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Genetic control of the circulating concentration of transforming growth factor type β1

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The concentration of transforming growth factor β (TGF-β) in plasma has been correlated with the development of several diseases, including atherosclerosis and certain forms of cancer. However, the mechanisms that control the concentration of TGF-β in plasma are poorly understood. In a study of 170 pairs of female twins (average age 57.7 years) we show that the concentration of active plus acid-activatable latent TGF-β1 [(a+l) TGF-β∴ is predominantly under genetic control (heritability estimate 0.54). Single strand conformation polymorphism (SSCP) mapping of the TGF-β1 gene promoter has identified two single base substitution polymorphisms. The two polymorphisms (G→A at position –800 bp and C→T at position –509 bp) are in linkage disequilibrium (correlation coefficient Δ = 0.215, *P* < 0.01). The C–509T polymorphism is significantly associated with the plasma concentration of (a+l) TGF-β1, explaining 8.2% of the additive genetic variance of (a+l) TGF-β1 concentration. It is therefore possible that predisposition to atherosclerosis, bone diseases or various forms of cancer may be correlated with the presence of particular alleles at the *TGFB1* locus.

INTRODUCTION

TGF-β is a multifunctional cytokine which regulates the proliferation and differentiation of a wide variety of cell types *in vitro*. Recently, pathological misregulation of the TGF-β pathway has been implicated in the development of several major disease groups, including cancer (1–5), atherosclerosis (6–8), fibrotic disease (9) and auto-immune disease (10).

Despite these correlations between plasma TGF-β concentrations and disease, the mechanisms which control the concentration of TGF-β in circulating plasma are poorly understood. In a previous study no correlations were identified between the concentration of TGF-β in plasma and any environmental parameters, such as age, body mass index or drugs taken (7). We have therefore examined whether there is genetic regulation of plasma TGF-β concentration. TGF-β in man is derived from three unlinked genetic loci, *TGFB1*, *TGFB2* and *TGFB3*, which encode three protein isoforms, TGF-β1, β2 and β3.

Studies using isoform-specific ELISA assays have demonstrated that human blood contains <0.2 ng/ml TGF-β2 (11) and TGF-β3 has been detected in platelet-poor plasma and serum from only ~20% of individuals tested (D.J.Grainger, unpublished observations). The majority of the TGF-β present in blood from most individuals is therefore the TGF-β1 isoform. TGF-β is secreted as a latent proprotein, in which the propeptide (termed LAP) remains non-covalently associated with the active 25 kDa dimer. This latent TGF-β complex can be activated either by acidification *in vitro* (hence the term 'acid-activatable latent TGF-β', to distinguish it from platelet forms of TGF-β which are not activated by acidification in the absence of urea) or by further proteolytic cleavage of the LAP propeptide. To determine the genetic contribution to control of plasma levels of TGF-β1, an ELISA was used which detects both active and acid-activatable latent TGF-β [termed (a+l) TGF-β] (12,13). The classical twin study by which the correlation within monozygous twins (MZ) is compared with the correlation in dizygous twins (DZ) was used to determine the genetic contribution to plasma levels and to quantify the contribution of candidate genes to the genetic variation using the variance components modelling approach.

RESULTS

The concentration of (a+l) TGF-β1 was determined by ELISA (see Materials and Methods) in serum samples from 170 pairs of twins (84 MZ and 86 DZ pairs). The baseline characteristics of these twins are shown in Table 1. The MZ twins were slightly older than the DZ twins (mean age 58.7 versus 56.6 years) and a significantly higher proportion were post-menopausal (87 versus 75%). However, the concentration of (a+l) TGF-β1 was not correlated with age nor differed significantly between pre- and post-menopausal subjects. After normalization of the dataset by logarithmic transformation, the intra-class correlation coefficient for (a+l) TGF-β1 [*r*MZ = 0.58, *r*DZ = 0.22] was indicative of a substantial genetic effect on circulating TGF-β1 levels.

