, which are published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

Group-level data

A49G polymorphism. The analyses included a total of 4848 cases with GD, 866 with HT, and 7314 controls (Table 1) (the complete list of cases can be found in supplemental Table A2, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). The frequency of the *G* allele was 48.9% among control subjects (62.0% and 36.9% among Asian and Caucasian descent, respectively). The overall prevalence was 26.5% (40.5% and 14.0%, respectively) for *G/G* homozygosity and 42.0% (40.2% and 42.7%, respectively) for *G/A* heterozygosity.

The summary OR of published and unpublished data suggested a 1.49-fold increase in susceptibility to GD among

TABLE 1. Meta-analyses of group-level data for various contrasts of *A49G* and *CT60* polymorphism in the *CTLA-4* gene and susceptibility to GD and HT

Contrast and racial descent group	GD					HT				
	No. of studies	Total sample size (n)	Random effects	95% CI	I^2 (%)	No. of studies	Total sample size (n)	Random effects	95% CI	I^2 (%)
A49G: G vs. A allele										
All	32	22,038[a]	1.49	1.34–1.66	64	12	8,958[a]	1.29	1.11–1.50	54
Asians	15	9,800[a]	1.54	1.36–1.75	38	7	4,530[a]	1.31	1.02–1.68	71
Caucasians	11	10,210[a]	1.55	1.33–1.80	66	4	4,158[a]	1.30	1.11–1.52	0
G/G vs. A/A genotype	30	5,778[b]	2.13	1.72–2.62	51	12	2,447[b]	1.95	1.50–2.52	19
G/G vs. G/A and *A/A* genotypes	30	10,338[b]	1.70	1.43–2.01	64	12	4,490[b]	1.29	1.01–1.64	58
G/G and *G/A vs. A/A* genotype	30	10,338[b]	1.62	1.36–1.92	55	12	4,560[b]	1.64	1.25–2.15	47
CT60: G vs. A allele										
All	15	14,492[a]	1.45	1.28–1.64	56	6	6,172[a]	1.64	1.18–2.26	83
Asians	8	7,588[a]	1.60	1.27–2.00	72	4	3,780[a]	1.54	1.24–1.92	39
Caucasians	6	6,620[a]	1.44	1.31–1.60	0	1	2,124[a]	2.83	2.28–3.52	–
G/G vs. A/A genotype	14	4,192[b]	2.24	1.68–3.00	47	6	1,763[b]	2.83	1.42–5.66	76
G/G vs. G/A and *A/A* genotypes	14	6,948[b]	1.62	1.35–1.94	64	6	3,087[b]	1.87	1.22–2.88	82
G/G and *G/A vs. A/A* genotype	14	6,948[b]	1.84	1.46–2.32	33	6	3,087[b]	2.18	1.34–3.54	59

[a] Total sample size refers to the total number of alleles for cases (GD or HT) and controls.
[b] Total sample size refers to the total numbers of cases (GD or HT) and control.

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subjects with the G allele ($P = 6 \times 10^{-14}$) but with evidence of significant between-study heterogeneity (Q = 85.6; $P < 0.001$ for heterogeneity; $I^2 = 64\%$) (Table 1 and Fig. 1A). Effect sizes were consistent across subgroups of different racial descent (Table 1)

When the analysis was limited to studies in which published or unpublished data were available for both *A49G* and *CT60* polymorphisms, the summary OR for *A49G* became 1.40 ($I^2 = 74\%$). When the two studies that deviated significantly from HWE (36, 38) were also excluded, the random-effects OR remained 1.49 ($I^2 = 66\%$). A sensitivity analysis limited to published data yielded a summary OR of 1.45 ($I^2 = 50\%$).

The G allele conferred an almost 1.3-fold increase in the susceptibility of HT that was significant ($P = 0.001$), and there was substantial between-study heterogeneity ($I^2 = 54\%$; Table 1 and Fig. 1B). Similar effects were found for different ethnic groups (Table 1), with heterogeneity only in the Asian studies. When the analysis was limited to studies in which published or unpublished data were available for both *A49G* and *CT60* polymorphisms, the summary OR for the G allele was still 1.31 ($I^2 = 0\%$). Sensitivity analysis excluding studies with unpublished data yielded similar results (OR 1.28; $I^2 = 65\%$). No studies had significant deviations from HWE in their control groups.

The magnitude of the overall OR diminished modestly over time for GD (from 1.64 in 1996, to 1.49 in the final analysis) and HT (from 1.57 in 1997, to 1.29 in the final analysis). There was no evidence that more precise studies showed more conservative results for the association of the G allele with GD or HT either, than less precise studies ($P = 0.24$ and $P = 0.68$ for GD and HT, respectively).

CT60 polymorphism. Group-level data included 3047 GD cases, 839 HT cases, and 3741 healthy controls (Table 1) (the complete list of cases can be found in supplemental Table A5,

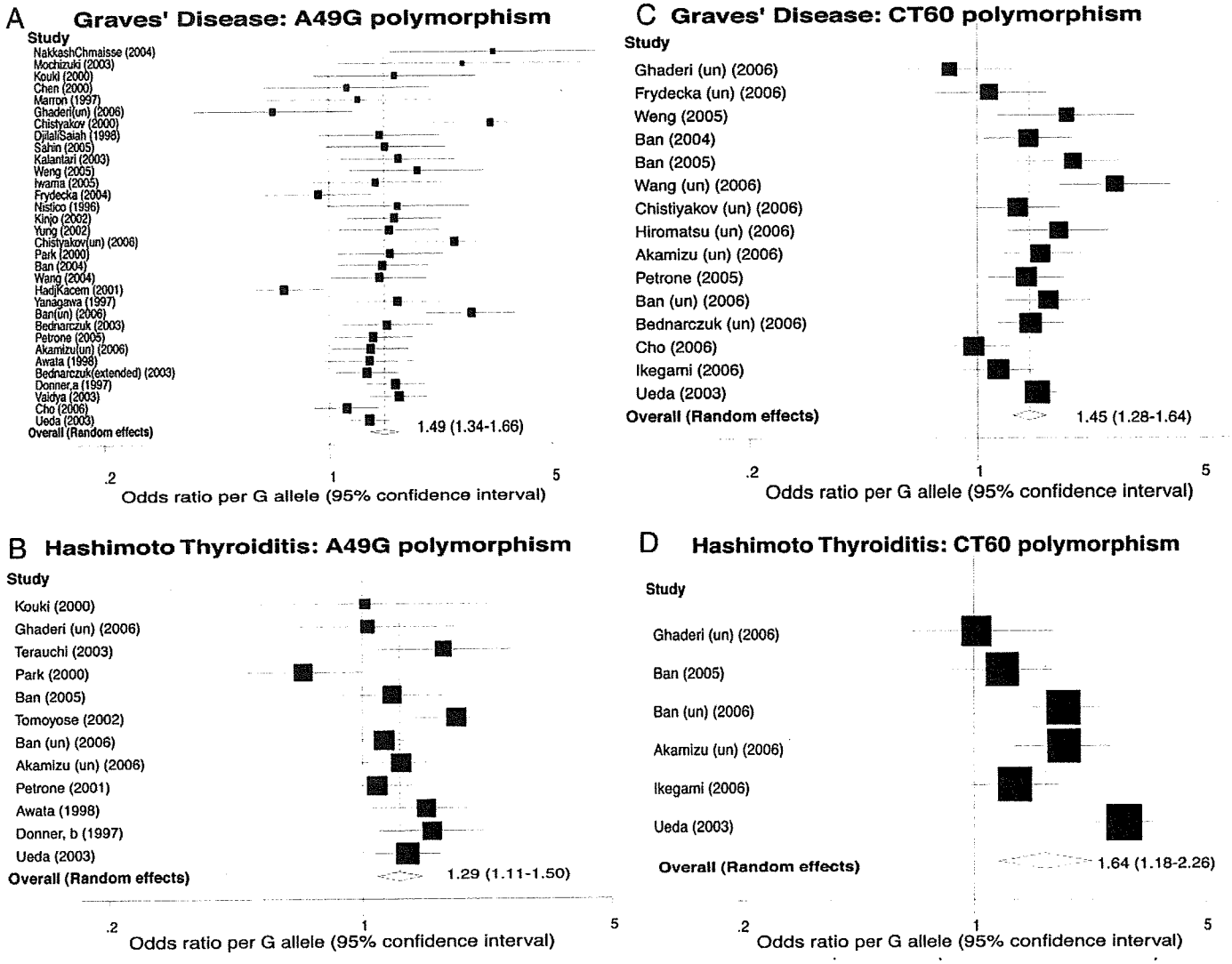

FIG. 1. Odds ratios for the association between the G allele of the *A49G* (A and B) and the *CT60* (C and D) polymorphisms in the *CTLA-4* gene and susceptibility to GD (*upper panel*) and HT (*lower panel*). Individual studies [published and unpublished (un)] (*boxes*) are listed by increasing sample size from top to bottom. The *diamond* shows the summary random-effects OR estimate from a meta-analysis. *Horizontal lines* indicate 95% CI.

