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SHORT REPORT

Promoter polymorphism influences the effect of dexamethasone on transcriptional activation of the LTC_4 synthase gene

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The molecular mechanisms of corticosteroid action in asthma are gradually being elucidated. The LTC4S gene encodes for LTC_4 synthase, the terminal enzyme in the generation of cysteinyl-leukotrienes (cys-LTs), which are key mediators in the pathogenesis of asthma. We have identified a novel promoter polymorphism in LTC4S at position −1072 (G/A) and a −444 (A/C) polymorphism has previously been reported. We hypothesised that the LTC4S gene promoter may be a potential site of regulation by corticosteroids and that genetic polymorphism may determine their effects at this locus. Using *in vitro* transfection of promoter–reporter constructs, dexamethasone was shown to increase transcription of LTC4S by more than 50% for the −1072G/−444A, A–C and G–C haplotype constructs ($P<0.02$), but to have no effect on the A–A haplotype ($P=0.27$). These data identify an interesting phenomenon that requires validation in a human study examining *ex vivo* production of LTC_4 in cells from genotyped asthmatic and nonasthmatic subjects. The 9% of the Caucasian asthmatic population with the A–A haplotype may have genetically predetermined lower cys-LT levels in the presence of corticosteroids compared to other patients. These findings have potential implications in the evaluation of combined corticosteroid and antileukotriene therapy in asthma.
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Introduction

Corticosteroids remain the most effective anti-inflammatory treatment for persistent asthma of all degrees of severity. Their mechanisms of action involve regulation of gene transcription and mRNA stability. The cysteinyl leukotrienes (cys-LTs) have been identified as important bronchoconstrictor mediators in asthma and corticosteroids have variable effects on leukotriene generation in blood leucocytes *in vitro*, but they do not appear to suppress cys-LT production *in vivo* in either normal or asthmatic subjects, even at significant systemic doses.[1,2] This may be the basis of the additional clinical benefit observed when oral leukotriene receptor antagonists (LTRA) are added to inhaled or oral corticosteroid therapy.[3] The failure of corticosteroids to suppress cys-LT synthesis *in vivo* may arise from paradoxical upregulation of the enzymes of the cys-LT synthetic pathway.[4,5]

The terminal enzyme in the production of cys-LTs is LTC_4 synthase. In a study of aspirin-intolerant asthmatics, levels of cys-LTs in bronchoalveolar lavage (BAL) fluid and bronchial responsiveness to aspirin challenge correlated exclusively with LTC_4 synthase immunoexpression in the bronchial wall.[6] This correlation may involve a transcriptional mechanism because of the presence of gene

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promoter polymorphism.[7] Factors including IL-5 and TGFβ have been shown to regulate LTC4S transcription and cys-LT levels *in vitro*, providing further evidence for the importance of transcriptional regulation at this locus in asthma. We and other workers have identified a correlation between asthma patient responses to LTRAs and patient genotypes at this locus.[8,9]

The leukotriene C_4 synthase (LTC4S) gene contains -1072 G/A[10] and -444 A/C[7] promoter polymorphisms. The LTC4S -444 A to C substitution is predicted to generate an H4TF-2 transcription factor binding site (GGTCC) and remove a glucocorticoid response element (GRE) (GGGACA), an NF-Elc (nnnnnTGATAnnnnn) and an NF-E site (CTGTC) (transfac/ transcription factor database (TFD)). The -1072 G to A polymorphism is predicted to generate an additional LF-A1 (GGGCA) and alpha-IFN-2 (AARKGA) site.

We hypothesised that LTC4S transcription may be differentially regulated in the presence of corticosteroids depending on which specific polymorphism haplotype was present.

Materials and methods

Subjects and genotyping

Ethical approval was obtained from the Southampton and SW Hants Joint Ethics Committee. Nonasthmatic Caucasian individuals were recruited from the Southampton area (mean age = 42.3 years, SD 10.6, $n = 174$). DNA was extracted from 10 ml whole blood using a Genomic DNA Maxi-Prep kit (Qiagen, Crawley, England). The -444 A/C polymorphism was genotyped using *Msp*I restriction fragment length polymorphism (RFLP)- microplate-array diagonal-gel assay (MADGE) as described previously.[11] The -1072 G/A polymorphism was genotyped using oligonucleotide ligation assay (OLA) essentially as described.[12] LTC4S haplotype frequencies were estimated using the 'Estimate Haplotype-frequencies (EH)' program.[13]

Plasmid construction

Fragments (1.3 kb) of the LTC4S promoter corresponding to -1359 to -55 including all reported transcription initiation sites were generated by PCR using primers that engineered *Mlu*I and *Bgl*II restriction sites at the 5′ and 3′ ends of the product, respectively. DNA previously sequenced was used to generate G–C, G–A and A–A haplotypes. PCR product was cloned into the pGL3 basic vector (Promega, Southampton, UK) using established molecular techniques. The A–C haplotype was engineered by replacing the '-444 A allele *Apa*I/*Nhe*I cassette' from the A–A construct with the C allele cassette from the G–C construct. All constructs were verified by sequencing the entire DNA insert.

