

The functional –374T/A polymorphism of the receptor for advanced glycation end products may modulate Crohn’s disease

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Däbritz J, Friedrichs F, Weinhage T, Hampe J, Kucharzik T, Lügering A, Broeckel U, Schreiber S, Spieker T, Stoll M, Foell D. The functional –374T/A polymorphism of the receptor for advanced glycation end products may modulate Crohn’s disease. *Am J Physiol Gastrointest Liver Physiol* 300: G823–G832, 2011. First published February 10, 2011; doi:10.1152/ajpgi.00115.2010.—The receptor for advanced glycation end products (RAGE) is involved in innate immune mechanisms. Polymorphisms of the RAGE gene have been described as a factor amplifying inflammation in susceptible patients, but the association with Crohn’s disease (CD) is not known. The coding RAGE polymorphism G82S (rs2070600) and two promoter polymorphisms, –374T/A (rs1800624) and –429T/C (rs1800625), were studied in two samples from Germany and the United States consisting of 421 and 317 CD patients and 549 and 218 controls, respectively. To test the functional relevance, additional data on serum soluble RAGE (sRAGE), tissue RNA, and protein levels were collected and immunohistochemical stainings of bowel tissue of CD patients and healthy controls as well as models of experimental (dextran sodium sulfate-induced) colitis in RAGE knockout and wild-type mice were performed. The –374T/A RAGE promoter single nucleotide polymorphism (SNP) was negatively associated with CD (odds ratio = 0.708, 95% confidence interval = 0.535–0.938, $P = 0.016$) and with stenosis (OR = 0.627, $P = 0.04$) in the German sample. Transmission disequilibrium testing confirmed an undertransmission of the –374A allele. Serum sRAGE levels were higher in patients in complete remission of the –374AA/TA group ($1,975 \pm 299$ pg/ml; –374TT group: $1,310 \pm 153$ pg/ml SE, $P < 0.05$) and showed a trend toward decreased levels in CD patients with active disease compared with CD patients in remission. Further in vitro and in vivo studies indicated that an increase of sRAGE ameliorates inflammation. The –429T/C and the G82S polymorphism were not associated with CD. The –374T/A RAGE polymorphism leading to facilitated RAGE gene transcription may to some degree protect from developing a structuring subphenotype of CD, most likely by increasing levels of sRAGE, which neutralizes proinflammatory mediators.

colitis; innate immunity

THE ETIOLOGY AND PATHOGENESIS of inflammatory bowel disease (IBD) are still not entirely clear. However, it seems likely that Crohn’s disease (CD) and ulcerative colitis result from complex interactions among susceptibility genes, the environment and the immune system (4). The intestinal epithelium comprises the largest surface proportion of our outer physiological

barrier and regularly encounters numerous challenges. Interactions between the host and pathogens lead to a cascade of events that initially activate nonspecific inflammatory cells, enhance production of inflammatory mediators, and facilitate recruitment of lymphocytes into inflamed tissue. Under normal conditions these first-line defense mechanisms protect the host from infection and damage. It is hypothesized that in case of CD an imbalance between innate and adaptive immune mechanisms leads to a perpetuation of inflammation resulting in chronically active bowel disease (9). One explanation could be an overwhelming activity of adaptive immune mechanisms, which is supported by the clinical occurrence of autoimmune phenomena, as for example the prominence of lymphoid tissue proliferation in inflamed tissue in CD and the effects of immunosuppressive therapies. Another explanation could be a nonsufficient activity of innate immune mechanisms without appropriate reaction to diverse challenges, which is supported by the finding of several pathogens associated with CD and by the beneficial effects of immunostimulation with granulocyte macrophage colony-stimulating factor (6, 28, 43).

The epithelial barrier comprised by the gastrointestinal mucosa harbors immunocompetent cells and is central to natural resistance. The epithelium forms a cellular network that stands at the interface between environmental factors and both the innate and the adaptive immune systems. As a result it needs to integrate the different signals and has to respond to them (5). Receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily of surface molecules (42). Several molecules have been described to bind to this multiligand receptor, and recently RAGE has been proposed as a pattern recognition receptor for damage-associated molecular patterns (DAMP) (35). Those patterns involve molecules like advanced glycation end products, which accumulate in diabetes or oxidative stress, proinflammatory S100A12, which is secreted by activated granulocytes, or amphotericin B (high-motility group box protein 1; HMGB-1) (13, 16).