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which is published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). The *G* allele frequency was 64.3% in controls (76.0% in subjects of Asian descent and 52.7% in those of Caucasian descent), *G/G* homozygosity had a frequency of 44% (57.6 and 29.3%, respectively), while *G/A* heterozygotes were 41.9% (36.8 and 47.3%, respectively) of the controls.

The *G* allele increased 1.45-fold the odds of GD ($P = 2 \times 10^{-9}$); there was large heterogeneity ($I^2 = 56\%$) (Table 1 and Fig. 1C). The results were similar in subjects of Asian and Caucasian descent (Table 1), with between-study heterogeneity only in the Asian descent studies. Analyses limited to studies in which both *A49G* and *CT60* had been genotyped yielded an OR of 1.45 ($I^2 = 56\%$) per allele. A sensitivity analysis including only previously published studies yielded similar results (OR 1.40; $I^2 = 57\%$). After excluding one study (52) that significantly deviated from HWE, neither the effect size (OR 1.43) nor the heterogeneity ($I^2 = 58\%$) changed.

For HT, the *G* allele of the *CT60* polymorphism increased the odds 1.64-fold ($P = 0.003$) with very large heterogeneity ($I^2 = 83\%$) (Table 1 and Fig. 1D). The data came from Asian descent studies, with the exception of a single study on Caucasian descent subjects, in which a very strong effect was seen (OR 2.83). A sensitivity analysis including only previously published studies yielded similar results (OR 1.68 per *G* allele; $I^2 = 91\%$). All studies conformed to HWE.

Compared with the first study, the magnitude of the OR diminished slightly for GD (from 1.53 in 2003, to 1.45 in the last analysis) and more prominently for HT (from 2.83 in 2003, to 1.64 in the last analysis). No evidence was found that more precise studies showed more conservative results than less precise studies ($P = 0.40$ and $P=0.19$ for GD and HT, respectively).

Individual-level database

Individual-level data were available for 2306 GD cases, 657 HT cases, and 2530 controls for the *A49G*, and 2276 GD cases, 662 HT cases, and 2469 controls for the *CT60* polymorphism. The allele and genotype frequency for each polymorphism were similar to those of the larger group-level database (the complete list of results can be found in supplemental Tables A8–A11, which are published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). Controls did not deviate significantly from HWE in any study teams. Moreover, the summary effect using group-level information from these studies on each polymorphism was similar to the aforementioned analyses, including all studies (the complete list of results can be found in supplemental Tables A12 and A13, which are published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

The database included a total of 5586 subjects: GD, 2334; HT, 680; and controls, 2572. The haplotype frequencies among controls for the: *GG* haplotype was 47.9% (63.1% in Asian descent controls, 38.4% in Caucasian descent, and 29.5% in Iranian descent, respectively); *AG* was 14.3% (11.7, 16.0, and 16.0%, respectively); *GA* was 1.6% (1.4, 1.6, and 2.0%, respectively); and *AA* was 36.2% (23.8, 44.0, and 52.5%, respectively) (the complete list of frequencies can be found in supplemental Tables A14 and A15, which are published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

For GD (Table 2 and Fig. 2A), the *GG* haplotype increased the odds by 1.49-fold per copy [95% confidence interval (CI) 1.31–1.70; $P = 2 \times 10^{-9}$; $I^2 = 48\%$)] compared with the *AA* haplotype. The result was consistent in Asian and Caucasian descent subjects (1.57 and 1.52, respectively). The *AG* haplotype also increased the odds of GD by 1.35-fold (Table 2 and Fig. 2B). No differences were found for Asian and Caucasian descent subgroups (1.30 and 1.42, respectively). The *GA* haplotype did not differ significantly in the GD risk overall (OR 0.78; Table 2 and Fig. 2C). After stratifying for *CT60*, the OR per copy of *G* allele of *A49G* was 1.13 (95% CI 0.98–1.31).

The *GG* haplotype increased the odds of HT by 1.36-fold (OR 1.36; 95% CI 1.16–1.59; $P = 0.001$; $I^2 = 0\%$; Table 2 and Fig. 3A). No effect was shown for the *AG* haplotype, but the CIs cannot exclude a modest effect (OR 1.02; Table 2 and Fig. 3B). For both *GG* and *AG*, the effects were stronger in the

TABLE 2. Meta-analyses of individual-level data for the association of different haplotypes reconstructed of the *A49G* and *CT60* polymorphisms of the *CTLA-4* gene with GD and HT patients

Disease, polymorphism, and contrast	GD					HT				
	No. of teams	Total sample size (n)[a]	Random effects	95% CI	I^2 (%)	No. of teams	Total sample size (n)[a]	Random effects	95% CI	I^2 (%)
GG haplotype										
All	10	5,128	1.49	1.31–1.70	48	5	2,419	1.36	1.16–1.59	0
Asians	5	2,819	1.57	1.33–1.85	0	3	1,516	1.26	1.01–1.57	0
Caucasians	4	2,229	1.52	1.24–1.85	68	1	819	1.59	1.22–1.98	–
GA haplotype										
All	6	122	0.78	0.48–1.27	12	2	47	4.62	0.05–404.7	93
Asians	3	39	0.51	0.21–1.23	12	–	–	–	–	–
Caucasians	3	79	0.97	0.58–1.63	53	–	–	–	–	–
AG haplotype										
All	10	1,426	1.35	1.16–1.55	9	5	666	1.02	0.71–1.47	35
Asians	5	473	1.30	1.00–1.69	13	3	266	0.86	0.62–1.21	0
Caucasians	4	906	1.42	1.20–1.69	6	1	358	1.62	1.21–2.16	–

All analyses represent contrasts of the respective haplotype *vs.* the *AA* haplotype.

[a] Total sample sizes comprise both cases' (GD and HT, respectively) and controls' total number of chromosomes with the respective haplotype. One study had subjects of Iranian descent having different haplotype frequencies from both Asian and Caucasian descent subjects, and, thus, are not shown in the racial subgroup analyses.

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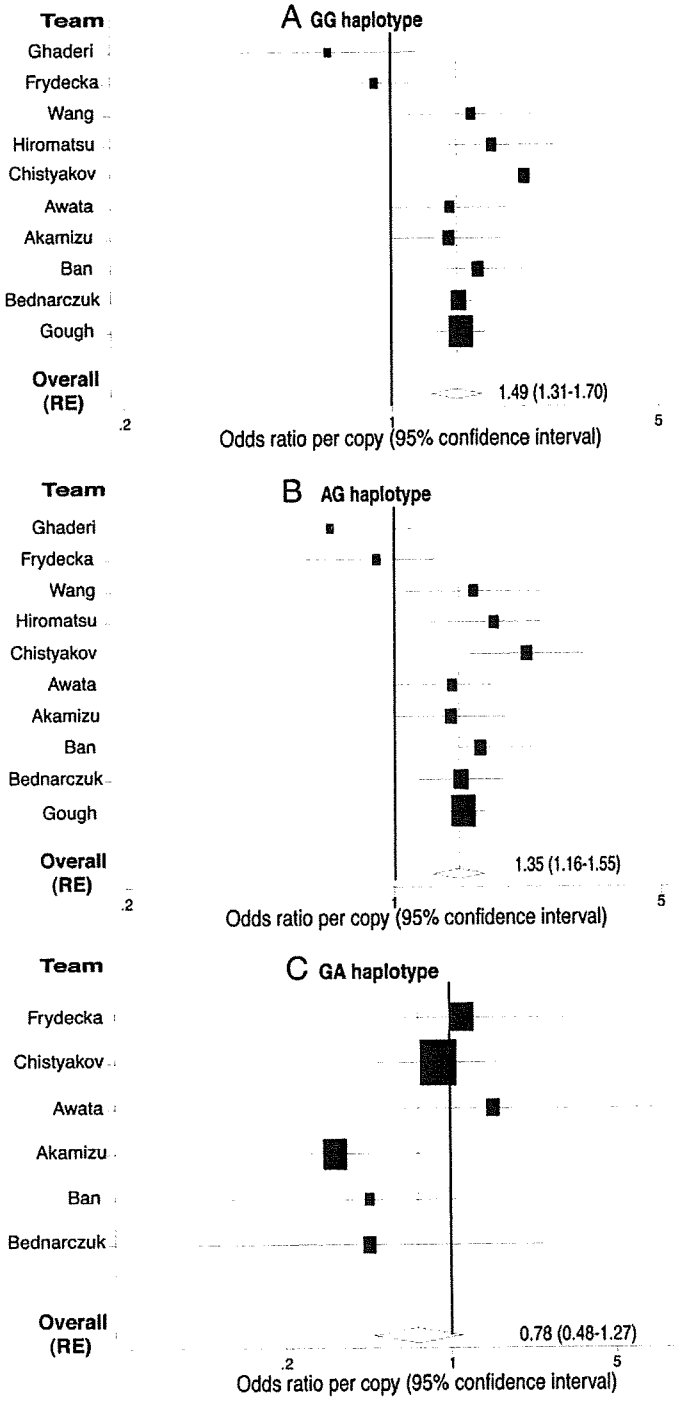

