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The Coding ECP 434(G>C) Gene Polymorphism Determines the Cytotoxicity of ECP but Has Minor Effects on Fibroblast-Mediated Gel Contraction and No Effect on RNase Activity[1] ⊘

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The Coding ECP 434(G>C) Gene Polymorphism Determines the Cytotoxicity of ECP but Has Minor Effects on Fibroblast-Mediated Gel Contraction and No Effect on RNase Activity[1]

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Eosinophil cationic protein (ECP) is a secretory protein of the eosinophil granulocyte, a cell involved in innate immunity. Functional studies have implicated ECP in numerous processes, such as tissue remodeling in allergic inflammation and cytotoxicity toward a variety of pathogens. Recent genetic studies have suggested that the ECP 434(G>C) polymorphism resulting in an arg97thr substitution would alter the function of ECP in vivo. Functional (in vitro) studies of ECP up until now have either been conducted with native preparations containing an unknown mixture of the ECP[97arg] and ECP[97thr] variants, or with recombinant proteins. Therefore, we have now for the first time extracted the native ECP[97arg] and ECP[97thr] variants from healthy blood donors and tested them functionally in vitro. Our results show that the arg97thr shift dramatically alters the cytotoxic capacity of ECP in vitro; the tested ECP[97arg] variants were cytotoxic toward the small-cell lung cancer cell line NCI-H69, whereas ECP[97thr] was noncytotoxic. RNase activity was unaffected by the arg97thr substitution. Both ECP[97arg] and ECP[97thr] stimulated fibroblast-mediated collagen gel contraction, an experimental model, which depicts wound healing, in a dose-dependent manner. In conclusion, our results demonstrate that the ECP 434(G>C) gene polymorphism affects the functional properties of native ECP, but also that there is a dissociation between different biological activities; the arg97thr substitution impairs the cytotoxic potential of ECP but less the gel contraction and not at all the RNase activity. *The Journal of Immunology*, 2009, 183: 445–451.

osinophil cationic protein (ECP)[3] is a basic eosinophilic protein stored in the secondary granules of the cell. ECP is also known as RNase 3; it has weak RNase activity and is a member of the RNase A superfamily (1). ECP has been shown to have a number of different functional properties and the toxic ability of this protein has been studied to a high extent, as reviewed by Boix et al. in Refs. 2 and 3. Several studies show that ECP efficiently kills several pathogens such as parasites (4, 5), but that the toxic properties of the protein may also lead to tissue damage within the host in conditions such as asthma (6).

The cytotoxic ability of ECP has been ascribed to its membrane disrupting capacity (7), which was shown to be dependent on both hydrophobic and cationic residues (8, 9).

ECP consists of a single polypeptide chain with a molecular mass of 15.5 kDa, but the native protein is a very heterogeneous molecule and the molecular weights range from 16 to 22 kDa as measured by gel electrophoresis (SDS-PAGE) (10). This is mainly depending on glycosylation of the protein; there are three potential sites for N-linked glycosylation (11) and several studies showed the presence of N-linked carbohydrates on ECP (12–14). In a recent study, we showed that these N-linked carbohydrate chains are in part made up of sialic acid, galactose, and N-acetylglucoseamine (15). Another reason for the heterogeneity of ECP is single nucleotide polymorphisms (SNPs) in the ECP encoding gene (*RNASE3*). Several SNPs have been identified in the human ECP gene (16) and some have been linked to function or cellular content of ECP. In a study by Jönsson et al. the 562(G>C) polymorphism in the 3′UTR region of the ECP gene correlated to the cellular levels of ECP (17). In another study the intron polymorphism −38(C>A) that is positioned in a putative GATA-1 binding site was correlated to serum ECP levels (18). The coding 434(G>C) polymorphism is located in exon 2 of the ECP gene and this SNP results in a substitution of arginine at position 97 in the mature protein to threonine (arg97thr) (16, 19). A study on recombinant ECP[97arg] and ECP[97thr] showed that the two variants of the protein differed significantly with respect to cytotoxic activity; rECP[97arg] displayed cytotoxic properties toward the mammalian small-cell lung cancer cell-line NCI-H69 whereas rECP[97thr] was noncytotoxic (20).

In a recent study, we showed a correlation of ECP 434(G>C) genotypes and infection with the *Schistosoma mansoni* parasite (21). Subjects with the GG genotype (ECP[97arg]) were found to be infected by the parasite to a lower extent, implicating a role for ECP in the primary defense reaction. Also, the infected subjects

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carrying the G-allele (ECP[97arg]) displayed a higher frequency of peri-portal fibrosis, suggesting a role for ECP, and in particular ECP with arginine at position 97, in the development of fibrosis. The involvement of ECP in fibrotic process was suggested in previous studies; incubation of fibroblasts in the presence of ECP results in an enhanced proteoglycan production by the fibroblasts (22), and also, ECP has been shown to augment fibroblast-mediated collagen-gel contraction in vitro (23) and to promote migration of fibroblasts (24) and TGF-β production (25). Activated eosinophils in the lung of asthmatics are proposed to release granule proteins such as ECP, leading to remodeling and epithelial damage (26).

Previously, in vitro studies of ECP function have either been conducted with native ECP preparations containing an unknown mixture of the ECP[97arg] and ECP[97thr] variants, or with recombinant ECP. Recombinantly produced proteins, whether produced by bacteria, yeast, or insect cells will differ from the native human proteins in terms of posttranslational modifications, such as glycosylation (27). And although sometimes small, these differences can greatly influence the function of the protein.