RAGE is expressed constitutively in relatively large amounts in the lung and only at low levels in other tissues including the intestinal epithelium (8). However, its expression may be significantly enhanced in endothelial cells, epithelial cells, and leukocytes (3). On the one hand, RAGE signaling promotes inflammation (21), whereas on the other hand the receptor may be shed from cells or be expressed as a soluble protein after alternative splicing and can thereby neutralize ligands in the circulation (26, 36, 41, 52, 53). Soluble RAGE (sRAGE) has been proposed to physiologically serve as a protective

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Table 1. Characteristics of the German and US-American study samples

	Germany	USA
CD patients, no.	421	317
Age, mean \pm SE	34.5 \pm 8.5	12.1 \pm 3.4
Male, %	29.0	59.6
Ileal, %	80.8	70.8
Colon, %	87.1	85.6
Inflammatory, %	N/A	88.1
Perforating, %	60.2	14.4
Strictureing, %	64.1	12.3
Control, no.	549	218
Age, mean \pm SE	40.4 \pm 12.5	10.0 \pm 4.8
Male, %	49.4	47.7

CD, Crohn's disease; N/A, not applicable.

factor (14, 19, 40). In RAGE knockout ($-/-$) mice initiation and perpetuation of inflammation is normal in disease models involving predominantly the adaptive immune response. However, in a sepsis model RAGE deletion protected against sepsis being largely dependent on the innate immune system. In contrast to wild-type mice, most RAGE $-/-$ mice survived the septic shock (31). These data emphasize the role of RAGE in innate defense mechanisms. Furthermore, overexpression of RAGE did not enhance lethality in murine models of sepsis or modulated adaptive immune responses, which again points to the generally beneficial role of innate immune mechanisms.

The G82S RAGE polymorphism leads to an amino acid exchange within the putative binding site of the receptor and has been reported to amplify arthritis due to enhanced ligand binding (31). Besides arthritis, this polymorphism was found to be associated with Alzheimer disease (30) and diabetic complications (27), but several subsequent studies revealed contradictory results (25, 33). Additionally, no correlation to cardiovascular disease was found in the Framingham offspring study (23). The $-374T/A$ RAGE promoter polymorphism leads to reduced binding of a nuclear factor to a regulatory element of the RAGE gene promoter, thus resulting in enhanced transcriptional activity (24, 29). Reports on disease associations have previously focused on diabetic complications and resulted in contradictory data ranging from positive to negative associations (11, 20, 32, 38, 39). Fewer data exist for the $-429T/C$ promoter polymorphism with also inconsistent results mainly focusing on diabetic complication (24, 37). So far, it is not known whether RAGE polymorphisms influence susceptibility for IBD. Since RAGE has a prominent role in innate immune responses, which may be disturbed in IBD, we studied the frequency of the three most relevant RAGE polymorphisms

(G82S; $-374T/A$, $-429T/C$) in healthy controls and in patients suffering from CD and analyzed the association of the polymorphisms with disease phenotype.

PATIENTS AND METHODS

IBD study samples. Individuals with CD were included and two different study samples from Germany and the United States were compared (Table 1). Individuals of the German sample with CD were recruited through the Charité University Hospital Berlin, Germany, and at the Department of Internal Medicine I, University Hospital, Kiel, Germany. Diagnosis of CD and subsequent classification into CD was determined by standard diagnostic criteria. All individuals were of European descent. We analyzed trios consisting of father, mother, and child affected with CD, in which one parent or neither parent was affected with CD. These trios have been described before (45). For case-control association, we compared the same individuals with CD (singletons) with 549 age- and sex-matched volunteers from the Kiel University blood donation program. All study participants gave informed, written consent. The recruitment protocols and study procedures were approved by the ethics committees of the Charité University Hospital Berlin, Germany, and the Schleswig-Holstein University Hospital, Campus Kiel, Germany, respectively.