FIG. 2. Odds ratios derived for the association among the *GG* (A), *AG* (B), and *GA* (C) haplotypes of the *A49G* and *CT60* polymorphisms in the *CTLA-4* gene and susceptibility to GD. The *AA* haplotype is the reference. Individual studies (*boxes*) are listed by increasing sample size from top to bottom. The *diamond* shows the summary random effects (RE) OR estimate from a meta-analysis of individual participant data. *Horizontal lines* indicate 95% CI.

subjects of Caucasian descent than in subjects of Asian descent (1.59 *vs.* 1.26 and 1.62 *vs.* 0.86, respectively). The *GA* haplotype had a nonsignificant effect, but data were very sparse (only 14 HT cases and 33 controls had the *GA* haplotype).

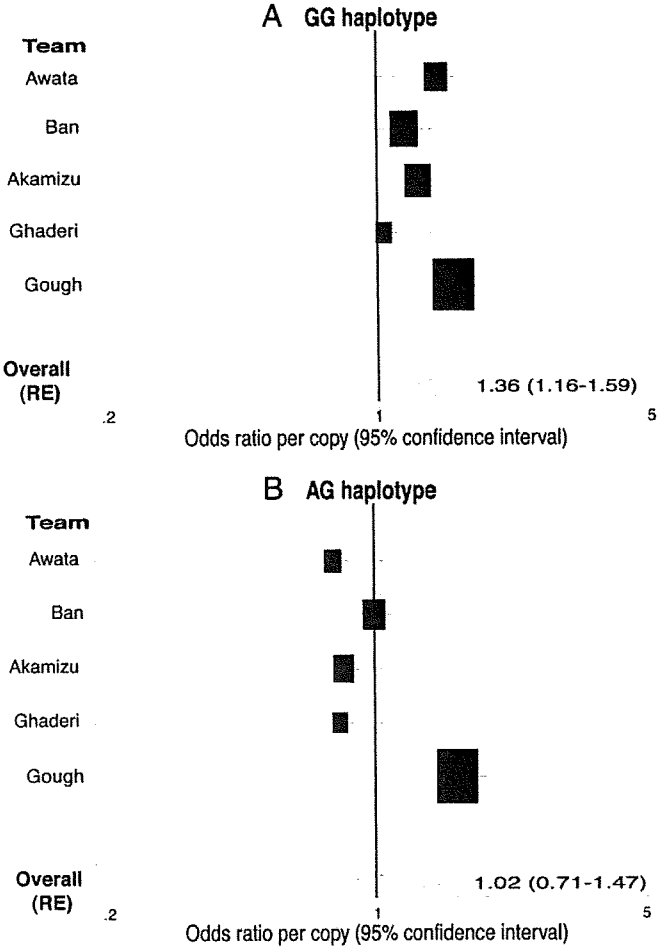

FIG. 3. Odds ratios derived for the association between the *GG* (A) and *AG* (B) haplotypes of the *A49G* and *CT60* polymorphisms in the *CTLA-4* gene and susceptibility to HT. The *AA* haplotype is the reference. Individual studies (*boxes*) are listed by increasing sample size from top to bottom. The *diamond* shows the summary random effects (RE) OR estimate from a meta-analysis of individual participant data. *Horizontal lines* indicate 95% CI.

The secondary analyses were consistent with a dose-response effect for the *GG* haplotype. With one copy, the OR was 1.29 for GD (95% CI 1.07–1.55; $P = 0.007$; $I^2 = 18\%$), while with two copies, the OR became 1.97 (95% CI 1.48–2.63; $P = 3 \times 10^{-6}$; $I^2 = 48\%$) (the complete list of ORs can be found in supplemental Table A16, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). The respective effects for HT were 1.48 (95% CI 1.15–1.91; $P = 2 \times 10^{-3}$; $I^2 = 0\%$) and 1.71 (95% CI 1.25–2.33; $P = 10^{-3}$; $I^2 = 0\%$). The data were also consistent with a dose-response for the *AG* haplotype in GD (OR 1.06 and 1.39 with one and two copies, respectively), while data were sparse to examine this for HT (only 12 HT subjects and 43 controls had two copies of *AG* in the entire database).

The AF according to the primary analysis was 32% for Asian descent and 29% for Caucasian descent subjects for GD, and 19 and 28%, respectively, for HT. Results were similar in the secondary analysis (AF = 24–39%).

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Other polymorphisms

No formally significant results were seen overall for the 104-bp allele of the *(AT)n* microsatellite, the *C(-318)T* polymorphism, or the *JO27_1* polymorphism, either for GD or HT (the complete list of results can be found in supplemental Appendix Tables A15–A17, A20, and A21, which are published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). The *G* allele of the *JO31* polymorphism [four studies (5412 alleles)] and the *G* allele of the *JO30* polymorphism [three studies [2796 alleles)] showed a possible association to GD, but the effects were modest (OR 1.40, 95% CI 1.15–1.72, $P = 0.01$, $I^2 = 57\%$; and OR 1.25, 95% CI 0.99–1.59, $P = 0.06$, $I^2 = 68\%$, respectively) (the complete list of results can be found in supplemental Tables A20, A21, and A23, which are published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). Moreover, *JO31* and *JO30* were in LD with the *CT60* polymorphism ($r^2 = 0.65$ and 0.79, respectively) and among themselves ($r^2 = 0.55$).

Discussion

This meta-analysis examined the association of *CTLA-4* polymorphisms with AITD (GD and HT), and included group-level data from more than 13,000 subjects and individual-level data from more than 5,000 subjects. The group-level data clearly showed highly significant associations of both GD and HT with each polymorphism separately, with *P* values in the range of 10^{-3} to 10^{-16} in the main analyses. The individual-level data allowed the consideration of haplotypes, including both polymorphisms. The association with *A49G* polymorphism is probably mostly the result of LD with *CT60*. The *G* allele of the *CT60* polymorphism increases the odds of both GD and HT by 1.4-fold. A dose-effect association was also demonstrated as the presence of two copies of the susceptible haplotype *GG* almost doubled the odds compared with one copy of *GG*.

Although environmental agents are undoubtedly important for the development of AITD in susceptible individuals, it has been estimated in twin studies that almost 80% of the predisposition to GD is due to genetic factors (60). The genetics do not represent a simple Mendelian model (61). Several genes may be associated with AITD. The human leukocyte antigen region, *CTLA-4* gene, and *PTPN22* gene have shown the strongest results to date (62–65). Given the observed OR and high allele frequencies in the examined populations, the AF for *CT60* is in the range of 20–30%. We should acknowledge that genetic effects may occasionally be overestimated due to biases, however, this is one of the largest AFs ever shown in the genetics of complex diseases (66).