Because the only previous in vitro study of the arg97thr substitution of ECP was performed with recombinant protein, the aim of our study was to characterize the functional properties of native ECP[97arg] and ECP[97thr], with emphasis on cytotoxicity.

To accomplish this, ECP was purified from large numbers of subjects of the various 434(G>C) genotypes and then tested in three functional assays; RNase activity, cytotoxic activity toward a mammalian cell-line, and impact on tissue remodeling in a three-dimensional fibroblast-mediated collagen gel contraction assay.

Materials and Methods

DNA extraction

Buffy coats of deidentified healthy blood donors were supplied by the blood bank at the Academic Hospital in Uppsala, Sweden. Two hundred microliters was collected from each buffy coat and DNA was extracted using the QIAamp DNA blood mini kit (Qiagen) according to manufacturer's instructions.

ECP genotyping

DNA was genotyped for the ECP 434(G>C) polymorphism by TaqMan analysis as described in detail in Ref. 21. In brief, each 10 μl reaction contained 2 μl of genomic DNA, 900 nM of each primer, (forward) 5'-GCC TTT ACT CCA CTG TGA CCT CAT-3' and (reverse) 5'-TGC AAC TAC ATA GAA CCT CCT TCC T-3', 200 nM of each oligonucleotide probe labeled with fluorescent reporter dyes VIC and FAM, 5'-VIC-AAA CTG CAG GTA TGC AGA-3' and 5'-6-FAM-AAA CTG CAC GTA TGC AG-3', 1×Universal PCR Master Mix (Applied Biosystems), 200 μg/mL BSA (Sigma-Aldrich), and sterile water. Cycling took place in an ABI Prism 7000 and cycling conditions were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The genotypes were determined by allelic discrimination according to the ABI Prism 7000 SDS software.

Isolation of granules

ECP was purified from buffy coats of healthy blood donors using the method described by Peterson et al. in Ref. 28 with some modification. Buffy coats of the same ECP 434(G>C) genotype were pooled together and the pools of different genotypes were treated separately throughout the extraction and purification process. Buffy coats were mixed with an equal volume of 2% Dextran T-500 (GE Healthcare Biosciences) in NaCl/PBS (Invitrogen) in a measuring cylinder and the erythrocytes were left to sediment for 60 min in room temperature. The granulocyte-rich plasma was collected from the cylinders and the cells were washed twice with PBS and once with 0.34 M sucrose (Merck), with each wash centrifugation was performed for 10 min at 400 × g at room temperature. Finally, the cells were suspended in five times the volume of 0.34 M sucrose. Three hundred milliliter cell suspension was mixed with an equal amount of cold 0.34 M sucrose and was pressurized with N$_2$ in a nitrogen bomb (Parr Instrument Company) at 750 psi, 4°C with constant stirring for 30 min. The homogenate was then collected in 400 ml 0.34 M sucrose, 0.3 M NaCl and cen-

trifuged at 450 × g for 20 min in 4°C. The supernatant was collected and centrifuged for 20 min at 10,000 × g at 4°C. The pellet containing granules was collected and frozen at −70°C until further purification.

Extraction of granules

The granule preparations were thawed and five volumes of 50 mM HAc was added; extraction was performed at 4°C for 1 h with constant stirring. The double amount of 0.4 M NaAc (pH 4.0) was added and the granules were extracted for another 3 h at 4°C with constant stirring. The extract was centrifuged at 12,000 × g for 30 min at 4°C and the supernatant was collected. The granule extract was then concentrated to ~3 ml using YM-10 filters (Amicon Corporation).

ECP purification by gel filtration and ion exchange chromatography

Gel filtration chromatography was performed using the Sephadex G-75 superfine column (GE Healthcare Biosciences) calibrated with 0.2 M NaAc (pH 4.5). The eluted fractions from one granule extract were divided into ten pools, of which two contained ECP (as shown in Fig. 1A for GG-genotype donors) (ECP[97arg]). The first ECP-containing G-75 pool contained high m.w. ECP and the second contained low m.w. ECP (LMW-ECP). The eluted fractions were kept at −70°C until further purification.

The LMW-ECP pools were chosen for further purification because they have been shown to harbor the ECP with cytotoxic activity (20). Ion-exchange chromatography of the low m.w. ECP pools was performed using the ÄKTAprime system and a Mono-S column (GE Healthcare Biosciences). The column was equilibrated with 50 mM MES (Merck), 2% betaine (Sigma-Aldrich), 0.1 M LiCl (Merck) (pH 6.0), and the proteins were eluted with a linear gradient from 0.1 to 2.0 M LiCl (pH 6.0). Eluted fractions were pooled together according to the chromatograms in Fig. 1, B and C. As displayed in Fig. 1B, the low m.w. ECP from GG-genotype subjects (ECP[97arg]) generated four distinct pools, whereas the low m.w. ECP from GC-genotype subjects (ECP[97arg/thr]) generated five pools (Fig. 1C).

Protein determination and concentration

The purity of ECP in the pools was assessed by analysis on NuPAGE 10% Bis-Tris gels (Invitrogen) and the purity was >95% (Fig. 1, D and E). The concentration of ECP was determined by the absorbance at 280 nm using the extinction coefficient (E$^{1\%.1\ cm}$) of ECP of 15.45 (28) and by immunoassay in the UniCAP system (Phadia). ECP containing fractions were concentrated using YM-10 filters (Amicon) and buffer change was accomplished using a Sephadex G-25 column (GE Healthcare Biosciences) and the ÄKTAprime system (GE Healthcare Biosciences). Purified ECP was stored in 0.2 M NaAc buffer (pH 5.5) at −70°C.