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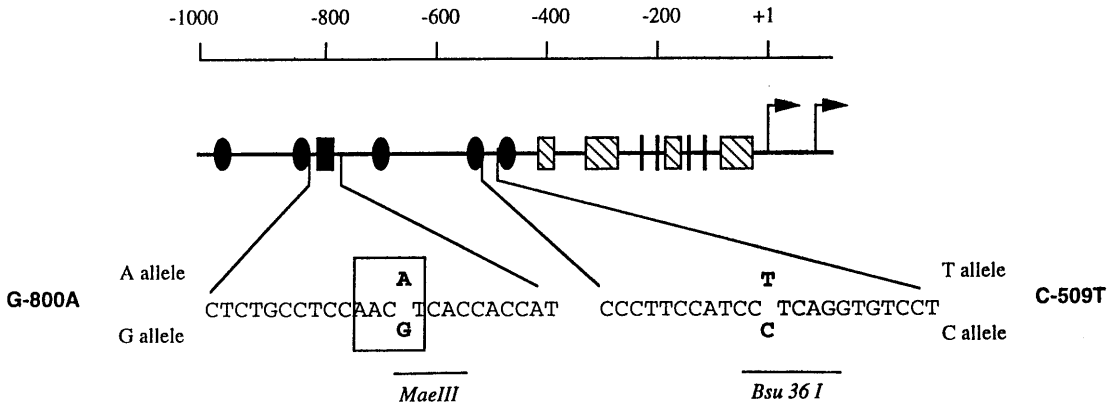

Figure 1. Location of polymorphisms in the *TGFB1* promoter. Diagramatic representation of the putative transcription factor binding sites in the *TGFB1* promoter. Hatched box, one or more consensus sp1 binding sites; vertical line, consensus ap2 binding site; stippled oval, consensus nuclear hormone binding element; filled box, consensus CREB half-site. The sequence of the CREB half-site is shown (boxed) with the single base polymorphism at –800 bp marked in bold script. The *Mae*III consensus sequence present in the G allele (GTNAC) is underlined. The sequence surrounding the single base polymorphism at –509 bp (bold) is also shown, with the *Bsu*36I consesus sequence present in the C allele (CCTNAGG) underlined. All nucleotide positions are related to the 5′-most transcriptional start site of the *TGFB1* gene described in Kim *et al*. (14).

Table 1. Baseline characteristics of the population

	MZ (n = 168)	DZ (n = 172)	P-value
Age (years)	58.7 ± 6.6	56.6 ± 8.3	**0.006**
Height (cm)	160.2 ± 6.1	161.3 ± 5.9	0.078
Weight (kg)	62.9 ± 9.7	65.0 ± 11.0	0.063
No. (%) post-menopausal	131 (87)	112 (75)	**0.007**
No. (%) current smoker	24 (14)	33 (19)	0.325
No. (%) on no alcohol	23 (13)	15 (9)	0.121
No. (%) previous HRT use	32 (18)	31 (18)	0.889
No. (%) hysterectomy	35 (20)	37 (21)	0.791

Only age and the number of post-menopausal individuals differed significantly between the MZ and DZ groups (P-values in bold). None of the individuals studied was currently on hormone replacement therapy (HRT) or taking other hormonally active medications.

Twin model fitting was performed (see Materials and Methods) to estimate the contribution of genetic and environmental factors to the observed variance in TGF-β1 levels. Fitting of specific sub-models showed that the shared environment and genetic dominance components could be dropped from the model without significant deterioration in fit. Thus, a model including only additive genetic and unique environmental effects gave the most parsimonious explanation of the data. The heritability estimate from this model was 0.54 [95% confidence interval (CI): 0.39–0.66] for (a+l) TGF-β1.

