A (G→C) transversion in the 3′ UTR of the human ECP (eosinophil cationic protein) gene correlates to the cellular content of ECP

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Abstract: Eosinophil cationic protein (ECP) is a cytotoxic protein produced by and secreted from human eosinophil granulocytes. ECP may be involved in the injury of epithelial cells in allergic diseases such as asthma. The objectives were to determine the prevalence of the ECP gene polymorphism 562(G>C) in apparently healthy subjects and subjects with allergy and relate the prevalence to clinical disease and to serum and cellular levels of ECP. The 562(G>C) ECP gene polymorphism was determined by gene sequencing of the ECP gene from DNA prepared from 163 apparently healthy subjects and 151 subjects with allergic and nonallergic asthma or other diseases. ECP was measured by a sensitive radioimmunoassay. A polymorphism was detected at position 562, which mapped to the 3′ untranslated region (UTR) of the gene encoding the ECP (RNase 3). Sixty-nine percent of the population had the 562GG genotype and 4%, the 562CC genotype. The cellular content of ECP in peripheral blood eosinophil granulocytes was significantly lower in cells from subjects with the 562GC (4.6±1.5 µg/10⁶ eosinophils) and 562CC (3.2±0.7 µg/10⁶ eosinophils) genotypes as compared with those with the 562GG genotype (6.0±1.9 µg/10⁶ eosinophils; P<0.001). A close link was found to the 434(G>C) ECP gene polymorphism. Associations between the 562(G>C) polymorphism and the 434(G>C) polymorphism was also found. The 562(G>C) polymorphism in the 3′-end of the UTR of the ECP gene may determine the ECP content in human eosinophils, but unlike the 434(G>C) polymorphism, the 562(G>C) polymorphism is not related to allergy J. Leukoc. Biol. 79: 846–851; 2006.

Key Words: gene polymorphism · allergy · granule

INTRODUCTION

The human eosinophils contain an abundance of basic proteins located in secretory granules. One of these granule proteins is the eosinophil cationic protein (ECP) [1], which is a truly multifunctional protein with cytotoxic and noncytotoxic capabilities. ECP belongs to the superfamily of RNases [2, 3], and indeed, ECP has some RNase activity, although it is weak as compared with its sister protein, eosinophil protein x/eosinophil-derived neurotoxin, with which it shares ~70% sequence homology [4, 5].

Recently, we and others showed the presence of single nucleotide polymorphisms in the ECP gene [6, 7]. Two of these alter the protein-coding region of the gene, and the others, the noncoding regions in the 3′- and 3′-ends. The 277(C>T) polymorphism gives rise to the replacement of arginine at position 47 with cysteine [8] and the 434(G>C) polymorphism, to the replacement of arginine at position 97 with threonine. The 277(C>T) polymorphism is fairly uncommon, and no disease relationships with this polymorphism have been described. The 434(G>C) polymorphism, conversely, was found closely related to allergy, as among those with 434CC, none had allergic symptoms, whereas among those with the 434GG genotype, we saw an over-representation of subjects with allergic symptoms [7]. The 434(G>C) polymorphism was also related to disease severity in Hodgkin’s lymphoma [8, 9]. Among the polymorphisms affecting the noncoding regions, the 562(G>C) polymorphism attracted our particular interest, as preliminary studies suggested a close association between this polymorphism and the 434(G>C) polymorphism described above.

In this report, we have investigated the relationship between the 562(G>C) and 434(G>C) polymorphisms further and also show that the 562(G>C) polymorphism is related to the content of ECP in peripheral blood eosinophils.

Subjects

Three groups of subjects were included in the study. One group included 139 medical students, 60 women and 79 men. The subjects were included after their written, informed consent. They were asked whether they considered themselves allergic or not. Ninety-nine subjects considered themselves nonallergic and 34 allergic; in six cases, no information was obtained. A second group was hospital employees (n=24), five men and 19