Functional evidence supports the role of CTLA-4 in autoimmunity. CTLA-4 was recently described as a gatekeeper of conjugation timing (67). Reduced conjugation might protect against prolonged contact periods of cytotoxic T lymphocytes with autoantigen-defined targets. *CT60 G* haplotypes produce less soluble *CT60* transcript than *A* haplotypes (3). However, the disease-implicated haplotypes may extend over the long costimulatory receptor region of chromosome 2 consisting of *CD28*, *CTLA-4*, and *ICOS* (68). The *CTLA-4 CT60 A* protective allele haplotype goes with the most common extended haplotype (15-2-4) in Caucasians. The relative role of other polymorphisms and extended haplotypes may be further clarified in additional large-scale studies.

Some caveats should be discussed. Most of the group-level data showed large between-study heterogeneity. This could be due to bias, chance, or genuine diversity of genetic effects. We found no evidence of differences according to racial descent, even though the allele frequencies differed across racial subgroups. The smaller published studies did not show different results compared with the larger ones. However, there was a suggestion that the first studies may have yielded somewhat stronger effects (15). This is consistent with a "winner's curse phenomenon" in which early data show exaggerated effects. Thus, the group-level derived estimates may be modestly inflated, as suggested also by the trend for relatively smaller effects sizes for the *G* allele of *CT60* in the individual-level data. The latter may provide more reliable estimators and more options for deciphering the relative contribution of each polymorphism, but they are also not necessarily devoid from potential biases. Practical considerations did not allow shipping of specimens for re-genotyping at a central facility. Nevertheless, genotyping error for SNPs should be low at experienced facilities. All control genotype frequencies were consistent with HWE in the individual-level database, but this was not so in the group-level data. However, some analyses have significant between-study heterogeneity, even in the individual-level data. Besides biases, this could be attributed to differences in terms of disease phenotype (*e.g.* presence of ophthalmopathy and/or of other autoimmune diseases) among AITD cases. AITD is rare in men to allow evaluation of gender differences. Moreover, data on age of onset were not sufficiently standardized across studies to allow meaningful investigation of age-related effect modification.

In conclusion, despite these caveats, our collaborative analysis shows consistent associations between GD and HT with *CT60*. This association crosses ethnic barriers, and we can make a reasonable estimate of the implicated OR. Although we still cannot identify a single etiological polymorphism, our study confirms the important role of the *CTLA-4* locus in determining the risk of AITD.

Glossary of statistical terms

Effect size: The magnitude of the association (*e.g.* odds ratio).

"Fixed effects" model: Considers that the variability of the results between studies of the meta-analysis is exclusively due to random variation (chance). Therefore, if all the studies were infinitely large they would give identical results.

"Random effects" model: Assumes a different underlying effect for each study of the meta-analysis and takes this into consideration as an additional source of variation, leading to wider confidence intervals than the fixed effects model. The model tries to measure the mean and dispersion of these study-specific effects.

Heterogeneity: Denotes the diversity in a meta-analysis due to clinical differences (participants, interventions, outcomes) or methodological differences (study design, quality, analysis) among the included studies.

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I^2 (I-square): Describes the percentage of total variation across studies included in a meta-analysis that is due to heterogeneity rather than chance. I^2 lies between 0% and 100%. A value of 0% indicates no observed heterogeneity, and larger values show increasing heterogeneity.

Attributable fraction: Denotes the proportion of a disease (or other outcome of interest) in the community that can be explained by the presence of a risk factor. It is mainly influenced by the prevalence of the risk factor, thus common risk factors can have high attributable fractions even when the effect size is not large.

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A Case of Subclinical Hypothyroidism Developing Marked Pleural Effusions and Peripheral Edema with Elevated Vascular Endothelial Growth Factor

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Abstract. A 69-year-old woman was admitted for the treatment of marked pleural effusions and peripheral edema. Analytical studies of the pleural effusion revealed exudates. Culture for bacterial organisms and tuberculosis were negative, and cytology was normal. She had a mediastinal tumor at the age of 61 and regular follow-up showed no evidence of malignancy. She underwent the mediastinal tumor resection, because we thought this was the cause of her symptoms. However, her clinical symptoms persisted after surgery. Next, we noticed subclinical hypothyroidism, in which serum TSH level was elevated with concomitant normal thyroid hormone levels. In addition, serum vascular endothelial growth factor (VEGF) levels, which have been reported to be related to the pathophysiology of the extravascular volume overload, were elevated. Although her TSH level was slightly elevated (15.4 μU/ml), we started thyroid hormone replacement therapy. This therapy gradually ameliorated her clinical manifestation and abnormal laboratory data, including elevated VEGF levels. These observations indicate that even subclinical hypothyroidism may cause severe clinical manifestations. Furthermore, elevated VEGF may be a contributing factor in the pathogenesis of extravascular volume overload in hypothyroid patients.

Key words: Primary myxedema, Hypothyroidism, VEGF, Estradiol, Pleural effusion

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SUBCLINICAL hypothyroidism is defined as a condition with an elevated TSH and normal free T_4 (FT_4), despite the confusing nomenclature [1–3]. The overall prevalence has been reported to range from 4%–10% in large general population screening surveys and from 7%–26% in studies of the elderly [1]. The condition may be associated with cardiac dysfunction, increased risk for the development of atherosclerosis, elevation in total and low-density lipoprotein (LDL) cholesterol, systemic hypothyroid symptoms, neuropsychiatric symptoms, and progression to overt, symptomatic hypothyroidism [3]. The measurement of TSH is the most sensitive test for early diagnosis of primary hypothyroidism; however, TSH may be a poor measure for estimating the clinical and metabolic severity of overt hypothyroidism [4].

Effusions in serous body cavities, including peritoneal, pleural and pericardial, are frequently recognized in hypothyroidism [5, 6]. Although increased capillary permeability with leakage of plasma proteins was reported in hypothyroid patients [7], the mechanism by which these effusions develop is not well understood. Vascular endothelial growth factor (VEGF) is an angiogenic and mitogenic substance that seems to be active in vascular endothelial cells [8–10] and plays an important role in tumor growth and in the metastatic

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process [11]. VEGF is also known as a vascular permeability factor and induces a rapid and reversible increase in vascular permeability [12, 13]. Recently, an association of edema with increased VEGF levels has been demonstrated in many pathological conditions, such as POEMS (polyneuropathy, organomegaly, endocrinopathy, M-protein and skin changes) syndrome [14], ovarian hyperstimulation syndrome [15], preeclampsia [16], and diabetic patients with troglitazone treatment [17].

Here we report a case of primary hypothyroidism with severe clinical manifestations, though the biochemical thyroid function showed subclinical hypothyroidism. She had marked pleural effusions and peripheral edema, with elevated VEGF.

Case Report

A 69-year-old woman suffered from cough and dyspnea in June 2000 and bilateral pleural effusion was observed. Her past medical history included the following conditions. At 37 years of age she underwent a total hysterectomy for rupture of the uterus during labor. She had no history of estrogen treatment. At the age of 50, she developed leg edema; however, she ignored the condition. At 61, she had a mediastinal tumor and regular follow-up showed no evidence of malignancy.

She was hospitalized for the treatment of pleural effusions in thoracic surgery in our hospital in July 2000. Chest radiography demonstrated massive bilateral pleural effusions (Fig. 1A) and echocardiography showed pericardial effusions. Analytical studies of the pleural effusion revealed exudates with protein 3.6 g/dL and cell count of 400/mm^3. Lymphocytes were predominant (90%). Culture for bacterial organisms and tuberculosis were negative and cytology was normal. F-18-fluorodeoxyglucose positron emission tomography (FDG-PET) showed a high-uptake lesion, which coincided with the anterior mediastinal tumor but no other abnormal uptake could be found. In August, she underwent both mediastinal tumor and pericardium resection. The pathological report of the surgical specimen was consistent with a benign thymoma of the noninvasive type. Her clinical symptoms persisted after surgery and it was noticed that this was not the cause of her symptoms.