Cell culture, transfection and dual luciferase assay

HeLa cells and KU812F cells were maintained as directed by ATCC. Cells were grown to 50–60% confluency, harvested, washed with D-PBS and resuspended at a density of 10^7 cells/ml in ice-cold D-PBS. Water, pGL3basic (–) negative, and pGL3-SV40 positive controls were included. 10 μg of pGL3 construct and 0.2 μg pRL-SV40 were combined with 0.8 ml cells. Cells were transiently transfected using electroporation (single pulse 300 V, 500 μF) using a GenePulser (BioRad, Hemel Hempstead, England). Each transfection was resuspended in 10 ml of the appropriate media with or without 10^{-6} M dexamethasone and grown for 24 h.

Cells were harvested and luciferase activity was determined using the Dual Luciferase Assay™ (DLA) (Promega), as described by the manufacturer, using a dual injector Lucy 1 Luminometer (LabTech, Andover, MA, USA). Luciferase activity was measured in triplicate and the mean of these values taken for analysis. Transfection efficiencies were normalised using cotransfection of pRL-SV40 and *Renilla* luciferase activity.

Promoter database analysis

Allele-specific transcription factor binding sites were identified using BioInformatics & Molecular Analysis Section and TFSearch (http://bimas.cit.nih.gov/molbio/signal/) (http://pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html).

Data analyses

Relative firefly luciferase activity was presented as fold increase over pGL3 basic control following normalisation to the pGL3-SV40 positive control level. The second analysis used firefly luciferase activity compared to the -1072 G/-444 A haplotype (designated 100%). Data represent the mean of triplicate values from independent experiments ($\pm$SEM). Differences in the luciferase activity for different constructs were compared by a Mann–Whitney *U*-test (Minitab Inc., State College, PA, USA). A *P*-value <0.05 was considered significant.

Results

We have previously reported that the genotype frequency of the -444C allele is 0.29 and that of the -1072A allele is 0.09 in the Caucasian population.[10] The inferred haplotype frequencies from nonrelated adult Caucasian volunteers are G–A 0.597754, G–C 0.313165, A–A 0.089027 and A–C 0.000053 ($n = 174$). Data were generated on the assumption that allelic association was allowed ($P = 0.045$). These data were representative of those previously observed in patients with asthma or nonasthmatic control subjects.[10]

Transient transfection followed by DLA™ did not identify a functional role for any combination of polymorphisms in determining basal transcription. However, an activation mechanism for the G–A, G–C and A–C haplotypes, but

not for the A–A haplotype, was observed in the presence of dexamethasone (Figure 1a). This was most prominent in the HeLa cell line, which resulted in a greater than 50% increase in transcriptional activity compared to basal levels ($P<0.02$, Figure 1a). For the A–A construct, transcriptional activity was not significantly different from basal levels ($P=0.272$, Figure 1a). When expressed as a percentage of the G–A haplotype, the level of transcriptional activity of the A–A haplotype in the presence of dexamethasone was $75.0\pm4.1\%$ ($n=7$). The 25% reduction in transcriptional activity compared to the 'wild-type' G–A haplotype was statistically significant ($P=0.01$, Figure 1b). Similar results were obtained when these experiments were repeated in the human basophilic cell line KU812F, with RLU values of: G–A 9.142 ± 0.786, A–A 7.849 ± 0.787, G–C 9.174 ± 0.293, and A–C 9.074 ± 0.482. The level of transcriptional activity of the A–A haplotype was $86.2\pm2.9\%$ of that in the G–A haplotype ($n=6$, $P=0.06$). A major effect of one of the two polymorphisms was not apparent following analysis of all combinations of haplotypes.