The second study sample of 317 white children (Caucasian descent) with CD was recruited through the Children's Hospital of Wisconsin, Milwaukee, WI (2). The parents from the CD patients from the United States were also available for transmission disequilibrium testing (TDT) analysis. For case-control association, we compared these CD patients with 218 nonaffected controls. The respective institutional review boards and the ethics committees approved the study. The methods and criteria for diagnosis (based on well-defined endoscopic, radiological, and histological criteria) and phenotyping of CD have been previously published.

Genotyping. CD patients, their available parents, and controls were genotyped for RAGE SNPs G82S (rs2070600), $-374T/A$ (rs1800624), and $-429T/C$ (rs1800625) by using Applied Biosystems (Foster City, CA) Assays-by-Design TaqMan technology on an ABI 7900HT sequence detection system. Genotyping was performed with a 98% successful call rate overall.

Case-control study. Hardy-Weinberg equilibrium in cases and controls was tested by using the exact test as implemented in the program Haploview 3.2 at a significance level of 0.05 (1). Logistic regression was used to model the relationship between a binary outcome variable and one or more predictor variables. In this study the outcome variable is the CD vs. control variable unless indicated otherwise. Predictors are the genotype variables, which were defined as follows: RAGE $-374T/A$: one or two copies of the A allele, coded as 1, no A allele, coded as 0; RAGE G82S: one or two copies of the G allele, coded as 1, no A allele, coded as 0; RAGE $-429T/C$: one or two copies of the T allele, coded as 1, no T allele, coded as 0. All ORs for genotype variables were adjusted for age and sex of study participants. Logistic regression analyses were performed by use of

Table 2. Association of RAGE promoter and coding SNPs with CD: case control study results

SNP	Cohort	Minor Allele	No. of Cases	No. of Controls	MAF, Affected	MAF, Control	OR (95% CI)	P Value
$-429T/C$	Germany	T	335	488	22.8	21.5	1.103 (0.814–1.493)	ns
	USA	T	310	202	18.4	18.6	1.005 (0.617–1.636)	ns
$-374T/A$	Germany	A	389	525	24.7	29.1	0.708 (0.535–0.938)	0.016
	USA	A	312	216	28.5	28.2	0.844 (0.533–1.334)	ns
G82S	Germany	G	323	526	3.3	2.6	1.383 (0.742–2.579)	ns
	USA	G	312	214	4.7	3.3	1.353 (0.554–3.301)	ns

Minor allele frequencies (MAF), P values, odds ratios (OR), and 95% confidence intervals (CI) are shown. The ORs shown are adjusted for age and sex. All genotypes were analyzed by the dominant inheritance model (1 or 2 copies of the minor allele vs. no copy). RAGE, receptor for advanced glycation end products; SNP, single nucleotide polymorphism; ns, not significant.

Table 3. Association of RAGE promotor and coding SNPs with CD: family study results

SNP	Cohort	Minor Allele	No. of Trios	Transmitted / Undertransmitted	P Value
-429T/C	Germany	T	468	164/123	0.016
	USA	T		14/27	0.040
-374T/A	Germany	A	304	108/166	0.001
	USA	A		28/19	ns
G82S	Germany	G	459	26/20	ns
	USA	G		1/2	ns

STATA statistical package (version 9); *P* values are given according to the Wald statistic.

Family studies. Mendelian inheritance checks were performed and trios with Mendelian inheritance errors were excluded from further analysis. TDT was used to analyze association within the CD trios. The TDT is proposed as a family-based association test for the presence of genetic linkage between a genetic marker and a trait. A specificity of the TDT is that it detects genetic linkage only in the presence of genetic association. Whereas genetic association can be caused by population structure, genetic linkage will not be affected, which makes the TDT robust to the presence of population structure.

Analysis of RAGE expression in human bowel tissue. Quantitative real-time PCR (qRT-PCR) was used to evaluate RAGE expression at the mRNA level in patients with CD and healthy controls. All patients were included in other ethics committee-approved studies that even allowed additional research using tissue samples. Total RNA from diverse bowel tissues was prepared by use of TRIzol (Invitrogen, Carlsbad, CA). One microgram of RNA was reverse transcribed by use of the RevertAid H Minus Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany) and oligo(dT18) primer into cDNA. PCR amplification was performed by using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with a Power SYBR Green PCR Kit (Applied Biosystems), according to the manufacturer's instructions. Thermocycling was performed with 40 cycles of amplification (15 s at 95°C, 15 s at 60°C, and 15 s at 72°C). Gene expression was normalized to the endogenous housekeeping control gene GAPDH, and relative expression of respective gene was calculated by the comparative threshold cycle method (34). Primers used were as follows: RAGE forward primer 5'-CCA-GGAGGAAGAG-GAGGAG-3', reverse primer 5'-GCTGATGGATGGGATCTGTC-3'; GAPDH forward primer 5'-GCAAATTCATGGCACCGT-3', and reverse primer 5'-GCCCACTTGATTTTGGAGG-3'.