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PCR product was amplified by the 5 sequenc- ing quick start kit (P/N 608120). The complementary strand of the dideoxy chain termination method using the CEQ™ dye terminator cycle cation kit (Qiagen GmbH, Hilden, Germany) and sequenced in directions by A total of 100 ng DNA was used in a 50/H9262 DNA sequencing/H9262 Venous EDTA blood at a volume of 200/H9262 DNA preparation/H9262 Venous blood was drawn in blood collection tubes (Becton Dickinson Vacu- /H11032 granulocytes. Serum was obtained from venous blood collected in BD Vacu- /H11006 EDTA blood (100 μL), fresh or frozen, was diluted with 900 μL 0.9% (v/v) N- cetyl-N,N,N-trimethylammonium bromide (CTAB) in 0.15 M NaCl in 1.5 mL Eppendorf tubes and mixed on a cyclomixer [11]. Mixing was repeated after 30 and 60 min. After a total incubation time of 60 min, the samples were centrifuged at 600 g for 10 min at room temperature. The supernatant was removed, centrifuged, and removed again before stored at −20°C until assayed for ECP or EPO. The concentration of ECP in the blood samples was expressed as ECP per 10⁶ eosinophils after correction for the assumed average content in the neutrophils, which was set to 0.123 μg/10⁶ neutrophils [12]. The concentra- tion of EPO in the blood samples was expressed as EPO per 10⁶ eosinophils. In the calculation of the cellular content, subjects on per oral corticosteroids were excluded, as they showed significantly lower levels (P<0.05) of neutro- phil-corrected ECP per eosinophil than untreated subjects.

**Imprecision**

EDTA blood from one subject was suspended in 15 different Eppendorf tubes, after which, they were extracted as above. ECP and EPO in the extracts were analyzed in duplicates at one occasion. The variations in this experiment were 3.0% and 3.3% coefficient of variation, respectively.

**Immunoassays of ECP and EPO**

ECP, in whole blood extracts and serum, was analyzed by a double-antibody competition radioimmunoassay (Pharmacia Diagnostics AB, Uppsala, Sweden). EPO was assayed in whole blood extracts by a prototype fluoroimmunoassay (Pharmacia Diagnostics AB). Both assays were run according to the instructions of the manufacturer.

**Statistics**

In the calculations of P values for differences in proportions, the Difference test was used. In calculations of differences between more than two groups, ANOVA was used, and in the case of statistical differences, Student’s t-test was used to calculate differences between pairs of groups. Statistical differ- ences between groups were assumed for P values <0.05. The statistics were performed with the Statistica 6.0 software, Statsoft (Tulsa, OK).

**RESULTS**

The distribution of the 562(G>C) polymorphism was investigated in a group of apparently healthy students and hospital employees (n = 163). The distribution of the genotypes is shown in Figure 1. The 562CG genotype (G at position 562 on both chromosomes) was clearly the most prevalent and carried by 69% of the subjects as compared with 4% having the 562CC

**MATERIALS AND METHODS**

**Blood sampling**

Venous blood was drawn in blood collection tubes (Becton Dickinson Vacu- tainer Systems, Plymouth, UK) containing K² EDTA blood for DNA prepara- tion, blood cell count, and determination of the total amount of ECP in granulocytes. Serum was obtained from venous blood collected in BD Vacu- tainer™ SST™ serum separation tubes allowed to clot for 60 min at room temperature, followed by 10 min centrifugation at 1300 g. The supernatant was recentrifuged to ensure complete cell removal and then stored at –20°C until analysis.

**DNA preparation**

Venous EDTA blood at a volume of 200 μL was used, as has been described by Kawasaki [10], with minor modifications. The blood was mixed with 500 μL 10 mM Tris-HCl, 20 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 0.5% Tween 20, 100 μg/mL proteinase K and incubated at 56°C for 2 h. Subsequently, the samples were heated to 95°C for 10 min to inactivate the proteases. DNA concentration and purity were measured at 260 and 280 nm in a spectrophotometer, SPECTRAmax™ 250 (Molecular Devices, Sunnyvale, CA).

**DNA sequencing**

A total of 100 ng DNA was used in a 50-μL polymerase chain reaction (PCR) containing 1 U Taq DNA polymerase, buffer, and 1.5 mM MgCl₂ from Invitro- gen (Groningen, the Netherlands), 0.05 mM deoxynucleoside triphosphate, and 0.2 μM each primer. The primer pair used was the 5’ primer, 5’-aatggagc- ccctgtgtggtgca-3’ [base pairs (bp) 442–462], and the 3’ primer, 5’-gatgagga- ccctgtgtggtgca-3’ (bp 1119–1143), amplifying a 702-bp fragment according to GenBank Accession Number X16545. The PCR reactions were subjected to GenBank Accession Number X16545.