Peptide mapping

The first pool from GC genotype donors corresponds in theory to the C-allele product and was selected for identification by peptide mapping. As a comparison, one pool from the GG-donors was also selected for identification. The two samples were enzymatically deglycosylated as described in Ref. 15 by the enzymes of the E-DEGLY kit (Sigma-Aldrich) and were then run on a NuPAGE 10% Bis-Tris gel (Invitrogen) at reducing conditions. Bands were cut from the gel and proteins in the bands were reduced and alkylated with iodoacetamide. The alkylated proteins were digested with trypsin (Promega modified porcine trypsin). Digests were analyzed by MALDI-TOF using an ultraflex Tof/Tof instrument, (Bruker Daltonics). Peptide maps were compared with theoretical tryptic peptide maps of ECP. The intensity of m/z corresponding to tryptic peptides different in the two ECP variants was used for estimation of cross contamination.

Affinity capture of ECP by surface enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF MS)

The nine low m.w. ECP pools from GG-and GC donors were analyzed by the ECP affinity capture assay as described in (15). In brief, 0.6 μg monoclonal anti-ECP Ab 614 (Diagnostics Development) in a 10 μl volume of PBS was added to each spot of PS20 ProteinChip arrays (Bio-Rad). After a 2.5-h incubation at room temperature and blocking of unspecific binding to the surface by incubation with 25 μl 0.5 M ethanolamine, the arrays were washed three times in 5 min with 0.5% Triton X-100 and three times 5 min by PBS. ECP (0.5 μg) was added to each spot and the arrays were incubated over night at 4°C. The following morning, the arrays were washed three times for 5 min with 0.5% Triton X-100 and three times for 5 min by PBS. Finally, the arrays were washed with 1 mM HEPES buffer before the addition of saturated sinapinic acid (Bio-Rad) dissolved in 0.5% trifluoroacetic acid (Sigma-Aldrich) and 50% acetonitrile (Merck).

A

B

D

C

E
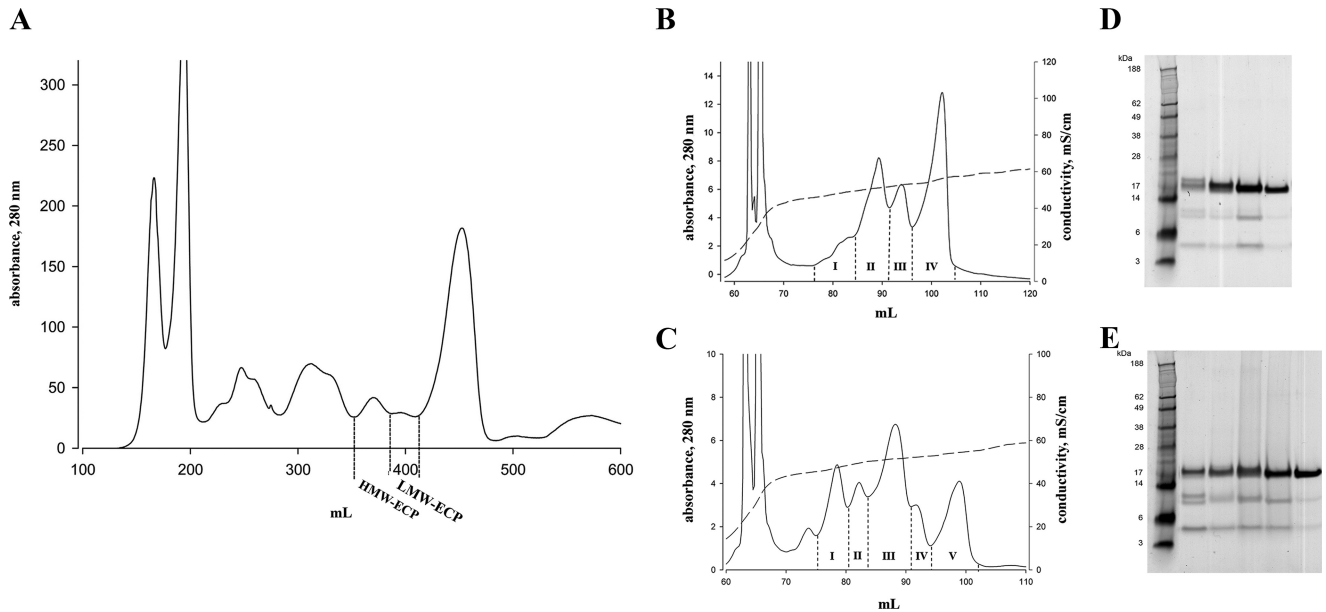
FIGURE 1. *A*, Gel filtration of the granule extract from blood donors with the ECP 434GG genotype. The gel filtration was performed on a Sephadex G-75 superfine column with granule extract corresponding to ~200 buffy coats. The absorbance of the proteins was measured at 280 nm. Eluted proteins in peaks were pooled together according to the dotted line in the chromatogram. Two pools contained ECP; first pool contained high m.w. ECP and the second pool low m.w. ECP. Gel filtration of the granule extract from blood donors with the ECP 434GC genotype resulted in an almost identical chromatogram, and is not shown. *B*, Ion exchange chromatography of the low m.w. ECP from blood donors with the ECP 434GG genotype. Ion exchange chromatography was performed on a Mono-S column and the absorbance of the proteins was measured at 280 nm. Proteins in peaks were pooled together according to the dotted lines in the chromatogram (I-IV). The hatched line shows the salt gradient expressed as conductivity. *C*, Ion exchange chroma-tography of the low m.w. ECP from blood donors with the ECP 434GC genotype. Ion exchange chromatography was performed on a Mono-S column and the absorbance of the proteins was measured at 280 nm. Proteins in peaks were pooled together according to the dotted lines in the chromatogram (I-V). The hatched line shows the salt gradient expressed as conductivity. *D*, Gel electrophoresis of the ECP pools from blood donors with the ECP 434GG genotype. Samples were analyzed on a NuPAGE 10% Bis-Tris gel (Invitrogen) with the SeeBlue Plus2 Pre-Stained Standard (Invitrogen) as a m.w. marker. First lane from the left is the m.w. marker, lanes to follow show GG-ECP I-IV in that order. *E*, Gel electrophoresis of the ECP pools from blood donors with the ECP 434GC genotype. Samples were analyzed on a NuPAGE 10% Bis-Tris gel (Invitrogen) with the SeeBlue Plus2 Pre-Stained Standard (Invitrogen) as a m.w. marker. First lane from the left is the m.w. marker, lanes to follow show GC-ECP I-V in that order.