Since most of the TGF-β in plasma is the TGF-β1 isoform we investigated whether mutations at the *TGFB1* locus influence the circulating concentration of TGF-β. Using SSCP analysis two polymorphisms were identified in the promoter region of the *TGFB1* gene within 1.5 kb upstream of the major transcriptional start site. Sequencing of the PCR fragments analysed by SSCP mapping identified two single base substitution polymorphisms. The first polymorphism occurred at –800 bp (from the 5′-most transcription start site), with adenine replacing guanine in the published genomic sequence (14) in ~10% of alleles (G–800A; Fig. 1). The second polymorphism was at –509 bp, with thymine replacing cytosine in ~30% of alleles (C–509T; Fig. 1). The genotype at these two sites in the *TGFB1* promoter region was determined for the majority of the individuals in the twin study. For the G–800A polymorphism we found 59/340 individuals were heterozygous (AG) and two individuals (an MZ twin pair) were homozygous AA, corresponding to an A allele frequency of 0.09 (95% CI: 0.06–0.11; Table 2). For C–509T, 152/322 individuals were heterozygous (CT) and 24/322 individuals were homozygous for thymine at this position (T allele frequency 0.31, 95% CI: 0.27–0.35).

Alleles at the two polymorphic sites were found to be in linkage disequilibrium (correlation coefficient $\Delta = 0.21$, $P < 0.01$). Only two of the estimated haplotypes have frequencies >10% (C-G and T-G, frequencies 60 and 31% respectively). The C-A haplotype is rare (9%) and the T-A haplotype was not observed. Since the two polymorphisms are physically close and the alleles at these sites are in linkage disequilibrium, further analysis of the effect of the polymorphisms on circulating concentration of TGF-β was restricted to the C–509T locus, which showed the stronger association with TGF-β concentrations. Constraining the mean (a+l) TGF-β1 concentrations to be equal across genotypes in the most parsimonious model led to a significant decrease in the quality of the fit ($\chi^2[2] = 13.47$, $P < 0.005$), implying a significant effect of the polymorphism on the phenotype. By comparing the non-standardized estimates of the additive genetic variance in the full model with that in the reduced model in which the means were set equal, the percentage of additive genetic variance explained by the genotype was derived (8.2%; Table 3). Estimates of genotype-specific mean (a+l) TGF-β1 concentrations (with 95% CIs) are shown in Table 4.

The results obtained from the twin modelling were confirmed by GEE (see Materials and Methods; Table 4). The presence of the T allele at –509 bp is associated with higher concentrations of (a+l) TGF-β1 and this increase in concentration is higher among individuals homozygous for T than in heterozygotes, suggesting a dose–response effect of the T allele on circulating concentrations of (a+l) TGF-β1.

Table 2. Genotype distribution of the G–800A and C–509T polymorphisms

	G–800A					C–509T				
	AA	AG	GG	Total	Allele frequency A	TT	CT	CC	Total	Allele frequency T
MZ[a]	2	32	134	168	0.11 CI (0.06, 0.16)	12	80	72	164	0.32 CI (0.27, 0.37)
DZ	–	27	145	172	0.08 CI (0.05, 0.11)	12	72	74	158	0.30 CI (0.25, 0.36)
All[b]	2	59	279	340	0.09 CI (0.06, 0.11)	24	112	110	322	0.31 CI (0.27, 0.35)

[a]Allele frequency in MZ is estimated by considering one of each pair of MZs since MZs have identical sets of genes.
[b]Allele frequency estimates for all the data (MZ and DZ) are computed by selecting one of each pair of MZs and all DZs.