Immunohistochemistry. Tissue samples were fixed in 4% formaldehyde and routinely embedded in paraffin. Immunohistochemical studies were performed by staining with a specific monoclonal RAGE antibody (Millipore, Billerica, MA), as described previously (8, 49). Sections were analyzed by using a Zeiss Axioskop connected to an AxioCam camera supply with software Axiovision 4.8 for Windows (Zeiss, Goettingen, Germany). All samples were assessed by independent analysts. RAGE expression was quantified by using a score from 0–3 (0, no expression; 1, low expression; 2, medium expression; 3, strong expression).

ELISA. Serum levels of sRAGE and S100A12 were determined by double-sandwich ELISA systems as described previously (16). The readers of the laboratory assay were blinded for the diagnosis and the inflammation score. The release of S100A12 and sRAGE from intestinal tissue of patients with CD and healthy controls was analyzed by using bowel specimens obtained during endoscopy. Subjects in

screening programs for early detection of polyps and colon cancer and having normal colonoscopic findings were chosen as normal controls. Endoscopic, histological, laboratory, and clinical disease activity measures were documented. After 24-h culture, S100A12 and sRAGE were analyzed in supernatants by using aliquots from identical samples.

Analysis of TNF- α expression in human monocytes. Human mixed donor monocytes were isolated from buffy coats (German Red Cross, Münster, Germany) as described before (12). Monocytes were isolated by Ficoll-Hypaque and Percoll (Biocoll, Biochrom, Berlin, Germany) density gradient centrifugation and cultured in McCoy's 5a medium supplemented with FCS (GIBCO-BRL Life Technologies, Eggenstein, Germany) in Teflon bags overnight. Cells were then incubated for 4 h with 10 μ g/ml S100A12 (\pm 40 μ g/ml sRAGE). Total RNA was prepared from cell lysates. The primers used for PCR analysis were as follows: TNF- α forward primer 5'-CTTCTCGAAC-CCCAGTGAC-3', reverse primer 5'-TGAGGTACAGGCCCTCT-GATG-3'; GAPDH forward primer 5'-GCAAATTCATGGCACCGT-3', reverse primer 5'-GCCCACTTGATTTTGGAGG-3'. Recombinant S100A12 and sRAGE were expressed in and purified from *Escherichia coli* as described previously (15).

Animals. RAGE^{-/-} mice were generated as described previously (31). The abrogation of RAGE expression at the mRNA level was analyzed by qRT-PCR of lung tissue, in which RAGE is constitutively expressed at high levels. No RAGE mRNA could be detected in RAGE^{-/-} mice with primers for the extracellular RAGE domain. To confirm the abrogation of RAGE expression, we used different primers covering the entire RAGE molecule and detected no transcripts. In addition, in tissues with low RAGE expression in wild-type mice (heart, liver, kidney, and brain), no RAGE message could be detected in RAGE^{-/-} mice. The absence of RAGE protein was demonstrated by Western blot and immunohistochemistry of lung tissue. RAGE^{-/-} mice were viable and displayed normal reproductive activity. No spontaneous disease development was observed in mice up to the age of 6 mo and mice displayed normal macroscopic pathology and histopathology. RAGE^{-/-} mice were backcrossed to generation F12. C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and served as control. All mice were housed under specific pathogen-free conditions and were used between 8 and 12 wk of age. All animal procedures in this study were reviewed and approved by the local authorities.