The arrays were analyzed in a PBS-IIC instrument (Bio-Rad) and a total of 192 transients were collected from each spot. For data analysis, back-ground was deducted and all mass spectra were externally calibrated with the All-in-one protein standard (Bio-Rad) using three calibrants covering the mass region of interest.

Cytotoxicity assay

A modification of the fluorometric microculture cytotoxicity assay de-scribed in Ref. 29 was used. The small-cell lung cancer cell line NCI-H69 was cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% heat inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, all from Sigma-Aldrich. The cells were pelleted and washed once with RPMI 1640 medium containing 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin but without FCS. The cells were separated by accumax treatment for 15 min at 37°C and then cells were washed another two times with RPMI 1640 medium containing 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were counted and seeded in V-shaped 96-well microtiter plates, 10,000 cells/well. ECP in a total vol-ume of 20 μl NaAc buffer (pH 5.5) was added in duplicates to the wells before the cells were seeded. ECP concentrations in the wells ranged from 0.07–0.57 μM (μM concentrations were calculated using the molecular mass of 17500 Da for ECP). Eight wells containing cells and NaAc buffer (pH 5.5) served as buffer control. Two wells of cells and 0.01% Triton X-100 served as positive control for cytotoxicity. Plates were incubated for 72 h at 37°C, 5% CO_2, and were then centrifuged 5 min at 200 × *g*. Medium was removed and the cells washed once with 200 μl/well PBS before the addition of fluorescein diacetate (Sigma-Aldrich) (10 μg/ml). Plates were incubated at 37°C, 5% CO_2, for 30 min before reading fluo-rescence with filters set at 485 and 538 for excitation and emission, re-spectively (Fluoroscan 2, Labsystems OY). The fluorescence generated is proportional to the number of cells with intact plasma membrane in the well, and data are presented as survival index (SI %). Survival index is calculated as: SI = mean fluorescence in 2 test wells − mean fluores-cence

in 12 blank wells/mean fluorescence in 8 control wells − mean fluores-cence in 12 blank wells × 100 (%).

Criteria for a successful assay included a fluorescent signal in control wells of >5 × mean blank values. Dose-response experiments were re-peated once so in total *n* = 4 at each tested concentration for each ECP pool. Data are presented in Fig. 3, *A* and *B* as the mean of one experiment (*n* = 2) ± SEM.

Activity staining gels

To determine the RNase activity of the ECP variants, the activity-staining zymogram technique on 15% SDS-PAGE was used. The gels contained poly(U) (Sigma-Aldrich) as substrate and samples were assayed according to the method described by Bravo et al. (30). The activity was tested with 100 pg of sample in each well. After electrophoresis, the relative intensity of the areas showing substrate degradation was analyzed by densitometry.

Collagen gel contraction assay

The fibroblast-mediated collagen gel contraction assay described in Ref. 23 was used to study the effects of ECP on fibroblasts. In brief, distilled water, type I collagen (rat-tail tendon collagen), 4× DMEM (Life Technologies and SOL, BRL Life Technologies), cultured human lung fibroblasts, and ECP were mixed together on ice to a final concentration of 0.75 mg/ml collagen, 3 × 10⁵ fibroblasts/ml, and physiological ionic strength of 1× DMEM. ECP was added in concentrations ranging from 0.01–0.29 μM. Five hundred and fifty microliters of each ECP-containing gel solution was cast into three wells of a 24-well tissue culture plate with a 2-cm² growth area (Falcon). After gelation (30 min at 37°C, 5% CO_2), the gels were transferred into 60-mm tissue culture dishes containing 5 ml serum-free DMEM using a sterile spatula. The ability of ECP to affect fibroblast-mediated gel contraction was determined by the slow contraction assay of Bell et al. (31). The floating gels were incubated at 37°C, 5% CO_2 for 96 h and the gel areas were measured daily by an image analyzer system (Leica