Table 3. Non-standardized estimate of variance components in the most parsimonious (AE) models

Models	V_A[a]	V_E[b]	V_P[c]	$-2\ln L$	df
AE different means	0.3723	0.3156	0.6880	716.70	605
AE means equal	0.4057	0.3196	0.7253	730.17	607
	8.2%		$\chi^2[2] = 13.47$, $P < 0.005$		

The estimated variance components (calculated as described in Materials and Methods) are shown for the most parsimonious (AE) model, either in the full model allowing different genotype-specific means (AE different means) or in a reduced model with genotype-specific means constrained to be equal (AE means equal). The percentage of additive genetic variance explained by the C–509T polymorphism is estimated from the difference between V_A for the full and reduced models and the significance determined from the difference in $-2\ln L$, which is distributed as χ^2 (see Materials and Methods).
[a]V_A, variance of additive genetic effects.
[b]V_E, variance of specific environmental effects.
[c]V_P, total phenotypic variance.

Table 4. Estimates of genotype-specific means and 95% CI from the most parsimonious model

Genotype	(a+l) TGF-β		
	Mean	95% CI	*P*-value
CC	3.83	3.26–4.50	–
CT	5.06	4.33–5.91	0.039
TT	7.62	5.25–11.08	0.002

The mean and 95% CI for (a+l) TGF-β1 plasma concentration for each genotype at the C–509T locus is estimated from the most parsimonious (AE) full model (see Materials and Methods). The *P*-values are linear contrasts relative to the CC genotype derived using the GEE technique (see Materials and Methods).

DISCUSSION

Taken together, these results demonstrate a significant genetic contribution to the control of (a+l) TGF-β1 concentration in plasma. The most parsimonious model explaining the phenotypic variance in (a+l) TGF-β1 concentration includes only the additive genetic (A) and unique environmental (E) components. Using this model, we estimate the heritability of (a+l) TGF-β concentration in plasma to be ~54%.

We have demonstrated an association between the TGF-β1 promoter genotype and plasma concentration of (a+l) TGF-β1. However, we have too few discordant DZ twin pairs for either polymorphism to be able to determine whether the polymorphisms we observe are directly responsible for the differences in plasma TGF-β concentration or are in linkage disequilibrium with other, as yet unidentified, polymorphisms. However, we note that the G–800A polymorphism is in a consensus CREB half-site and the A allele would be expected to have reduced affinity for the CREB family of transcription factors (15), which have been implicated in regulating transcription of other members of the TGF-β gene family. Additionally, both polymorphisms lie several bases from consensus DR1 or DR5 nuclear hormone receptor binding sites (16), which is of interest since ligands for nuclear hormone receptors have been shown to regulate TGF-β production *in vitro* (17) and *in vivo* (8). Estimates based on a reduced model with the genotype-specific means constrained to be equal suggests that almost 10% of the genetic variance in (a+l) TGF-β1 observed in our population is attributable to the C–509T polymorphism. This conclusion is confirmed by regression analysis using the GEE, which estimates the mean (a+l) TGF-β concentration to be approximately twice as high in TT compared with CC homozygotes.

The presence of polymorphisms in the *TGFB1* locus [such as those described here and elsewhere (18,19)] may indicate predisposition to diseases that have been linked to circulating levels of TGF-β, including atherosclerosis (7) and some forms of cancer (1,3). Consistent with this hypothesis, Cambien *et al*. (18) report an association between a G→C substitution at codon 25 and risk of myocardial infarction. Paradoxically, the same allele showed association with a *lower* systolic blood pressure. Therefore, the authors favour a cautious interpretation of these apparently inconsistent results. Several other polymorphisms were investigated in the same study (G–800A, C–500T, C insertion at +72, T→C at codon 10 and C→T at codon 263), but they showed no association with myocardial infarction or blood pressure.

Langdahl *et al*. (18) report association between an infrequent polymorphism in intron 4 of the TGF-β1 gene (a C deletion 8 bp upstream of exon 5) and the occurrence of spinal fracture in osteoporotic patients. Recently, a preliminary report of a novel TGF-β1 polymorphism in intron 5 has shown possible linkage to and association with hip bone density in women (20).