**Analysis of the 3’ UTR region of the ECP gene**

The 3’ UTR sequences of wild-type and 562(G>C) were compared using the Web-based tool transcription element search system (http://www.chil. upenn.edu/tess/).

**Cell counting**

Total blood cell counts in EDTA blood were analyzed by means of a Coulter STKS (Coulter Electronics, Hialeah, FL).

**Cellular content of ECP and eosinophil peroxidase (EPO)**

EDTA blood (100 μL), fresh or frozen, was diluted with 900 μL 0.9% (v/v) N- cetyl-N,N,N-trimethylammonium bromide (CTAB) in 0.15 M NaCl in 1.5 mL Eppendorf tubes and mixed on a cyclomixer [11]. Mixing was repeated after 30 and 60 min. After a total incubation time of 60 min, the samples were centrifuged at 600 g for 10 min at room temperature. The supernatant was removed, centrifuged, and removed again before stored at −20°C until assayed for ECP or EPO. The concentration of ECP in the blood samples was expressed as ECP per 10⁶ eosinophils after correction for the assumed average content in the neutrophils, which was set to 0.123 μg/10⁶ neutrophils [12]. The concentra- tion of EPO in the blood samples was expressed as EPO per 10⁶ eosinophils. In the calculation of the cellular content, subjects on per oral corticosteroids were excluded, as they showed significantly lower levels (P<0.05) of neutro- phil-corrected ECP per eosinophil than untreated subjects.

**Recovery**

A known amount of purified ECP or EPO was added to EDTA blood, after which, the mixture was extracted by CTAB as described above (sample a). As sample b, the same volume of buffer was added to the blood, and as sample c, the same amount of ECP or EPO was added to the assay buffer. Percent recovery of ECP or EPO in EDTA blood was calculated from the formula a/b + c × 100. The recovery of ECP varied between 96% and 104% (n=4) and the recovery of EPO, between 94% and 102% (n=3).

**Imprecision**

EDTA blood from one subject was suspended in 15 different Eppendorf tubes, after which, they were extracted as described above. ECP and EPO in the extracts were analyzed in duplicates at one occasion. The variations in this experiment were 3.0% and 3.3% coefficient of variation, respectively.

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The distribution of the 562(G>C) polymorphism was investigated in a group of apparently healthy students and hospital employees (n = 163). The distribution of the genotypes is shown in Figure 1. The 562CG genotype (G at position 562 on both chromosomes) was clearly the most prevalent and carried by 69% of the subjects as compared with 4% having the 562CC
genotype. No difference in genotype between the apparently healthy subjects and the diseased group was found. The prevalence of the 562 genotypes among subjects with or without allergy (n=299) showed no differences in the proportions, and 73% of the allergies had the 562GG genotype as compared with 65% of the nonallergic subjects. Among allergic asthmatics, 69% were 562GG, and 31% were 562GC, and none had the genotype 562CC; among nonallergic asthmatics, 59% were 562GG, 33% 562GC, and 8% 562CC. The prevalence of the 562 genotypes among those subjects having asthma, allergic or nonallergic, did not differ from those not having asthma.

We compared the prevalence of the 562(G>C) polymorphism and the 434(G>C) polymorphism as shown in Table 1. A close relationship between the two polymorphisms was found, as the 434GG genotype was only found together with the 562GG genotype, and the most heterozygous of one polymorphism was associated with most heterozygous of the other polymorphism. The 562GG genotype, however, may go together with any of the 434(G>C) genotypes, and the 562CC genotype always goes together with the 434CC genotype. The distribution of the haplotypes among the diseased population was similar to that of the healthy population (Table 1). As shown before [7], the 434(G>C) polymorphism is related to allergy, with a higher prevalence of the 434GG genotype among those with allergic asthma as compared with those with nonallergic asthma. We investigated whether the different haplotypes might have any relation to allergic disease. As expected from the previous study, none of the subjects with a haplotype, including the 434CC genotype, had allergic asthma. The distribution of the other haplotypes was not significantly different between allergic and nonallergic asthma. We also investigated the haplotype prevalence in relation to allergy or no allergy in the entire study group of apparently healthy and diseased subjects, but we found no statistical differences between those with or without allergy. In contrast, the prevalence of the 434(G>C) genotypes was related significantly to allergy ($P=0.017$, $\chi^2$-test).