A

B
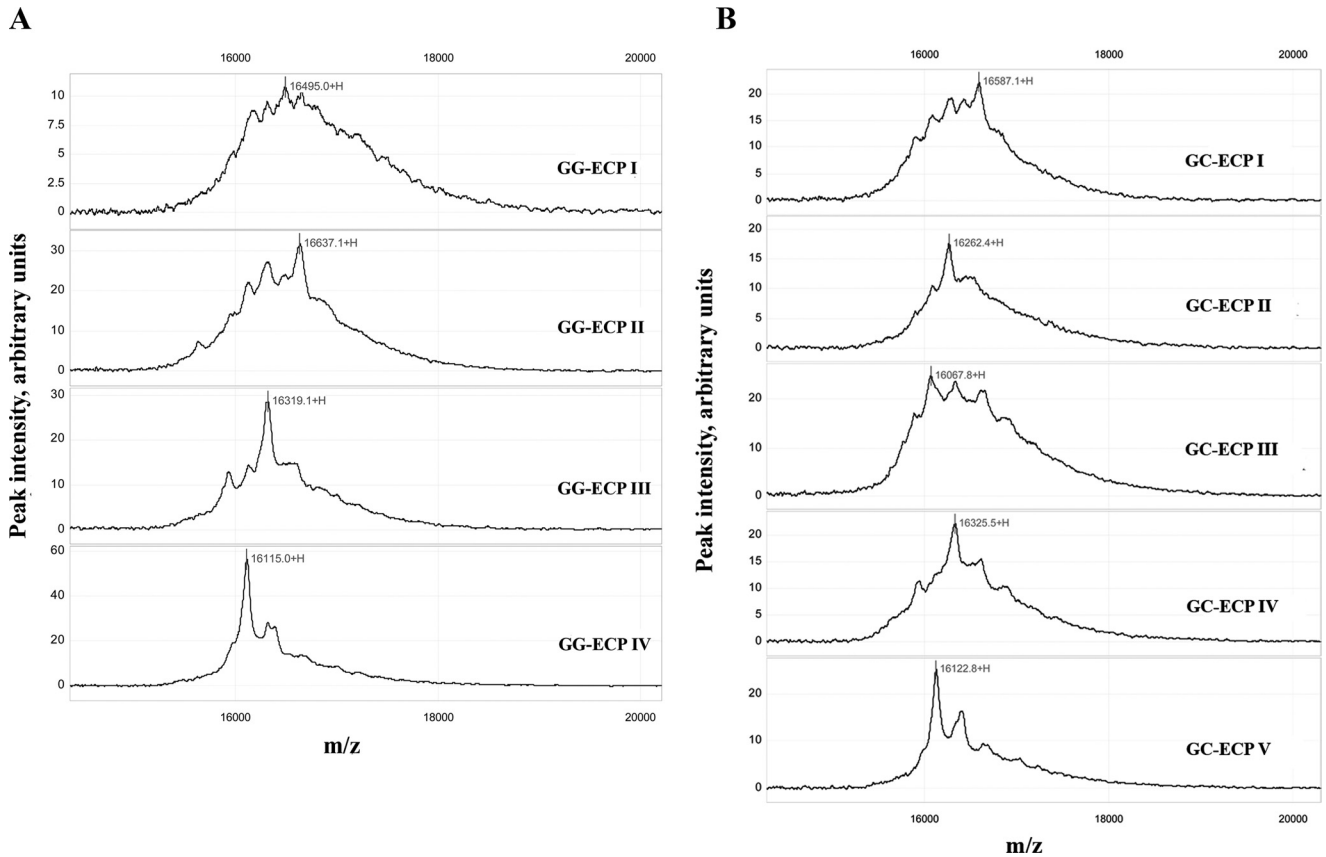
FIGURE 2. *A*, SELDI TOF analysis of GG-ECP I-IV with the 614 mAb. The same amount of protein (0.5 μg) was used for all experiments. The m.w. of the peak of highest peak intensity is indicated in each spectrum. Please note that the scale of peak intensity (*y*-axis) differs in the spectra. *B*, SELDI TOF analysis of GC-ECP I-V with the 614 mAb. The same amount of protein (0.5 μg) was used for all experiments. The m.w. of the peak of highest peak intensity is indicated in each spectrum.

Microsystems). The area of the gels was captured and processed by computer software from Leica Microsystems and collagen gel contraction in the horizontal axes was determined. Thickness was not measured and contraction in the vertical area was not assessed. Data are presented as means and SE of mean ($\pm$SEM) of three replicate gels for each condition.

Statistics

Statistical analyses were performed using the statistics function in Excel or the Statistica 7.0 Software for Windows (Statsoft). For the comparison of numerical variables between more than two groups, ANOVA was used. For the comparison of numerical variables between two groups, the Student's *t* test was used. $p < 0.05$ was considered significant.

Results

ECP purification and characterization

ECP was purified from pooled buffy coats of 200 blood donors with the ECP 434GG genotype (ECP^{97arg}) and 200 blood donors with the ECP 434GC genotype (ECP$^{97arg/thr}$). Only the gel filtration pool with LMW-ECP was further purified by ion exchange chromatography because the ECP with cytotoxic activity is found in this pool (20).

Ion exchange chromatography of the LMW-ECP revealed a difference in the chromatographic patterns of the two ECP-pools; ion exchange chromatography of the ECP-pool from GG-donors (ECP^{97arg}) resulted in four peaks in the chromatogram (Fig. 1*B*), whereas ion exchange chromatography of the ECP-pool from GC-donors (ECP$^{97arg/thr}$) resulted in five peaks in the chromatogram (Fig. 1*C*). Eluted fractions were pooled together as indicated in Fig. 1, *B* and *C*, with four pools from the GG-donors (GG-ECP I-IV) and five pools from the GC-donors (GC-ECP I-V).

The first ion exchange chromatography pool from the GC-donors (ECP$^{97arg/thr}$) was picked for peptide mapping based on the assumption that the threonine-containing C-allele product (ECP^{97thr}) having a lower charge than the arginine-containing G-allele product (ECP^{97arg}) would be present in this pool. The peptide mapping analysis verified that this was the C-allele product in an almost pure form (estimated to be <5% G-allele product in the preparation).