Acute DSS-induced colitis. RAGE^{-/-} mice and appropriate C57BL/6 wild-type mice were treated with 2% (wt/vol) dextran sodium sulfate (DSS; molecular mass 36,000–50,000 Da; MP Bio-medicals) dissolved in autoclaved drinking water for 5 days, followed by 8 days of recovery without DSS. Control mice were given drinking water only. Body weight was monitored daily. After 13 days mice were euthanized by CO₂ inhalation and their colons were collected,

Table 4. Association of RAGE polymorphism 374T/A with stricturing behavior in CD patients

Cohort	Number of Cases	Allele Frequency Stricturing	Allele Frequency Nonstricturing	OR, (95% CI)	P Value
Germany	369	22.4	28.7	0.627 (0.402–0.979)	0.04
USA	274	20.6	29.6	0.495 (0.229–1.067)	0.07

All genotypes were analyzed by the dominant inheritance model (1 or 2 copies of the minor allele vs. no copy).

Table 5. Association of RAGE promotor polymorphism -374T/A and serum sRAGE levels in CD patients

	374T/A		P Value	374TT Remission	374AA/AT Remission	P Value	374TT Active	374AA/AT Active	P Value
	Remission	Active							
No. of patients	14	22		10	4		9	13	
Age	35.8 ± 2.8	31.3 ± 2.9		35.6 ± 3.5	36.1 ± 5.7		27.9 ± 3.8	33.7 ± 4.2	
Male/female	6/8	12/10		4/6	2/2		5/4	7/6	
sRAGE, pg/ml	1,500 ± 142	1,414 ± 174	ns	1,310 ± 153	1,975 ± 299	0.049	1,201 ± 179	1,561 ± 240	ns
S100A12, ng/ml	178 ± 22	232 ± 23	ns	178 ± 28	180 ± 53	ns	196 ± 38	254 ± 24	ns

Values are means ± SE. sRAGE, soluble RAGE.

cleaned by rinsing with PBS, and measured. A small piece of the distal colon with a size of ~5 mm³ was immediately shock frozen in liquid nitrogen and stored at -80°C for further use. The remaining colon was sliced longitudinally, rolled to form a "Swiss roll," and fixed in 4% formalin.

Histology. Colon sections of 5 µm were stained with hematoxylin and eosin (H&E) by standard procedures. Disease severity was measured on the basis of a histopathological score (0-3) in the H&E-stained, sectioned intestinal tissue (0, normal tissues without inflammation; 1, mild inflammation in the mucosa with some infiltrating mononuclear cells; 2, moderate inflammation with more infiltrating cells, crypt glands have pulled away from the basement membrane, mucin depletion from goblet cells, and the epithelium begins to pull away from the mucosa into the lumen; and 3, severe inflammation with extensive infiltrating cells in the mucosa and submucosa area, crypt abscesses present with increased mucin depletion, and epithelial cell disruption). Ulcerations were quantified by a score from 0 to 3 (0, no ulcerations; 1, number of ulcerations up to 2 crypt widths; 2, number of ulcerations between 2 and 10 crypt widths; 3, number of ulcerations more than 10 crypt widths). Sections were assessed by two analysts by light microscopy at ×100 magnification, with the scorers blinded to the experimental groups.

Murine tissue RNA and qRT-PCR. The expression of proinflammatory mediators at the mRNA level was analyzed by qRT-PCR. Total RNA was isolated from distal colon specimens by using a Precellys24 (Bertin Technologies, Montigny-le-Bretonneux, France) and a NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Subsequent reverse transcription and amplification was performed as described above. Primers used were as follows: TNF-α forward primer 5'-AGAAACACAAGATGCTGGGACAGT-3', reverse primer 5'-CCTTTCGAGAACTCAGGAATGG-3'; IL-6 forward primer 5'-TGAGATCTACTCGGCAAACCTAGTG-3', reverse primer 5'-CTTCGTAGAGAACAACATAAGTCAGATAACC-3'; IL-1β forward primer 5'-TGCTTGGCCGAGGACTAAGG-3', reverse primer 5'-TGGGCTGGACTGTTTCTAATGC-3'; IL-11 forward primer 5'-TACAATGGCTGCGCCGTGCA3', reverse primer 5'-GGC-CAGGCGAGACATCAAGAGC-3; GAPDH forward primer 5'-GTCCACCAGCCTGTTGCTGTAG-3', reverse primer 5'-CCCCTCTTCCACCTTCGATG-3'.

