Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2008.03674.x

$(GT)_n$ Dinucleotide repeat polymorphism of haem oxygenase-1 promotor region is not associated with inflammatory bowel disease risk or disease course

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Accepted for publication 20 March 2008
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Summary

Haem oxygenase-1 (HO-1) up-regulation was suggested to reduce mucosal tissue damage in inflammatory bowel disease (IBD) and an up-regulation of HO-1 expression in patients with Crohn's disease (CD) and ulcerative colitis (UC) was demonstrated. A HO-1 gene promoter microsatellite $(GT)_n$ dinucleotide repeat polymorphism was associated with regulation of HO-1 in response to inflammatory stimuli. We therefore hypothesized that IBD patients might segregate into phenotypes with high or low HO-1 inducibility. Ethylenediamine tetraacetic acid blood samples were obtained from 179 CD patients, 110 UC patients and 56 control patients without inflammation. Genomic DNA was purified and the 5′-flanking region of the HO-1 gene containing the $(GT)_n$ dinucleotide repeat was amplified. Polymerase chain reaction (PCR) products were purified and the length of the PCR fragments was analysed. The number of $(GT)_n$ repeats in the population studied ranged from 13 to 42. The distribution of the allele frequencies was comparable in patients and controls for both the short and the long alleles. The frequencies of short-, middle- and long-sized alleles were not changed among the groups studied. No correlation was found between IBD and microsatellite instability detected in five individuals. Our data indicate that $(GT)_n$ dinucleotide repeats of the *HO-1* promotor region have no significance for the pathophysiology and disease course of IBD.

Keywords: dinucleotide repeat polymorphism, haem oxygenase-1, IBD, promoter

Introduction

Haem oxygenase-1 (HO-1) has attracted attention because of its anti-inflammatory property. Regarding inflammatory bowel disease (IBD), HO-1 up-regulation has been suggested to ameliorate recovery by reducing mucosal tissue damage caused by an enduring exposure to reactive oxygen metabolites provoking oxidative stress [1]. HO-1 is the rate-limiting enzyme in the conversion of haem into the potential anti-oxidants biliverdin/bilirubin, iron and carbon monoxide [2–4]. An up-regulation of HO-1 expression in patients with Crohn's disease (CD) and ulcerative colitis (UC) could be demonstrated [5]. However, differences in the expression pattern of HO-1 could be detected only in IBD patients with severely inflamed mucosa *versus* diverticulitis and intestinal ischaemia as non-specific inflammatory controls. HO-1 is one of the major acute phase proteins that is up-regulated by a variety of inducers, such as endotoxin, hydrogen peroxide, prostaglandins and cytokines (interleukin-1, tumour necrosis factor), known to be detectable during IBD.

Several *in vivo* models support strongly that HO-1 provides a potent cytoprotective effect. HO-1 knock-out mice do not survive to term and mice that do survive to adulthood are abnormal and die within a year, demonstrating signs of chronic inflammation in numerous organs [6,7]. A preventive HO-1 up-regulation in the established model of acute dextran sulphate sodium (DSS)-induced colitis caused an anti-inflammatory effect [5]. However, no protective effect could be detected in a therapeutic approach when HO-1 up-regulation was induced after the onset of acute DSS-induced colitis or in chronic DSS-induced colitis. The protective role of HO-1 has been demonstrated indirectly in the acute model of trinitrobenzene sulphonic acid (TNBS)-induced colitis in rats [8]. In these experiments inhibition of HO-1 prior to the induction of colitis

by TNBS increased the colonic inflammation significantly. The production of free radicals and inducible nitric oxide sythase expression was up-regulated significantly, suggesting that HO-1 plays a role in attenuating experimental colitis.

In vitro experiments support that HO-1 provides a mechanism in the protection of epithelial cells from apoptosis. Epithelial cell apoptosis has been demonstrated in DSS-induced colitis and is accompanied by significant expression of the pro-apoptotic molecules Fas, Bax and p53 [9]. Induced up-regulation of HO-1 in a colonic carcinoma epithelial cell line (HT-29) resulted in the down-regulation of Fas-mediated caspase-3 activation [5]. Similar results were obtained with freshly isolated murine islets cells and a pancreatic cell line [9,10].