The ECP pools were further characterized by SELDI-TOF MS analysis. Fig. 2*A* displays the SELDI spectra of GG-ECP I-IV and Fig. 2*B* displays the SELDI spectra of GC-ECP I-V. All nine ECP pools were quite heterogeneous with several molecular species present; each spectrum has a mass label at the peak of highest peak intensity. The first pools from the ion exchange chromatography were the most heterogeneous with several molecular species of ECP present whereas the later pools had a more homogeneous profile. Some molecular species of ECP were present in several pools. The smallest molecular variant of ECP was detected at ~15.6 kDa; this peak was seen in ECP pool GG-ECP III. The molecular variant of ECP with the highest m.w. was seen in pool GG-ECP I, at close to 17.2 kDa. The patterns of GG-ECP III and GC-ECP IV were almost identical and with similar mass distributions as were the patterns of GG-ECP IV and GC-ECP V. These comparisons suggested that the pools GC-ECP IV and V contained the G-allele products only. Peptide mapping of the GC-ECP I pool revealed that this was an almost pure C-allele product (ECP^{97thr}) and the SELDI analysis confirmed this finding. The mass difference of arginine and threonine is 55 Dalton; C-allele products are

A

B
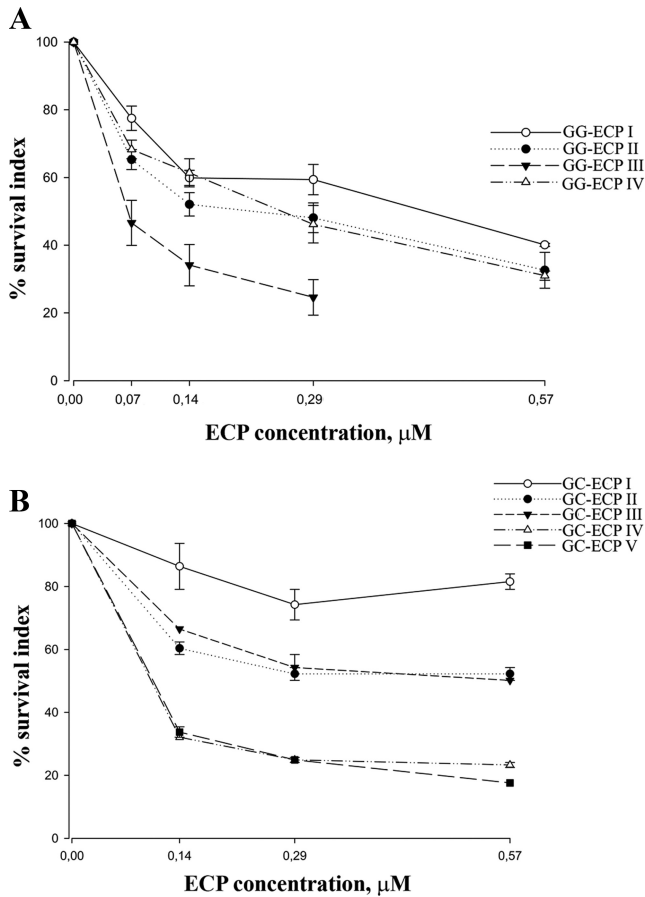
FIGURE 3. *A*, The cytotoxic activity of the GG-ECP pools, a representative dose-response experiment of 0.07–0.57 μM. All four ECP pools displayed potent cytotoxic activity over the entire concentration range. *B*, The cytotoxic activity of the GC-ECP pools, a representative dose-response experiment of 0.14–0.57 μM of ECP. GC-ECP I was not cytotoxic at any concentration tested (top curve) up to 0.57 μM. The other four ECP pools displayed potent cytotoxic activity in the entire concentration range. The cytotoxicity of the five pools differed significantly at 0.29 μM ($p < 0.001$, ANOVA), and pools GC-ECP I-III and V differed significantly at 0.57 μM ($p < 0.001$, ANOVA).

55 Da lighter than the G-allele products. The ECP^{97thr} peak of highest peak intensity in the GC-ECP I pool has a mass of ~16587 Da, which would correspond to the ECP^{97arg} peak with a molecular mass of 16637 in pool GG-ECP II minus ~55 Da. We previously showed that SELDI-TOF MS using monoclonal ECP Abs can distinguish the ECP$^{97arg/thr}$ variants (15).

Cytotoxicity

The nine ECP pools were tested in a dose-dependent manner regarding their cytotoxic properties toward the mammalian cell line NCI-H69 in the fluorometric microculture cytotoxicity assay. A representative dose-response experiment of the ECP pools from GG-donors (ECP^{97arg}) (GG-ECP I to GG-ECP IV) is shown in Fig. 3*A*, data are presented as the mean of one experiment $\pm$ SEM. All four ECP pools from GG-donors had similar cytotoxic activities, with clear effects at concentrations of 0.07 μM of ECP. IC$_{50}$ was ~0.29 μM of ECP for pools GG-ECP I-II and IV and ~0.14 μM for pool GG-ECP III.

Fig. 3*B* shows a representative dose-response experiment of GC-ECP I-V; data are presented as the mean of one experiment $\pm$ SEM. The GC-ECP I pool contained an almost pure C-allele product (ECP^{97thr}) (<5% G-allele product) and this ECP was noncy-
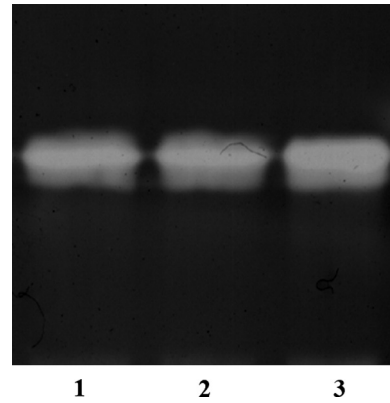

FIGURE 4. The RNase activity of recombinant ECP and native ECP^{97arg} and ECP^{97thr}, activity staining gel with poly(U) as substrate. Native ECP^{97thr} (*lane 1*), native ECP^{97arg} (*lane 2*), and recombinant ECP^{97thr} (*lane 3*); 100 pg ECP in each lane.

totoxic to the H69 cell line. At 0.57 μM of GC-ECP I, <20% of the cells were killed.