The cellular content of ECP in peripheral blood eosinophils obtained from the diseased population and the laboratory employees in relation to the 434(G>C) and 562(G>C) polymorphisms is shown in Table 2. The results show that the content was significantly lower in cells taken from subjects with the

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**TABLE 1. The Prevalence of the ECP Polymorphisms 434(G>C) and 562(G>C) in a Healthy and Diseased Population**

<table>
<thead>
<tr>
<th></th>
<th>434GG</th>
<th>434GC</th>
<th>434CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>562GG</td>
<td>Controls 56%</td>
<td>Patients 57%</td>
<td>Controls 1%</td>
</tr>
<tr>
<td>562GC</td>
<td>None</td>
<td>Patients 29%</td>
<td>Controls 4%</td>
</tr>
<tr>
<td>562CC</td>
<td>None</td>
<td>None</td>
<td>Patients 2%</td>
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</table>

The results are presented as percent of the total number in each cohort. Controls, $n=163$; patients, $n=146$. 

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TABLE 2. The Cellular Content of ECP in Eosinophils in Relation to the ECP Polymorphisms 434(G>C) and 562(G>C; μg/10⁶ cells)

<table>
<thead>
<tr>
<th></th>
<th>434GG</th>
<th>434GC</th>
<th>434CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>562GG</td>
<td>5.9 ± 1.7 (95)</td>
<td>6.6 ± 3.0 (12)</td>
<td>6.0 (1)</td>
</tr>
<tr>
<td>562GC</td>
<td>NA</td>
<td>4.7 ± 1.5 (41)*</td>
<td>4.0 ± 1.0 (2)</td>
</tr>
<tr>
<td>562CC</td>
<td>NA</td>
<td>NA</td>
<td>3.2 ± 0.7 (2)</td>
</tr>
<tr>
<td></td>
<td>5.9 ± 1.7 (95)</td>
<td>5.1 ± 2.1 (53)**</td>
<td>4.0 ± 1.3 (5)**</td>
</tr>
</tbody>
</table>

The results are presented as means ± s0. Statistical differences were calculated by Student’s t-test. * P < 0.01; ** P < 0.001; *** P < 0.05. The content of the haplotype 434GC/562GG was compared with the haplotype 434GC/562GG; the content of the 434GC and 434CC genotypes was compared with the 434GG genotype; and the 562GC and 562CC genotypes with that of the 562GG genotype. Also, the content of the haplotype 434GG/562GG was compared with the haplotype 434GC/562GC and found to be significantly different, P < 0.001. The number of subjects within each group is shown in parenthesis. NA, Not applicable.

562GC or 562CC genotypes as compared with the 562GG genotype (P<0.001 and P<0.05, respectively). The content of ECP in cells obtained from subjects with the 434CC genotype was significantly lower than the content of those with the 434GG or 434GC genotype (P<0.05). It is also noted that among subjects with the 434GC genotype, those with the 562GG genotype had significantly higher content than those with the 562GC genotype (P=0.004). These data suggest that the content of ECP in eosinophils is related to the polymorphism 562(G>C). To investigate this further, we investigated the content of ECP in relation to the 434 and 562 genotypes in cells obtained from the apparently healthy students. As shown in Figure 2, a similar pattern was found with significantly lower contents in cells of subjects with the 562GC or 562CC genotype as compared with the 562GG genotype. It is also shown in the inset in Figure 2 that the ECP contents of eosinophils obtained from subjects carrying the 562GG genotype were on average 50% of the content in cells from subjects carrying the 562GG genotype and that the levels were similar, irrespective of population. We also investigated any relationship between the different genotypes and serum levels of ECP but could not find any such relationships (data not shown). For comparison, we analyzed the cellular content of another eosinophil granule protein, EPO, in the whole blood extracts. The average content of EPO was 10.7 ± 2.5 μg/10⁶ eosinophils (mean±s0, n=153), with no differences among the different ECP562 genotype populations (562GG=10.7±2.7, n=105; 562GC=10.5±1.9, n=46; 562CC=10.8±1.2 μg/10⁶ eosinophils, n=2, respectively).