Thus, the currently available data suggest a protective effect of HO-1 induction and up-regulation of HO-1 expression in patients with inflamed intestinal mucosa. Interestingly, a HO-1 gene promoter microsatellite polymorphism was suggested to be associated with restenosis after angioplasty [11] and was identified as a vascular anti-inflammatory factor [12]. The $(GT)_n$ dinucleotide repeat polymorphism of the *HO-1* promotor region, that modulates the level of HO-1 inducibility, was investigated. Short GT repeats (< 25) were associated with highly significant up-regulation of HO-1 in response to inflammatory stimuli [11,12].

We hypothesized that patients may segregate into phenotypes with high and low HO-1 inducibility providing more or less anti-oxidants respectively. As HO-1 may present an anti-inflammatory factor regulating colonic inflammation, we investigated variants of the *HO-1* promotor region in 179 CD patients, 110 UC patients and 56 control patients without inflammation.

Materials and methods

Patients

Blood samples from age-matched individuals were obtained from 179 CD patients, 110 UC patients and 56 control patients without inflammation from the German IBD competence network serum bank (Core Facility, Regensburg). The study was approved by the University of Regensburg Ethics Committee.

DNA isolation

Genomic DNA was purified from human whole blood samples with the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Briefly, DNA was isolated following cell lysis and proteinase K digestion (56°C, 10 min). DNA was loaded onto a QIAamp spin column, washed twice and eluted in 200 µl distilled water.

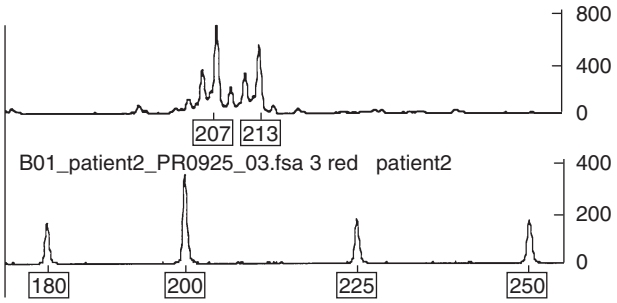

Fig. 1. Amplified region of the *HO-1* promotor containing the $(GT)_n$ dinucleotide repeat analysed by Genotyper® version 3·7. The signal strength is given as relative fluorescence in arbitrary units. The DNA standard (lower plot) allows reliable identification of the alleles from a patient with 207 and 213 base pairs according to 21 and 24 $(GT)_n$ dinucleotide repeats respectively (upper plot). The DNA standard is labelled with ROX; the amplified allele is carrying a 6-FAM fluorescent label.

Haem oxygenase-1 genotype assessment

The 5′-flanking region of the HO-1 gene containing the $(GT)_n$ dinucleotide repeat was amplified as described previously [13]. Briefly, a polymerase chain reaction (PCR) was performed using forward primer 1 µl 5′-CAG CTT TCT GGA ACC TTC TGG-3′ (5 µM), carrying a 6-FAM fluorescent label (MWG Biotech, Ebersberg, Germany) and downstream primer 1 µl 5′-GAA ACA AAG TCT GGC CAT AGG AC-3′ (5 µM). Genomic DNA and primers were mixed in a 50 µl reaction including 1 µl 2′-deoxynucleoside 5′-triphosphate (10 µM each; BD Biosciences Clontech, Heidelberg, Germany) and 1 µl Advantage™-HF2 polymerase mix (BD Biosciences Clontech) in the recommended buffer. After pretreatment at 94°C for 10 min the PCR comprised 30 cycles with denaturing at 94°C for 30 s, annealing at 60°C for 30 s, extension at 68°C for 30 s, and concluded with a extension step at 68°C for 30 min. The reactions were performed in a TRIO-Thermoblock (Biometra, Goettingen, Germany). PCR products were purified with the QIAquick PCR purification kit (Qiagen). Sequence analysis of the amplification product of an individual homozygous for the 211 base pair (bp) alleles showed correspondence with 23 $(GT)_n$ dinucleotide repeats (results not shown). Dinucleotide repeats were calculated as (PCR fragment length in bp − 165)/2 = numbers of $(GT)_n$. The length of the PCR fragments was analysed by the Medigenomix Corporation (Planegg, Germany; Fig. 1). The number of GT repeats was identified by sizing PCR products with the ABI 310 sequencer and Genescan Analysis 2·1 version software (Applied Biosystems, Foster City, CA, USA).