Pools GC-ECP II-V all displayed cytotoxic activity toward the cell line but to various degrees. All four pools had clear cytotoxic effects at 0.14 μM of ECP. The IC$_{50}$ was ~0.57 μM for pools GC-ECP II and III and <0.14 μM for pools GC-ECP IV and V. The cytotoxicity of the five pools differed significantly ($p < 0.001$; ANOVA) at 0.29 μM of ECP ($n = 4$). Pools GC-ECP I-III and V differed significantly ($p < 0.001$, ANOVA) at 0.57 μM of ECP ($n = 4$), pool ECP-GC IV was not tested at this concentration.

RNase activity

Several of the ECP pools were examined for their RNase activity on 15% SDS-PAGE gels containing poly(U) as substrate for activity staining. The native ECP pools were also compared with
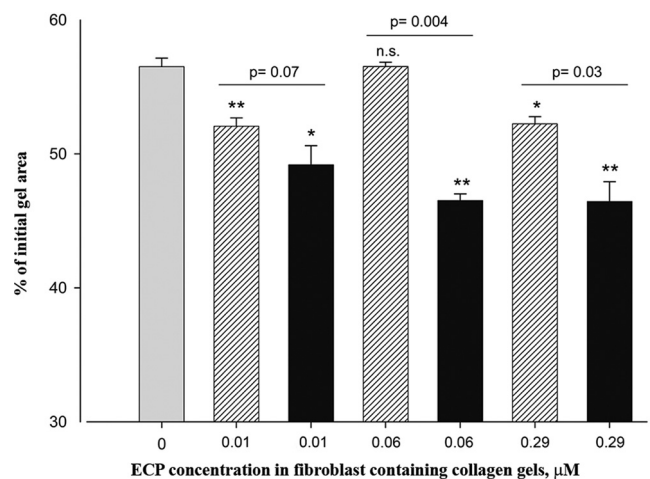
FIGURE 5. GG-ECP IV (ECP^{97arg}) and GC-ECP I (ECP^{97thr}) enhance fibroblast-mediated collagen gel contraction in a dose-dependent manner. ECP at increasing concentrations were added to the fibroblast-containing collagen gels. The area of the floating gels was measured after 1 day of culture. *Vertical axis*, Gel area expressed as percentage of original gel size. Data are shown as mean of triplicate gels $\pm$ SEM. Gray bar shows the results of the control gels containing NaAc buffer, hatched bars ECP^{97arg}, and black bars ECP^{97thr}. Both ECP pools differed significantly from the control at all concentrations tested except GG-ECP IV (ECP^{97arg}) at 0.06 μM. *, $p < 0.05$; **, $p < 0.01$; n.s. = not significant, as compared with the control. ECP^{97thr} enhanced fibroblast mediated gel contraction more than ECP^{97arg} at all concentrations tested.

recombinant ECP[97arg] and ECP[97thr]. All tested samples displayed similar RNase activities. In Fig. 4 recombinant ECP is displayed together with GG-ECP IV (ECP[97arg]) and GC-ECP I (ECP[97thr]). The OD of the area of each band was measured and there were no differences in activity between the samples.

Effect of ECP on fibroblast-mediated collagen gel contraction

Two ECP pools, representing the two allele products of the ECP (G>C) polymorphism, were selected for analysis in the collagen gel contraction assay; the cytotoxic GG-ECP IV (ECP[97arg]) and the noncytotoxic GC-ECP I (ECP[97thr]).

ECP was added to the fibroblast containing collagen gels in concentrations ranging from 0.01–0.29 μM and the gel areas were then measured on four consecutive days. Both ECP pools significantly enhanced gel contraction at all measured time points, data not shown. Fig. 5 shows a representative dose-response experiment of GG-ECP IV and GC-ECP I measured at day 1. GC-ECP I significantly enhanced gel contraction at all concentrations, whereas GG-ECP IV enhanced the contraction at 0.01 and 0.29 μM, respectively. It is also shown that after 1 day of culture, the enhancement of gel contraction by GC-ECP I was significantly higher ($p < 0.05$ to $p < 0.01$) than GG-ECP IV at all concentrations.

Discussion

ECP is a multifunctional and a very heterogeneous protein (10). Previous studies on the biological activities of ECP have either been conducted with native protein purified from healthy humans or patients with hypereosinophilia (12), or with recombinant protein produced in bacteria (8, 32) or insect cells (14, 33). With the new knowledge of the existence of genetic variants of ECP that may affect the activities of the protein and also with the knowledge that recombinant products may not be entirely representative of the native protein, it seemed important to characterize the activities of native ECP purified from genetically homogeneous populations. For the first time therefore, native ECP has been purified from healthy blood donors based on their ECP 434(G>C) genotype (arg97thr). We confirm findings made with recombinant proteins (20) that the cytotoxic activity of ECP is only associated with the variant of ECP containing arginine at amino acid sequence position 97. We also show that the capacity of the two gene products in the enhancement of fibroblast-mediated collagen gel contraction is quite similar, whereas the ECP 434(G>C) polymorphism (arg97thr) does not have any impact on the RNase activity of ECP. Thus, our results show a clear dissociation between biological activities of variants of ECP. From our findings, it is also quite obvious that many previous results on the activity of native ECP should be interpreted with caution, because the preparations were not controlled for the amounts of the two allele products.