After consultation with our peers, the possibility of hypothyroidism as a cause of her symptoms was considered. On physical examination, she had anasarca with pretibial pitting edema on her legs (Fig. 2A). Laboratory data showed mild decreased total protein and albumin levels (Table 1). Total cholesterol (T-Cho) and creatine kinase (CK) levels were within normal limit. Her thyroid function revealed subclinical hypothyroidism, such as slightly elevated TSH, and both free T_3 (FT_3) and FT_4 within normal range (Table 2). FT_3 and FT_4 were measured by immunoradiometric assays (Daiichi Radioisotope Laboratories Ltd., Tokyo, Japan). Antithyroid peroxidase antibodies, antithyroglobulin antibodies and anti-TSH receptor antibodies were all negative. Thyroid ultrasound examination demonstrated an atrophic thyroid gland, with multiple

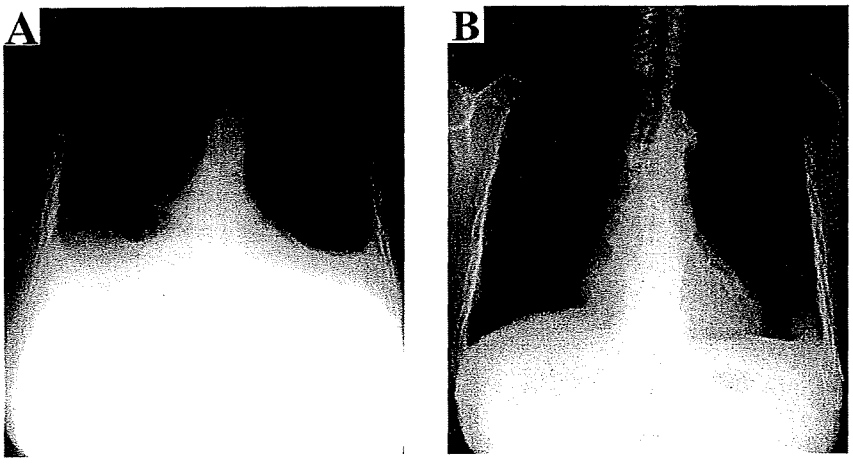

Fig. 1. Posteroanterior chest roentgenogram. A, in August 2000, demonstrating massive bilateral pleural effusions. B, in April 2001, demonstrating resolution of pleural effusions.

cysts in the thyroid. ^{99m}Tc pertechnetate thyroid scintigraphy showed a normal thyroid shape with even trapping and a normal uptake ratio [2.5% (normal range, 0.4–3.0%)]. Another endocrinological examination is shown in Table 2. Serum LH and FSH levels were 0.83 mIU/mL and 10.6 mIU/mL, with estradiol (E2) of 55 pg/mL.

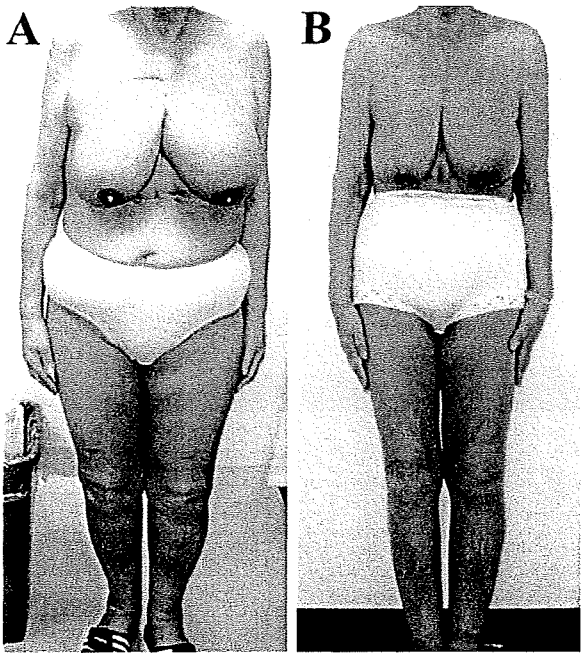

Fig. 2. A, Patient presented with marked peripheral edema in September 2000. B, Peripheral edema was improved after thyroid hormone replacement therapy in March 2001.

Initially, we suspected POEMS syndrome because she had marked pleural effusions and peripheral edema, even though her thyroid function showed a normal FT_4 and slightly elevated TSH. Furthermore, she had elevated E2 in spite of being postmenopausal. To evaluate polyneuropathy, motor nerve conduction velocity was performed, but was normal in both lower extremities. Abdominal computerized tomography scan showed a slightly enlarged liver, but neither splenomegaly nor apparent tumor lesion was found. Though serum IgG level was increased to 2185.7 mg/dL (normal range, 788.0–1841.0 mg/dL), serum immunoelectrophoresis did not reveal the possibility of IgG monoclonal protein, and bone marrow aspiration specimen showed no abnormality. Serum VEGF and interleukin-6 (IL-6) levels, which have been reported to increase in POEMS syndrome, were increased to 2289.9 pg/mL (normal range, 62.0–707.0 pg/mL) and 7.3 pg/mL (normal range, <4.0 pg/mL), respectively.

Clinical course of the patient

The patient did not have evidence of polyneuropathy, which was observed in 100% of POEMS syndrome [18]. In view of this, POEMS syndrome was ruled out. Though there was a slight elevation in TSH level, we started her on thyroid hormone replacement therapy. Levothyroxine sodium (T_4) was used at a dose of 25 μg/day from September and then gradually in-

Table 1. Laboratory data

Blood cell count		Normal range	Blood chemistry		Normal range
WBC	12000/μl	2700–8500	AST	22 IU/l	13–33
RBC	352 × 10^4/μl	319–494	ALT	11 IU/l	6.0–27.0
Hb	11.5 g/dl	10.2–14.9	ALP	238 IU/l	115–359
PLT	66.2 × 10^4/μl	11.0–34.7	TP	6.1 g/dl	6.3–8.1
			Alb	3.0 g/dl	3.9–5.1
			T-Cho	161 mg/dl	140–220
			Triglyceride	194 mg/dl	34–173
Urine			CPK	36 IU/l	35–141
Protein	(–)		BUN	10 mg/dl	8.0–22.0
Glucose	(–)		CRE	0.7 mg/dl	0.4–0.8
Ketone	(–)		Na	132 mEq/l	136–144
O.B.	(–)		K	3.5 mEq/l	3.6–4.8
			Cl	90 mEq/l	99–109
			Glu	107 mg/dl	78–110
			HbA1c	5.0%	4.8–5.8
			CRP	0.8 mg/dl	<0.2

Table 2. Endocrinological examination

	Reference interval			Reference interval	
TSH	15.4 μU/ml	0.41–4.0	LH	0.83 mIU/ml	4.2–79.6
FT_3	2.97 pg/ml	2.0–4.9	FSH	10.6 mIU/ml	12.6–235.7
FT_4	1.16 ng/dl	0.82–1.63	Estradiol	55 pg/ml	<16
TPOAb	<0.3 U/ml	<0.3	Testosterone	0.27 ng/ml	0.13–0.69
TgAb	<0.3 U/ml	<0.3	17α-OHP	0.2 ng/ml	0.1–3
TRAb	7.9%	<15	DHEA-S	774 ng/ml	50–1000
ACTH	11.9 pg/ml	7–56		Urinary hormone excretion	
Cortisol	10.2 μg/dl	5.0–15.0	17-OHCS	3.5 mg/day	2.2–7.3
GH	1.7 ng/ml	0.17–1.41	17-KS	3.4 mg/day	2.4–11.0
IGF-I	25 ng/ml	121–436	Estradiol	6 μg/day	<2.1
PRL	11.3 ng/ml	1.0–13.9			

17α-OHP: 17α-hydroxy progesterone, DHEA-S: dehydroepiandrosterone sulfate, 17-OHCS: 17-hydroxy corticosteroid, 17-KS: 17-ketosteroid