TNF-α expression in bone marrow-derived cells. Mouse bone marrow cells (5 × 10⁶) from RAGE^{-/-} and wild-type mice were isolated and cultured as described before (46) and stimulated with 10 µg/ml S100A12 immediately after isolation for 4 h. Total RNA was prepared from cell lysates, and TNF-α expression (relative to unstimulated control) was analyzed as described before.

Statistics. All data are expressed as means ± SE derived from at least three independent studies. The means of groups were compared by analysis of variance by Student's *t*-test for unpaired data and the Mann-Whitney *U*-test as a nonparametric significance tests. A probability *P* < 0.05 was considered to be statistically significant. All calculations were performed by using the Statistical Package for the Social Sciences (SPSS, version 11.0, Chicago, IL).

RESULTS

Polymorphisms of the RAGE gene. We analyzed single-nucleotide polymorphisms (SNPs) of the RAGE gene in CD patients. The most relevant functional RAGE polymorphisms, namely the promotor polymorphism -374T/A (rs1800624), the polymorphism 429T/C (rs1800625), and a G→A polymorphism in exon 3 causing an amino acid exchange (G82S; rs2070600), were studied in two study samples from Germany and the United States consisting of 421 and 317 CD patients and 549 and 218 controls, respectively. Furthermore, the parents from the CD patients from Germany and the United States were available for TDT analysis. Localization and behavior of disease as well as age and sex distribution in the two samples are given in Table 1.

The -374A/T SNP was analyzed in 389 and 312 CD patients and 525 and 216 control individuals of the German and US-American cohort, respectively (Table 2). The -374A allele of the RAGE promotor SNP was associated with CD in the German sample [odds ratio (OR) = 0.708, 95% confidence interval (95%CI) = 0.535-0.938, *P* = 0.016]. Furthermore, we found that the -374T/A promotor SNP was independently associated from well-established CD associated polymorphisms in the NOD2 gene (OR = 0.697, 95%CI = 0.517-0.940, *P* = 0.018, odds ratio adjusted for the presence of at least one risk allele of NOD2 SNP8 [rs2066844], SNP12 (rs2066845) or SNP13

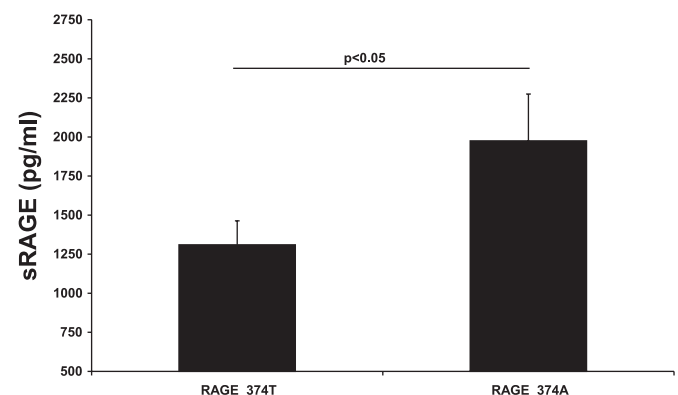


Fig. 1. Serum levels of soluble receptor for advanced glycation end products (sRAGE) of 36 patients with Crohn's disease (CD) were determined by a double sandwich ELISA system. Shown are the results of patients in complete remission according to the presence or absence of soluble receptor for advanced glycation end products (RAGE) polymorphism -374T/A. Serum sRAGE levels were significantly higher in patients in complete remission of the -374AA/AT group (-374A group: 1,975 ± 299 pg/ml SE) compared with patients in complete remission of the -374TT group (-374T group: 1,310 ± 153 pg/ml SE, *P* = 0.049).