Montreal classification

Allele frequencies in different phenotypic disease presentations were analysed according to the Montreal classification

[14], as follows: age at diagnosis: A1 < 16 years; A2 16–40 years; A3 > 40 years; location: L1, terminal ileum; L2, colon; L3, ileo-colon; L4, upper gastrointestinal tract involved; behaviour: B1, non-stricturing non-penetrating; B2, stricturing; and B3, penetrating. Perianal involvement (P) was not determined.

(GT) repeat classification

To record the distribution of the GT repeats in the *HO-1* promotor region, sized PCR products were subdivided into three groups as described by Chen *et al.* [15]. The three allele subclasses according to the number of GT repeats were set as follows: short-sized alleles (S) with less than 23 repeats, middle-sized alleles (M) with 23–32 GT repeats and long-sized alleles (L) with 33–42 repeats.

Statistical analysis

For statistical analyses the SigmaPlot 2002 software package (version 8·02; Systat Software Inc., San Jose, CA, USA) was used. Percentages of allele frequencies are presented as box-plots graph data with the median, 25th and 75th percentiles. Whiskers above and below the box indicate the 90th and 10th percentiles. Differences between two data groups were calculated with Student's unpaired *t*-test and were considered significant at a *P*-value of < 0·05.

Results

Allele frequencies of the HO-1 $(GT)_n$ dinucleotide repeat polymorphism

The number of $(GT)_n$ dinucleotide repeats in the human *HO-1* promotor were analysed via length determination of a corresponding PCR fragment (Fig. 1). The number of repeats in the population studied ranged from 13 to 42 (Fig. 2). The distribution of the allele frequencies was comparable in patients and controls for both the short and long alleles. Allele frequencies of the HO-1 promotor $(GT)_n$ dinucleotide repeats were analysed in different disease phenotypes according to the Montreal classification. No significant association was found between $(GT)_n$ dinucleotide repeats and the groups of the Montreal classification.

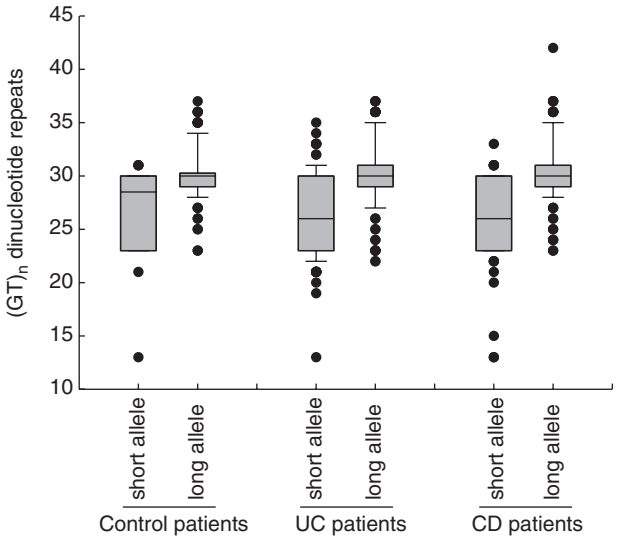

Fig. 2. Allele frequencies of the *HO-1* promotor $(GT)_n$ dinucleotide repeats (range 13–42).