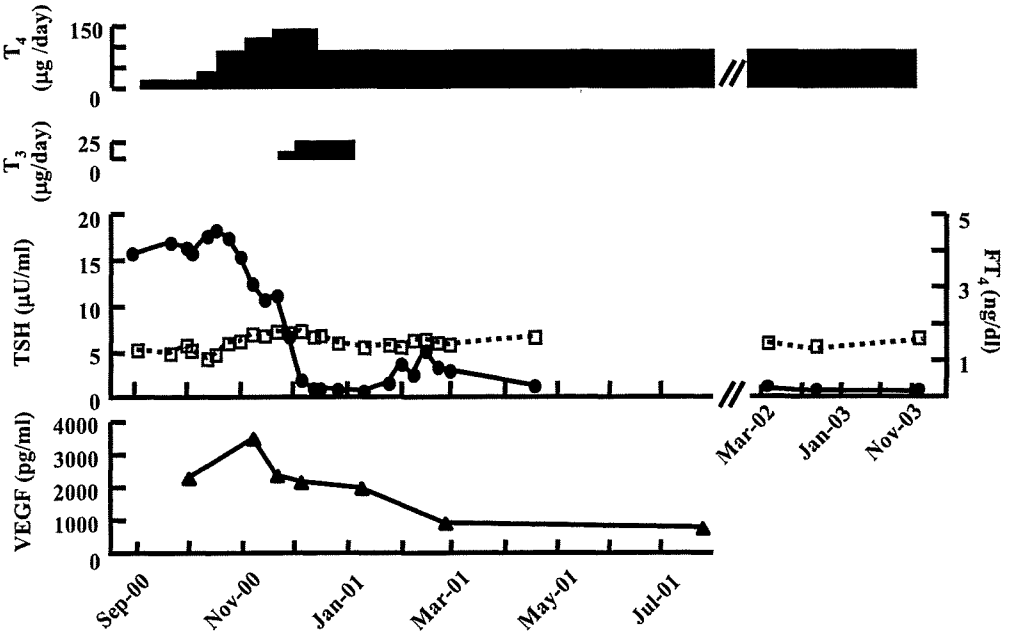

Fig. 3. Changes in TSH (●), FT_4 (□) and VEGF (▲) concentrations over time. Thyroid hormone treatment is indicated by the upper graph.

Table 3. Changes in TSH, gonadotropin, E2 and cytokines

	Oct 2000	Nov	Dec	Jan 2001	Feb	Jul	Reference interval
TSH (μU/ml)	15.4	12.0	0.25	0.04	2.7		0.41–4.0
LH (mIU/ml)	0.83		37.0	28.9		30.0	>4.2
FSH (mIU/ml)	10.6		49.0	47.0		78.0	>12.6
E2 (pg/ml)	55	42	19	<16		<16	<16
VEGF (pg/ml)	2289.9	3492.6		1974.0	896.6	754.8	62–707
IL-6 (pg/ml)	7.3			5.7		1.6	<4.0

creased to 150 μg/day. Because her TSH had not been normalized by 150 μg/day T_4, liothyroninesodium (T_3) was added at a dose of 8.3 μg/day in addition to T_4 from November and then gradually increased to 25 μg/day. In December, her TSH moved into normal range, and thereafter TSH and thyroid hormone levels were maintained within normal range at a dose of only 100 μg/day T_4 (Fig. 3).

Pleural effusions and peripheral edema were remarkably improved after the decrease of TSH (Fig. 1B, 2B). In addition, the abnormality of LH, FSH and E2 were ameliorated following the improvement of thyroid function (Table 3). Similarly, VEGF and IL-6 levels were decreased. Almost all symptoms and signs were improved after thyroid hormone replacement therapy. Her clinical condition has remained stable for several years.

Discussion

The biochemical state characterized by an elevated serum TSH level with a concomitant normal FT_4 level has received a variety of designations, including mild thyroid failure, as well as compensated, early, latent, mild, minimally symptomatic, and preclinical hypothyroidism [2]. The most widely applied designation for this biochemical state is subclinical hypothyroidism, despite the fact that the meaning is somewhat ambiguous [1–3]. Although it is commonly believed that subclinical hypothyroidism usually represents mild clinical signs, our patient had severe clinical manifestations such as marked pleural effusions and peripheral edema. Pleural effusions have been reported to be associated with the duration rather than the degree of biochemical hypothyroidism [6, 19]. Furthermore, tissue hypothyroidism at the peripheral target organs may be different in the individual patient. Zulewski *et al.* showed that some patients with severe biochemical hypothyroidism had only mild clinical signs, whereas other patients with minor biochemical changes had quite severe clinical hypothyroidism [20]. On the other hand, although her thyroid function showed slightly elevated TSH, she needed 100 μg/day T_4 to maintain TSH within normal range, which is the dosage usually used for moderate to severe hypothyroid patients. TSH measurement has a high diagnostic accuracy for the early detection of primary hypothyroidism. However, TSH may be a poor measure of the clinical and metabolic severity of hypothyroidism, because TSH is maximally stimulated in the early stages of primary thyroid failure, with no further increase occurring with greater severity of hypothyroidism [4]. In addition, TSH levels are reduced in prolonged critical illness, compared with the acute situation [21]. These findings suggest that a major change in thyroid hormone set point regulation may occur. Our observation indicates that even biochemical subclinical hypothyroidism causes severe clinical manifestations and the judgment of severity in subclinical hypothyroidism should be guided by clinical presentation and not only by serum TSH concentration.

The accumulation of fluid in serous body cavities in hypothyroidism is frequently recognized, the most common sites being the pleural, peritoneal and pericardial cavities. It was reported that pleural effusions secondary to hypothyroidism were small, noninflammatory effusions that have characteristics between transdates and exudates [6] and are frequently associated with marked ascites [19]. The precise mechanism by which these effusions develop is not well known. Parving *et al.* demonstrated the combination of increased extravasations of plasma proteins and lack of a compensatory increase in lymph flow and protein return rate [22], and suggested this as the cause of exudates in serous cavities. Lange demonstrated that the capillary permeability increased with leakage of plasma proteins in state of hypothyroidism and that the permeability rapidly returned to normal with thyroid hormone therapy [7]. Recently, elevation of VEGF has been reported in POEMS syndrome, which often accompanies extravascular volume overload [14]. VEGF induces a rapid and reversible increase in vascular permeability, and it is thought that these functions may cause the development of clinical manifestations, including ascites, pleural effusion, peripheral edema and organomegaly in this syndrome. An association of edema with increased VEGF levels has also been demonstrated in other pathological conditions, such as ovarian hyperstimulation syndrome [15], preeclampsia [16], and diabetic patients with troglitazone treatment [17]. In our case, VEGF was elevated in the initial course of the episode and was then reduced with improvement of pleural effusions and peripheral edema after thyroid hormone replacement therapy. Therefore, it seems that VEGF has, at least partly, contributed as a primary pathogenic factor of pleural effusions and peripheral edema in our case, as well as in POEMS syndrome.

VEGF is known to be expressed in a number of normal adult tissues, including the kidney, lung, uterus, ovary, brain, heart, skin, pituitary gland, and macrophages [23]. It has been demonstrated *in vitro* that VEGF is produced by thyroid follicular epithelial cells in response to stimulation of the TSH receptor [24–27]. Secreted VEGF stimulates VEGF receptors (Flt family) on endothelial cells of thiouracil-fed rats in a TSH-dependent paracrine mechanism, leading to proliferation of endothelial cells and hypervascularity of the thyroid gland [24, 26]. Recently, Klein *et al.* demonstrated that recombinant human TSH (rhTSH) stimulation for 3 weeks induced local VEGF expression in normal human thyroid, which were grafted into nude mice [28]. Moreover, Iitaka *et al.* showed that serum VEGF levels are positively correlated with TSH levels in patients with Hashimoto's thyroiditis [29]. However, conflicting results have been reported both *in vitro* and *in vivo* [30–33]. Sorvillo *et al.* demonstrated that a short-term administration of rhTSH in patients induces a significant reduction in serum VEGF levels [31], whereas, Tuttle *et al.* did not observe any difference in serum VEGF levels in patients after short-term stimulation rhTSH [32]. Therefore, the duration of TSH stimulation *in vivo* may be critical in determining the response of VEGF production. Furthermore, Sorvillo *et al.* showed that TSH *in vivo* might be able to regulate VEGF production from many extrathyroidal tissues [31]. A possible explanation of this case is that VEGF might be increased by prolonged stimulation of TSH, and that it might then be produced from tissues other than the thyroid gland because her thyroid gland was atrophic. Secreted VEGF might subsequently stimulate VEGF receptors on endothelial cells, leading to increase in vascular permeability, and develop pleural effusions and peripheral edema.