The exact mechanisms by which misregulation of TGF-β might lead to disease is unclear and complex (reviewed in ref. 21). Moses and colleagues showed that local expression of a constitutively active TGF-β1 transgene prevented the development of mammary carcinoma induced either by transgenic overexpression of TGF-α or by the chemical carcinogen DMBA (4). However, it is clear that the role of TGF-β is more complex than simply suppressing tumour development. Studies from the groups of Akhurst and Balmain have demonstrated that while low levels of TGF-β1 staining is prognostic for a high risk of malignant conversion of benign tumours in the *p53* knockout mouse (2), TGF-β promotes progression of carcinoma cells from the squamous phenotype to the more aggressive spindle phenotype (22). Markowitz *et al*. (5) have identified somatic mutations of the TGF-β type II signalling receptor, which would be likely to render it non-functional, in human colon cancer biopsies. In contrast, Arteaga *et al*. (23) have shown that transfecting a transformed cell line with a construct expressing active TGF-β

rendered the cells more tumorigenic *in vivo* (24). Consistent with this observation, elevated levels of plasma TGF-β in patients with malignant prostatic tumours (3) and hepatocellular carcinoma (1) have been reported. It is likely that alterations in TGF-β expression play an important role in the development of cancer, but as yet it is unclear whether increased or decreased TGF-β activity (or perhaps both at different times during tumorigenesis) promotes the development of tumours (reviewed in ref. 21).

In studies on the role of TGF-β in atherogenesis, we have shown that mice expressing the apolipoprotein(a) transgene develop diet-induced lipid lesions resembling early human atherosclerotic plaques at sites in the vessel wall where TGF-β activity is locally depressed by high concentration of apolipoprotein(a) (6). It has also been shown that tamoxifen, which elevates TGF-β activity both in the vessel wall and serum of mice, prevents diet-induced lipid lesion formation in three different mouse models of atherosclerosis (8,25,26). Consistent with these observations we have shown that the concentration of active TGF-β is depressed 5-fold in individuals with severe coronary atherosclerosis compared with individuals with normal coronary arteries determined by angiography (7). Taken together, these studies suggest that decreased TGF-β activity, either in the vessel wall or in the circulation, may be an important step in the development of atherosclerosis (reviewed in ref. 21). Recently, the gene responsible for hereditary haemorrhagic telangiectasia type 1 (27) has been indentifed as endoglin (a TGF-β receptor protein), suggesting the likely importance of the TGF-β family for the maintenance of normal vessel wall structure (28).

In conclusion, we have shown that certain common polymorphisms influence blood levels of TGF-β1. As it is likely that TGF-β1 has a role in a number of common important diseases, predisposition to these conditions may be associated with alleles at the *TGFB1* locus.

MATERIALS AND METHODS

Subjects

All of the subjects were female (age range 39–70 years, mean 57.7 years) and the majority were post-menopausal. Twins were recruited following a national media campaign and were broadly representative of the normal UK population as described previously (29). None of the subjects was taking HRT or other hormonally active medications. Serum was prepared and stored for TGF-β1 analysis as described previously (7,12,13). Lymphocyte DNA was also prepared for each subject using the standard phenol extraction method and zygosity status was determined by questionnaire and multiplex fingerprinting.

TGF-β analysis

Active plus acid-activatable latent (a+l) TGF-β was measured using the BDA19 capture ELISA as described previously (7,13). A single determination of each sample was made. The intra-assay coefficient of variation of the assay used is 6.8% and the sensitivity (defined as 2 SD above the mean of 16 blank determinations) was ~0.1 ng/ml.