Analysis of the 3’ UTR of the ECP gene showed that several transcription factor-binding sites were disrupted by the 562 polymorphism (Fig. 3). Thus, potential binding sites for transcription factors: RXR β, GTCTCT; PR, AGTCTC; and ATBP1/AT-BP2, AGTCCTCA [13], were lost by the polymorphism.

DISCUSSION

This study suggests that the intracellular content of ECP in peripheral blood eosinophils is influenced by the polymorphism 562(G>C) in the 3’ UTR region of the ECP gene. The polymorphism is only found together with the previously found allergy-related polymorphism, 434(G>C) [7], but it is interesting that it is not linked to allergy.

The linkage dependence between the 434(G>C) and 562(G>C) polymorphisms is not strictly bilateral, as the 562(G>C) polymorphisms may occur together with any of the
34(G>C) genotypes, whereas the 34GG genotype was only found together with the 562GG genotype. Conversely, however, the 562CC genotype was only found together with the 344CC genotype. This linkage pattern applies to the studied Scandinavian populations. Investigations are currently in progress to determine whether this linkage pattern is present in other ethnic populations.

Our finding of a close relationship between the cellular content and the 562(G>C) polymorphism is novel and of major interest, as this suggests a dependence of this region in the gene for the production of ECP. The mechanism behind this is at present not clear, but one possibility could be that this 3′ UTR region in the ECP gene contains a binding site for an enhancer element. We identified several potential transcription factor-binding sites, of which the interaction with the sequence could be prevented by the 562(G>C) polymorphism. Others have recently shown a relationship between serum ECP levels and a polymorphism in the noncoding 5′-end of the ECP gene containing the promoter region [14], but our demonstration of a possible enhancer response element in the noncoding 3′ end in the ECP gene is novel. Whether there is linkage between the promoter polymorphism and our 562(G>C) polymorphism in the noncoding 3′-end is not known but is under investigation. Response elements in the 3′ UTR region have been shown in other genes, such as the interleukin-12 gene, and have been related to increased expression of mRNA [15]. Previously, an intronic enhancer element for the transcription factor nuclear factor of activated T cells-1 was identified in the promoter region of the ECP gene [16]. The potential importance of the 3′ UTR region in regulation of gene expression has been emphasized recently [17, 18]. An alternative explanation could be alterations in the mRNA stability induced by the alternative G/C sequence [19].

In our studies about the cellular content of the protein, we used cells from several different populations. Overall, we did not find any obvious differences between the diseased population and the healthy employees. However, the cellular content of the healthy students was systematically lower than that of the two other groups, although the relative contents in relation to the 562(G>C) genotypes were identical. The explanation to the lower content is likely due to different procedures of extraction used, as the cells of the patient population and the healthy employees were extracted fresh, whereas the cells of the students were extracted after storage in the freezer for some time. The recommendation is therefore to extract cells before freeze-storage obviously results in a loss of the ECP antigen.

In a previous report, we showed that the 344(G>C) polymorphism in the coding region of the ECP gene is closely related to the expression of allergic symptoms [7]. Recently, we showed that this polymorphism affected the biological activity of ECP, as the protein coded for by the 344CC genotype had lost its cytoxic activity completely (to be published). This finding led us to speculate that the presence of a cytoxically active ECP is a prerequisite for the development of allergic symptoms, possibly by the injury of epithelial cells. Therefore, our findings of a relationship between the cellular content of ECP and the 562(G>C) polymorphism gave rise to the hypothesis that haplotypes of the 344 and 562 gene polymorphisms would show an even closer relationship to allergy development, as not only the function but also the availability of ECP might be of importance. This hypothesis, however, was not supported by our results, and the clinical consequences of the 562(G>C) polymorphism, if any, remain to be determined. In diseases heavily engaging the eosinophils and ECP, this polymorphism, however, might play a major role.

It is concluded that the 562(G>C) polymorphism in the ECP gene may be involved in the regulation of the production of ECP and that it is closely linked to the 344(G>C) polymorphism. Any clinical consequences of the polymorphism remain to be identified, as the polymorphism, unlike the 344(G>C) polymorphism, does not seem to be related to allergy.

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