In our patient, estrogen levels were increased in spite of being postmenopausal and decreased after thyroid hormone replacement. Thyroid hormone has been demonstrated to affect the clearance rate of estrogen and the peripheral aromatization of androgen [34, 35]. Longcope *et al.* demonstrated that hypothyroidism results in a decrease in the metabolic clearance rates of androstenedione (A) and estrone (E1), and an increase in peripheral aromatization of A to E1 [35]. The peripheral aromatization of androgens is a major source of estrogens in men and postmenopausal women, and an increase in peripheral aromatization might have led to the elevation of serum estrogen levels in our patient. An estrogen-responsive element in the promoter region of the gene for VEGF has been identified [36], that interacts with both ER-α and ER-β. Indeed, serum VEGF levels are higher in premenopausal women compared with postmenopausal women [37]. Furthermore, estrogen replacement therapy increased serum VEGF levels in postmenopausal women [37–39]. Therefore, increased estrogen, caused by hypothyroidism, also might have partially contributed to the elevation of serum VEGF levels in our patient.

In summary, we present a case of subclinical hypothyroidism with severe clinical manifestations. After thyroid hormone replacement therapy, elevated VEGF levels were decreased with improvement of pleural effusions and peripheral edema. Our observations indicate that even biochemical subclinical hypothyroidism causes severe clinical manifestations. Furthermore, these observations lead us to speculate that elevated VEGF may be a potential factor in the course of pathogenesis of pleural effusions and peripheral edema in hypothyroid patients.

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Ghrelin differentially modulates glucose-induced insulin secretion according to feeding status in sheep

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Abstract

The present study was conducted to investigate roles of ghrelin in glucose-induced insulin secretion in fasting- and meal-fed state in sheep. Castrated Suffolk rams were fed a maintenance diet of alfalfa hay cubes once a day. Hyperglycemic clamp (HGC) was carried out to examine glucose-induced insulin response from 48 to 53 h (fasting state) and from 3 to 8 h (meal-fed state) after feeding in Experiment 1 and 2 respectively. Total dose of 70 nmol/kg body weight of D-Lys3-GHRP6, a GH secretagogue receptor 1a (GHS-R1a) antagonist, was intravenously administered at 0, 60, and 120 min after the commencement of HGC. In the fasting state, the ghrelin antagonist significantly ($P<0{\cdot}01$) enhanced glucose-induced insulin secretion. In the meal-fed state, i.v. administration of synthetic ovine ghrelin (0·04 μg/kg body weight per min during HGC) significantly ($P<0{\cdot}05$) enhanced glucose-induced insulin secretion. D-Lys3-GHRP6 treatment suppressed ghrelin-induced enhancement of the insulin secretion. In conclusion, ghrelin has an inhibitory and stimulatory role in glucose-induced insulin secretion via GHS-R1a in fasting- and meal-fed state respectively.

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Introduction

Ghrelin is a novel peptide that acts on the growth hormone secretagogue receptor (GHS-R) in the pituitary and hypothalamus to stimulate GH secretion (Kojima *et al.* 1999, Takaya *et al.* 2000). In some species, there is evidence that ghrelin also stimulates food intake and reduces energy expenditure (Tschop *et al.* 2000, Nakazato *et al.* 2001, Wren *et al.* 2001).

Apart from these actions in the brain, ghrelin has been reported to have a dual role in the regulation of pancreatic insulin secretion. Some studies in rats show that ghrelin stimulates insulin secretion *in vivo* (Lee *et al.* 2002) and *in vitro* (Date *et al.* 2002, Adeghate & Ponery 2002). Others show that ghrelin inhibits insulin secretion from rat pancreatic islets in a dose- and glucose-dependent manner (Colombo *et al.* 2003) and from mouse islets in the presence of glucose (Reimer *et al.* 2003). GHS-R antagonist and immunoneutralization of endogenous ghrelin enhance glucose-induced insulin release from perfused rat pancreas (Dezaki *et al.* 2006). In humans, ghrelin has been shown to cause hyperglycemia by reducing insulin secretion (Broglio *et al.* 2001). These discrepancies among the effects of ghrelin on insulin secretion have not been examined.

On the other hand, blood ghrelin levels are affected by nutritional states. Plasma ghrelin levels are increased after fasting and reduced after feeding in humans (Ariyasu *et al.* 2001, Cummings *et al.* 2001, Shiiya *et al.* 2002). Lee *et al.* (2002) showed that a high-fat diet decreases plasma ghrelin levels, whereas a low-protein diet increases plasma ghrelin levels in rats. Therefore, ghrelin secretion may be enhanced under negative energy balance but inhibited under positive energy balance.

Overall, it appears that ghrelin may play an important role in glucose metabolism, through modulation of insulin secretion, but this could be dependent on whether the organisms are in negative or positive energy balance. We observed that plasma levels of ghrelin were inversely related with those of insulin around feeding in sheep (unpublished data). Furthermore, we have demonstrated that ghrelin infusion stimulates glucose-induced insulin secretion in meal-fed sheep (Takahashi *et al.* 2006). These observations led us to hypothesize that ghrelin regulates insulin secretion dependent on energy balance. In the present study, we have explored this hypothesis by examining the involvement of GHS-R1a in glucose-induced insulin secretion in fasting- and meal-fed sheep.

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Materials and Methods

Experimental animals and treatments

Twenty two-year-old neonatally castrated Suffolk rams of 51·4±0·3 kg were placed in metabolism cages and held at 20 °C ambient temperature under a 12 h light:12 h darkness cycle (0730–1930 h light:1930–0730 h darkness). The animals were fed a maintenance diet of alfalfa hay cubes at 0900 h each day for 10 days prior to the experimental period, with free access to water. Bilateral jugular venous cannulas were inserted one day prior to the experimentation and closed with two-way taps and filled with heparinized (40 U/ml) normal saline for infusion and blood sampling. The animals were divided into two groups ($n=4$ per group) in Experiment 1 and into three groups ($n=4$ per group) in Experiment 2.

In Experiment 1, hyperglycemic clamp (HGC; see below) was conducted in both groups from 48 to 53 h after the last feeding (fasting state), when plasma ghrelin levels reached plateau (Sugino *et al.* 2002). Ghrelin antagonist-treated group received a total dose of 70 nmol/kg body weight D-Lys3-GHRP-6 (Sigma) in normal saline via the right jugular cannula every 60 min from 0 to 120 min after the commencement of a glucose infusion via the contralateral cannula. The dose of D-Lys3-GHRP-6 was determined according to several reports (Fujino *et al.* 2003, Dezaki *et al.* 2004, Dong *et al.* 2006). The control group received saline vehicle alone. In order to determine physiological effects of ghrelin as far as possible, we avoided administering ghrelin to the fasting animals in which plasma ghrelin levels had reached plateau (2·0 ng/ml).

In Experiment 2, HGC was conducted in all groups from 3 to 8 h after feeding (meal-fed state) when plasma ghrelin levels were nadir (Sugino *et al.* 2002). Concomitantly, two ghrelin-treated groups received synthetic ovine ghrelin (Peptide Institute Inc., Osaka, Japan) in saline (0·9% NaCl, 0·1% sheep serum albumin) at a rate of 0·04 μg/kg body weight per min through the left jugular cannula. The control group received saline vehicle alone. The ghrelin plus antagonist-treated group received a total dose of 70 nmol/kg body weight D-Lys3-GHRP-6 in normal saline (or saline vehicle alone) via the right jugular cannula every 60 min from 0 to 120 min after the commencement of a glucose infusion via the contralateral cannula. In order to determine physiological effects of ghrelin as far as possible, we avoided administering the antagonist alone to the fed animals in which plasma ghrelin levels had reached nadir (0·5 ng/ml).

Blood samples were collected through the right cannula, immediately placed into a heparinized tube with aprotinin (1000 KIU/ml of blood) and centrifuged for 10 min at 4 °C. Harvested plasma was stored at −80 °C until assay.

Hyperglycemic clamp

The HGC technique was used to determine insulin responsiveness to glucose. Glucose solution was prepared at 20% (wt/vol). Basal glucose concentrations were determined three times at 10-min interval before glucose infusion. In the HGC, blood glucose levels were raised to the desired hyperglycemia (100 mg/100 ml higher than the basal blood glucose) and were maintained at that plateau by infusing the glucose solution via the right cannula with a peristaltic pump (Mode AC-2120, Atto Co. Ltd, Tokyo, Japan). Blood glucose levels were measured with a glucose analyzer (GLU-1, TOA Electronics Ltd, Tokyo, Japan) at 5-min intervals throughout the experiment, and glucose infusion rate was empirically determined.