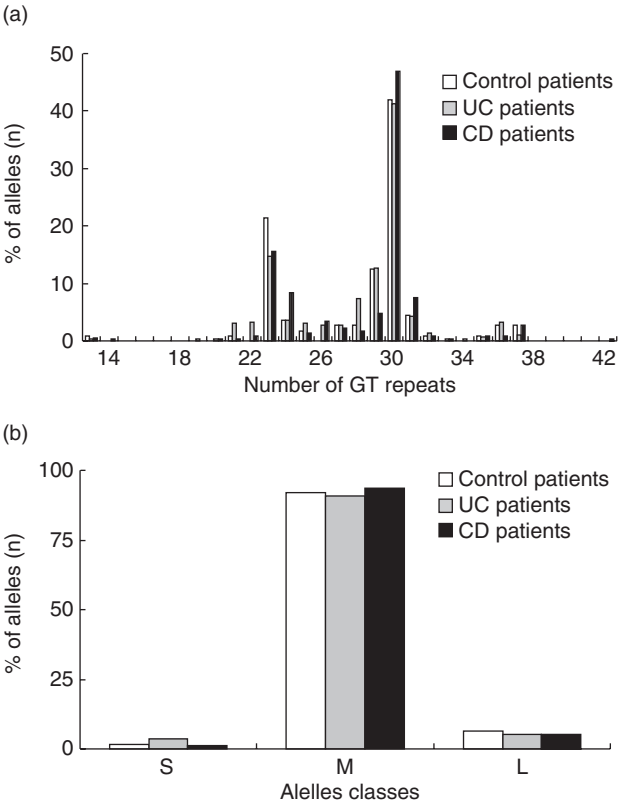

Fig. 3. Frequency distribution of HO-1 promotor $(GT)_n$ dinucleotide repeats. (a) Individual allele pattern; (b) distribution of short- (S), middle- (M) and long-sized (L) GT repeats. Control patients (white columns), UC patients (grey columns) and CD patients (black columns).

Allele frequency distribution

The distribution of GT repeats in the *HO-1* promotor region among the studied individuals is shown in Fig. 3a. The 23 and 30 GT repeats were the most common alleles. Three allele subclasses according to the number of GT repeats were set as S, M and L. The frequencies of different-sized alleles were compared between the groups studied (Fig. 3b). The middle-sized alleles are shown to appear more often but did not vary among the groups studied.

Microsatellite instability

Comparison of amplified DNA from blood samples of CD, UC and control patients allowed the identification of

microsatellite instability because of multiple PCR fragment lengths. Among the 179 CD patients analysed, four (2%) exhibited a microsatellite instability phenotype. The remaining 172 (98%) were classified as exhibiting a microsatellite stable phenotype. The number of GT repeats in the *HO-1* promotor region showed a distribution range from 15 to 31 (median 26·5 ± 3·9). This was not significantly different compared with stable phenotypes (median 28·5 ± 3·7). None of the 110 UC patients showed microsatellite instability. One control patient among the 56 tested (2%) displayed microsatellite instability (range 23–31, median 27 ± 2·7 compared with stable phenotypes 28 ± 3·9).

The frequencies of microsatellite instability were comparable in CD patients and controls. Individuals with microsatellite instability were excluded from allele frequency distribution analysis.

Discussion

In the present study we investigated $(GT)_n$ dinucleotide repeats of the *HO-1* promotor region in IBD patients to determine whether individuals segregate into phenotypes with high and low HO-1 inducibility. We found no relationship between the frequency distribution and IBD and a lack of influence on disease course. The distribution of the $(GT)_n$ dinucleotide repeats among studied groups is strikingly similar to previous findings [16] demonstrating the 23 and 30 GT repeats as the most common alleles. The range of GT repeats also found showed the expected allocation. Microsatellite instability was found but had no significance concerning IBD.

Lavrovsky *et al.* identified nuclear factor-kappa B (NF-κB) and AP-2-like binding sites upstream of the transcription start of the human *HO-1* gene and confirmed the binding of transcription factors by DNase I footprint analysis [17]. NF-κB is the main target of anti-inflammatory therapies in IBD and we could show the activation of this transcription factor during human mucosal inflammation *in situ* [18]. Accordingly, Paul *et al.* [5] could demonstrate an up-regulation of HO-1 expression in patients with CD and UC. Our data indicate that the different alleles of $(GT)_n$ repeats in the *HO-1* promotor region have no significance, at least with regard to IBD.

HO-1 is expressed widely in organs and tissues. It appears to be a novel protective factor with an indisputable potent anti-inflammatory and anti-oxidant effect. There has been increasing recognition of *HO-1* promoter polymorphisms leading to reduced HO-1 expression that may be associated with an increased risk of a variety of diseases. Longer $(GT)_n$ repeats were reported to be associated with higher susceptibility to the development of malignant tumours [19,20] or coronary artery atherosclerosis [15]. Contrary to the results for IBD presented in this work, certain other inflammatory diseases may be associated with the size of $(GT)_n$ repeats. Yasuda *et al.* [21] found an association of long-sized alleles with susceptibility to pneumonia in the older Japanese population. Yamada *et al.* [22] investigated the correlation between the length of the $(GT)_n$ repeats and susceptibility to the development of chronic pulmonary emphysema. He described that long-sized alleles may reduce HO-1 inducibility by reactive oxygen species in cigarette smoke, thereby resulting in the development of chronic pulmonary emphysema.

Acknowledgements

This study was supported by the Deutsche Forschungsgemeinschaft (Ro 1236/8-1) and the BMBF 'Kompetenznetz – Chronisch entzündliche Darmerkrankungen' (Core Facility, Regensburg).

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