As shown previously, ECP from healthy blood donors is separated by gel filtration roughly in a high-m.w. and a low-m.w. form, with the cytotoxic activity associated only with the low-m.w. ECP (20). For the purpose of this study, we therefore concentrated on the possible differences in activities of the low-m.w. ECP. The subsequent separation of the low-m.w. ECP obtained from the two genotyped populations i.e., the ECP 434GG (ECP[97arg]) and ECP 434GC genotypes (ECP[97arg/thr]), by ion-exchange chromatography showed distinct differences and similarities. Due to the lower isoelectric point of the ECP[97thr] (34), this molecular species was expected to be eluted at lower salt concentration. This fact was confirmed by peptide mapping of the material. Similarly, the ECP[97arg] was expected to elute at higher salt concentrations, which was clearly suggested by the differences in the chromatograms. The elution of pure G-allele product (ECP[97arg]) in the heterozygous

material was suggested by the striking similarities in the mass profiles of the two last pools in both chromatograms. However, on top of the genetic differences the molecular heterogeneity of both allele products is obvious and was shown recently to be due to complex glycosylation (15). So far we have not been able to collect enough material for purification of ECP from subjects carrying the ECP 434CC genotype (ECP[97thr]).

We found profound differences in cytotoxic activities between the different ECP pools, representing different allele products and mixtures thereof. Thus, the G-allele products (ECP[97arg]) purified from homozygous subjects all showed potent cytotoxic activities. These activities were similar to the two most basic pools of the heterozygous material and based on their mass similarities they are likely to be identical molecular species. The pools obtained from the heterozygous donors with intermediate activities probably contained a mixture of G- and C-allele products (ECP[97arg/thr]). The fact that the activity in these putative mixtures leveled off at higher concentrations is not readily explained at the time being, but could indicate interferences between the two allele products. Whether our results on the differences in cytotoxic activity are generally applicable to all cells is at present unknown, but is suggested by our recent findings in a *S. mansoni*-exposed population (21). In this population, we found significantly lower rates of infection in subjects carrying the G allele (ECP[97arg]), suggesting protection against the parasite by the cytotoxically active ECP.

In this study, we could demonstrate that the cytotoxic activity of ECP is only associated with ECP[97arg], since the purified ECP[97thr] was noncytotoxic. A potential mechanism for this change in activity could be that the ECP 434(G>C) polymorphism not only results in an amino acid shift, but a potential new glycosylation site is also created. It could be either O-linked glycosylation where the O-linked glycans are linked to the hydroxyl group of threonine, or N-linked glycosylation where the carbohydrate is attached to the asparagine in the tripeptide sequence Asn-x-Thr (27). Indeed, Trulson et al. (20) demonstrated that noncytotoxic rECP[97thr] regained the cytotoxic activity after deglycosylation. Further studies are needed to investigate whether this is the case also for native ECP.

Two ECP pools with opposing cytotoxic properties were selected for further functional characterization: the cytotoxic GG-ECP IV (ECP[97arg]) and the noncytotoxic GC-ECP I (ECP[97thr]). These two ECP pools both showed potent RNase activity, which seemed unaffected by the amino acid substitution at position 97. This would indicate that the arg97thr substitution does not interfere with the RNase activity of ECP. Previous studies on ECP showed that the amino acids Lys38 and His128 are important for the RNase activity of the molecule (33). Recombinant ECP that was mutated at these amino acid positions lost their RNase activity, but retained their bacteriostatic effect. These results support our findings that the cytotoxic properties of the protein do not depend on the RNase activity. However, some toxic activities of ECP such as the neurotoxic (35) and antiviral effects of the molecule (14, 36) seem to be dependent on the RNase activity.

In our previous study, we found a correlation of ECP 434(G>C) genotype and development of peri-portal fibrosis in the liver of subjects infected with the *S. mansoni* parasite (21). Infected subjects with the GG-genotype (ECP[97arg]) had a higher frequency of fibrosis as compared with subjects with the other genotypes, indicating that the arg97thr substitution of ECP is of importance in the fibrotic reaction. ECP has previously been implicated in fibrosis; ECP was shown to enhance fibroblast mediated collagen gel contraction (23), a model of wound healing, and also to enhance release of TGF-β1 by the fibroblasts (25). ECP was also shown to alter proteoglycan synthesis by fibroblast (22). Our hypothesis,

therefore, was that the amino acid substitution at position 97 with threonine would reduce the fibroblast-interfering activities. Our findings, however, demonstrated that both ECP[97arg] and ECP[97thr] mediated collagen gel contraction in a dose-dependent manner, and that the ECP[97thr] variant even had a higher impact on the fibroblast mediated collagen gel contraction than ECP[97arg]. Thus, our results showed a clear dissociation between this activity and the cytotoxic activity of ECP. The fact that the potency of ECP[97thr] was higher in the gel contraction assay might be due to the fact that the impact on fibroblasts in this regard was counteracted by the increasing cytotoxic effects on the cells by the ECP[97arg]. Our findings also led us to speculate on the actual mechanisms involved in the gel contraction model and how representative the model is for the mechanisms underlying the development of peri-portal fibrosis in patients infected by *S. mansoni*.

We conclude that the purification of the different ECP 434(G>C) gene products from large cohorts of healthy blood donors enabled us to show for the first time that the genetic variants of ECP determined by this gene polymorphism affect the activity of ECP differently in that the C-allele product (ECP[97thr]) has lost its cytotoxic properties, but retained the RNase activity and the capacity to enhance collagen gel contraction by fibroblasts.

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Disclosures

The authors have no financial conflict of interest.

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