Time-resolved fluoro-immunoassay of plasma ghrelin, insulin, and GH

Ghrelin An assay for bioactive ghrelin was done as described previously (Sugino *et al.* 2002). The ghrelin concentration was measured by competitive solid-phase immunoassay using Europium (Eu)-labeled synthetic rat ghrelin and polystyrene microtiter strips (Nalge Nunc Int., Tokyo, Japan) coated with anti-rabbit γ-globulin. Intra- and inter-assay coefficients of variation were 6·9 and 5·5% respectively. Least detectable dose and IC50 in this assay system were 0·025 and 0·831 ng/ml respectively.

Insulin Insulin assay was done as described previously (Takahashi *et al.* 2006). The insulin concentration was measured by competitive solid-phase immunoassay using Europium (Eu)-labeled synthetic bovine insulin and polystyrene microtiter strips (Nalge Nunc Int.) coated with anti-guinea pig γ-globulin. The anti-human insulin was kindly supplied by Dr. K. Wakabayashi (Biosignal Research Center, Institute for Molecular and Cellular Regulation, Gunma University). Intra- and inter-assay coefficients of variation were 3·2 and 3·1% respectively. Least detectable dose and IC50 in this assay system were 0·016 and 1·073 ng/ml respectively.

GH GH assay was done as described previously (Sugino *et al.* 2002). The GH concentration was measured by competitive solid-phase immunoassay using Europium (Eu)-labeled synthetic ovine GH and polystyrene microtiter strips (Nalge Nunc Int.) coated with anti-rabbit γ-globulin. Intra- and inter-assay coefficients of variation were 4·1 and 9·3% respectively. Least detectable dose and IC50 in this assay system were 0·158 and 8·738 ng/ml respectively.

Statistical analysis

The values of plasma ghrelin, insulin and GH concentrations, and glucose infusion rates were expressed as means±S.E.M. Repeated measures of two-way ANOVA was used to evaluate statistical significance of treatment effects on each parameter over time. Statistical comparisons for glucose, ghrelin, GH and insulin among the treatments at each time point was evaluated using the *post hoc* Fisher's test.

Results

In the HGC (Experiments 1 and 2), plasma glucose concentrations were clamped at 100 mg/100 ml above the initial level between 60 and 300 min after the start of glucose infusion (Figs 1a and 3a). There was no difference in basal plasma glucose concentrations before glucose infusion between the fasting (Fig. 1a) and meal-fed state (Fig. 3a).

In the fasting state (Experiment 1), average plasma ghrelin level was 1·8 ng/ml before glucose infusion (Fig. 1b). Plasma ghrelin levels were significantly ($P<0·01$) decreased after the commencement of glucose infusion in both groups (Fig. 1b). Plasma GH levels were significantly ($P<0·05$) increased after glucose infusion in both groups (Fig. 1c). Plasma ghrelin and GH levels were not affected by the ghrelin antagonist (Fig. 1b and c).

Changes in plasma insulin levels in the fasting state are presented in Fig. 2. Plasma insulin levels were significantly ($P<0·01$) increased by glucose infusion in both groups. There was a greater incremental increase ($P<0·01$) in plasma insulin concentrations in the D-Lys3-GHRP6 group when compared with the control beginning about 60 min after the third administration of the antagonist.

In the meal-fed state (Experiment 2), average plasma ghrelin level was 0·5 ng/ml before glucose infusion (Fig. 3b). Plasma ghrelin levels significantly ($P<0·01$) increased and reached a plateau within 10 min after the commencement of ghrelin infusion (Fig. 3b). There were temporal increases ($P<0·05$) in plasma GH levels during ghrelin infusion (Fig. 3c). Increase in plasma GH levels was significantly ($P<0·01$) depressed by the ghrelin antagonist between the first- and second administration of the ghrelin antagonist (Fig. 3c).

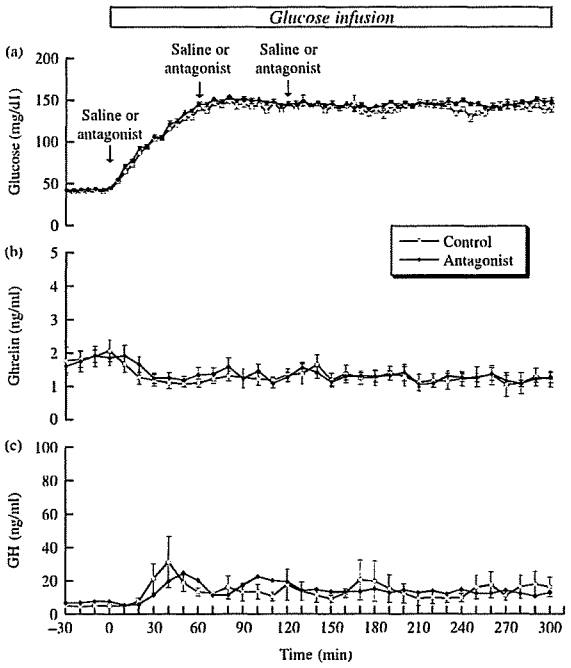

Figure 1 (a) Average glucose, (b) ghrelin, and (c) GH plasma concentrations in fasted sheep receiving saline (control) or D-lys3-GHRP-6 (antagonist, total dose of 70 nmol/kg body weight) every 60 min during the first half of hyperglycemic clamp.Values are means±S.E.M. ($n=4$).

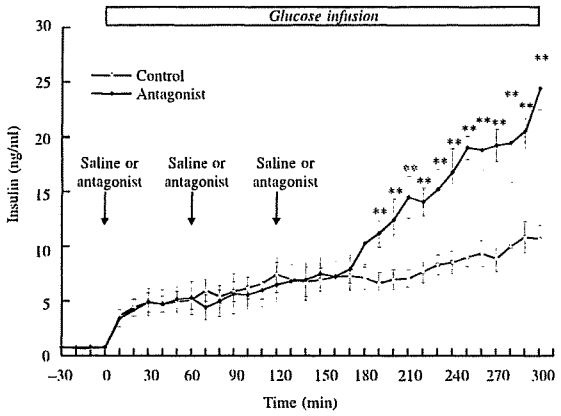

Figure 2 Average insulin plasma concentrations in fasted sheep receiving saline (control) or D-lys3-GHRP-6 (antagonist, total dose of 70 nmol/kg body weight) every 60 min during the first half of hyperglycemic clamp.Values are means±S.E.M. ($n=4$). $^{**}P<0·01$ versus control.

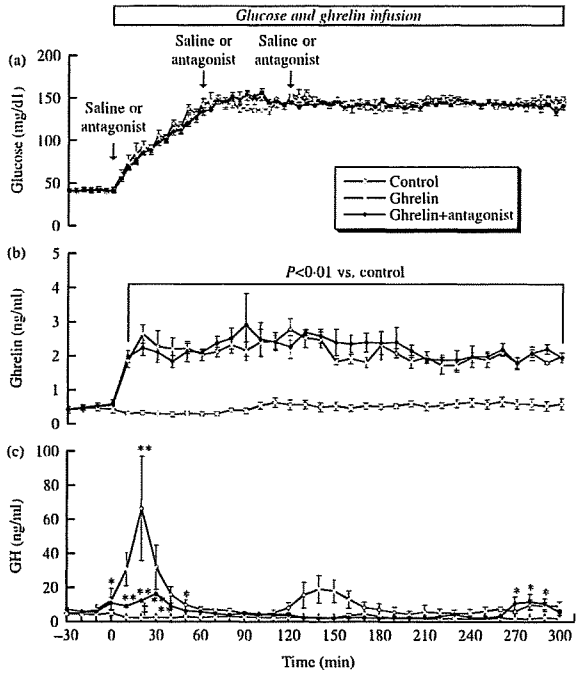

Figure 3 (a) Average glucose, (b) ghrelin, and (c) GH plasma concentrations in meal-fed sheep continuously receiving saline (control) or ghrelin (ghrelin, 0·04 μg/kg body weight per min) during hyperglycemic clamp. Saline vehicle or D-lys3-GHRP-6 (antagonist, total dose of 70 nmol/kg body weight) was administered every 60 min during the first half of hyperglycemic clamp.Values are means±S.E.M. ($n=4$). $^{*}P<0·05$ versus control, $^{**}P<0·01$ versus control, $^{‡}P<0·01$ versus ghrelin.