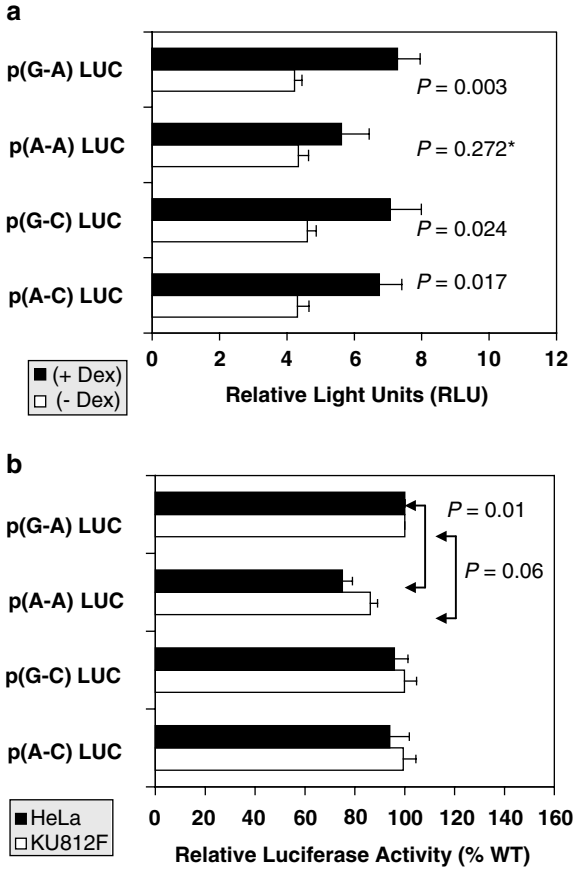

Figure 1 Transcriptional activation of the LTC4S promoter in the presence and absence dexamethasone. (Panel **a**) Mean luciferase activity of LTC4S constructs transfected into the epithelial HeLa cell line in the presence ($n=7$) and absence ($n=8$) of 10^{-6}M dexamethasone. (Panel **b**) Normalised luciferase activity of LTC4S constructs transfected into the HeLa and KU812F cell lines in the presence of dexamethasone. All data represent the mean of triplicate readings for independent experiments $\pm$SEM.

Discussion

This study demonstrates that corticosteroids upregulate transcriptional activity at the LTC4S locus in an *in vitro* gene-reporter system. We have identified that this transcriptional effect is modulated by the presence or absence of two promoter polymorphisms (−1072 G/A and −444 A/C), such that transcriptional upregulation of the LTC4S A–A haplotype by dexamethasone is less than that in other haplotypes. This haplotype is found at a frequency of 9% in the Caucasian population. The rationale of combining therapy with inhaled corticosteroids and oral LTRAs in asthma[3] is that corticosteroids fail to reduce leukotriene synthesis *in vivo*,[1,2] possibly because of a paradoxical upregulation of leukotriene biosynthetic enzymes including 5-LO, FLAP and LTC4S.[3,4] Our data allow the hypothesis that 9% of the asthmatic population may derive less additive benefit from the combination of corticosteroid and LTRA therapies because corticosteroids will fail to upregulate LTC4S activity in patients with the LTC4S A–A haplotype.

Previously, we have shown that the −1072 (G/A) and −444 (A/C) polymorphisms do not constitute genetic risk factors for the development of asthma and do not influence basal LTC4S transcription levels.[10] We did however identify a functional role for the −444 C allele in LTC_4 production by eosinophils from genotyped individuals following stimulation with calcium ionophore A23187 in the presence of indomethacin,[8] suggesting that gene–microenvironment interactions are important.

In the current study, the G–A, G–C and A–C haplotypes were shown to be upregulated in the presence of dexamethasone, while the A–A haplotype was upregulated but not to a statistically significant level compared to basal transcription. Differential numbers of glucocorticoid receptors and/or other cell-specific mechanisms may explain the differences in magnitude of these effects in the two cell lines. Dexamethasone was previously shown not to affect LTC4S mRNA production in the human HMC-1 cell line.[14] The improved sensitivity of promoter–reporter methods in the present study may explain this discrepancy.

Although the effects on LTC4S transcription that we have revealed are modest, small effects on gene transcription have been shown to affect dramatically physiological responses *in vivo*. Thus, in asthma patients, reductions of approximately 20% in reporter activity resulting from polymorphisms in the 5-lipoxygenase gene promoter controlling leukotriene production had a marked effect

on lung function (FEV_1) responses in a clinical trial of the 5-LO inhibitor ABT-761.[15]

The mechanism of differential regulation observed in the A–A haplotype does not support the hypothesis of a role for −444 A GRE in determining the response to dexamethasone because of the similar activity observed in the A–C, G–C and G–A constructs, suggesting a more complex mechanism. The true transcription complex can only be identified experimentally, although the role of transcription factors identified as allele specific at each polymorphic site and the role of corticosteroid-mediated transcription factors including AP-1 and NF-kB require investigation.

In the current study, we present evidence for a molecular mechanism in which polymorphisms in the gene encoding the terminal enzyme for cys-LT synthesis influence the effect of corticosteroids at that locus. These data have identified a specific role for the −444 and −1072 polymorphisms in a precise *in vitro* assay devoid of confounding factors associated with gene expression analysis from patients where other polymorphisms in the genome may influence the outcome measures. However, understanding the clinical significance of these observations requires a human-based study examining the effect of dexamethasone on *ex vivo* tissues/cells from genotyped individuals. Elucidation of the pharmacogenetic mechanisms influencing corticosteroid activity in asthma and their interaction with oral LTRAs will lead to a greater understanding of drug efficacy and to the better targeting of therapies to those patients most likely to gain clinical benefit.

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