SSCP

PCR was used to amplify fragments of the promoter that were ~400 bp long. Aliquots of 1.0 μg of genomic DNA were added to a 20 μl reaction consisting of 1× Taq polymerase buffer (Pharmacia, St Albans, UK), 50 nmol each dNTP and 6.25 pmol forward primer. The reaction was heated to 95°C for 30 s and then held at 80°C while 5 μl containing 6.25 pmol reverse primer and 0.5 U Taq polymerase in 1× Taq polymerase buffer was added. Samples were amplified for 35 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min, followed by an extension period of 10 min at 72°C. Primers were 5′-CCCGGCTCCATTTC-CAGGTG-3′ and 5′-TGCTCTTGACCACTGTGCCA-3′ for the G–800A polymorphism, and 5′-CAGACTCTAGAGACTGT-CAG–3′ and 5′-GTCACCAGAGAAAGAGGAC-3′ for the C–509T polymorphism. PCR products were denatured with 0.2 M sodium hydroxide and 0.5 mM disodium EDTA at room temperature. Single-stranded DNA bands were resolved on a 20% acrylamide gel (Phastsystem, Pharmacia; or XCell II system, Novex, San Diego, CA) and visualized by silver staining.

Genotyping

The G–800A polymorphism abolishes a *Mae*III restriction site. *Mae*III and *Mae*III buffer (Boehringer Mannheim, East Sussex, UK) were added directly to the PCR products to a final volume of 30 μl and incubated at 55°C for 5 h. Digests were resolved on a 1.2% agarose gel. C–509T polymorphism is in a *Bsu*36I restriction site. PCR products were precipitated with 3 vol of 96% ethanol and a 1/10 vol of 3 M sodium acetate, pH 5.2, at –20°C for 1 h, followed by centrifugation at 18 000 *g* for 10 min. The DNA was resuspended in a 20 μl *Bsu*36I digest containing 10 U *Bsu*36I (New England Biolabs, Hitchin, UK) and incubated at 37°C for a minimum of 12 h. Digests were resolved by 1.5% agarose gel electrophoresis.

Statistical analysis

Intra-class correlation coefficients (ICC) were used to measure the tendency of TGF-β levels in twin pairs to be more alike than two unrelated individuals, with a coefficient of 1 indicating complete concordance and 0 indicating no relationship. The F-ratio statistic of a one-way analysis of variance comparing variability between and within co-twins was used as a test of significance of the ICC. Calculation of ICC and one-way analysis of variance were performed after taking natural logs of the (a+l) TGF-β concentrations to obtain a more normal distribution.

Since parental genotype information was not available for any of the twins, haplotype frequencies for the sites, as well as linkage disequilibrium, were estimated by maximum likelihood using an expectation maximization algorithm on S-plus (30).

We used the twin model fitting approach to estimate the total influence of genetic factors, and tested for the effects of the C–509T polymorphism on (a+l) TGF-β1 concentration to quantify the contribution of this polymorphism to the total genetic variance (31). Model fitting has major advantages over the classic twin methodology (32,33), allowing more extensive separation of the observed phenotypic variance into additive (V_A) and dominant (V_D) genetic components as well as shared (V_C) and unique (V_E) environmental components. Model fitting was performed using Mx (31). Parameters were estimated by normal theory maximum likelihood, where the models were fitted to the raw data (31,34). A full model was specified which provided parameter estimates, as well as confidence intervals, for the variance components (A, D, C and E) and the genotype-specific means (CC, CT and TT). The significance of the variance components A, C and D was tested by dropping them in specific

sub-models, eventually leading to the most parsimonious fit to the data. Hierachical χ^2 tests were used to compare sub-models since the difference between minus twice the log-likelihood ($-2\ln L$) for a reduced model and that of the full model is approximately distributed as χ^2 with degrees of freedom (df) being the difference between the df for the full and reduced models.

The effect of the C–509T polymorphism on (a+l) TGF-β1 concentration was assessed by comparing the most parsimonious model, in which the means were estimated separately for each genotype, with the reduced model, in which the means were constrained to be equal across genotypes. This analysis of the effect of the C–509T polymorphism on plasma TGF-β concentration was validated using a regression analysis technique. As measurements on twins are not independent observations, we fitted the models using the generalized estimating equation (GEE) approach (35). The GEE procedure allows for dependency of observations in twin pairs and yields consistent estimators of the regression coefficients and the corresponding standard errors.

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