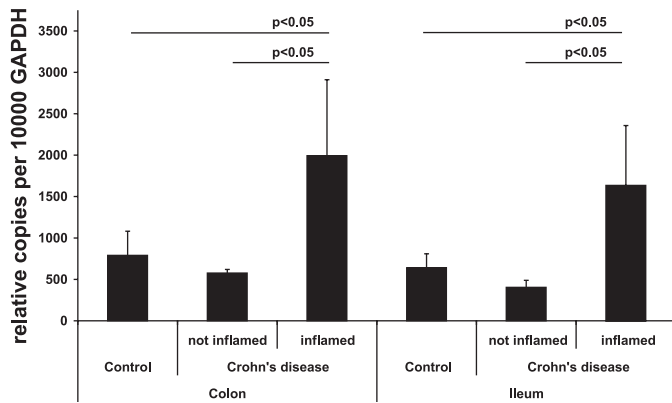


Fig. 2. Expression of RAGE was analyzed by quantitative real-time PCR (qRT-PCR) in noninflamed and inflamed tissue sections of the colon and ileum of 14 healthy controls and 4 patients with CD, respectively. RAGE expression was significantly increased in inflamed colon and ileum sections of CD patients compared with noninflamed colon ($P < 0.05$) and ileum ($P < 0.05$) sections of CD patients and colon ($P < 0.05$) and ileum ($P < 0.05$) sections of healthy controls. Data are expressed as means \pm SE.

(rs2066847)]. However, no significant association of the RAGE promoter SNP $-374T/A$ and CD was observed in the US-American study sample. Furthermore, TDT was used to analyze association of $-374T/A$ within the CD trios and showed a strong CD transmission disequilibrium in the German study sample ($P = 0.001$), whereas differences were not observed in the US-American cohort (Table 3). We analyzed the association of RAGE polymorphism $-374T/A$ with CD

subphenotypes of localization and behavior of inflammation in the patients sample and found an association with stricturing behavior for the $-374A$ allele in the German CD patients (OR = 0.627, $P = 0.04$), whereas a trend for an association was observed in the US-American patients ($P = 0.07$) (Table 4). Interestingly, the German study sample comprises 64% CD patients that suffer from stricturing disease whereas in the US-American samples most of the patients are not affected by stricturing phenotype of CD (12% US-American CD patients with stenoses).

The $-429T/C$ SNP was analyzed in 335 and 310 CD patients and 488 and 202 control individuals of the German and US-American cohort, respectively (Table 2). We found no association of the $429T/C$ RAGE polymorphism to CD in the case-control approach. However, transmission disequilibrium was observed in the TDT in the German and US-American sample (Table 3). Likewise, we observed no association of the G82S SNP and CD, which was analyzed in 323 and 312 CD patients and their parents (TDT), and 312 and 214 control individuals of the German and US-American cohort, respectively (Tables 2 and 3).

Serum levels of sRAGE. To confirm whether the $-374T/A$ SNP previously shown to lead to enhanced gene transcription and elevated sRAGE also leads to evaluated serum levels of sRAGE in an independent sample of CD patients treated at the Department of Medicine B (University Hospital of Münster, Münster, Germany), we included 36 patients (18 men, 18 women) and genotyped the $-374A/T$ RAGE polymorphism. We included 14 patients in remission and 22 patients with

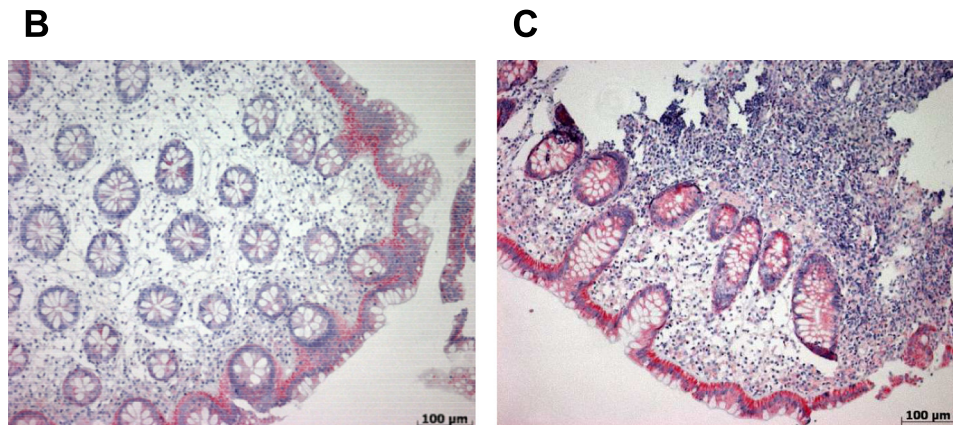
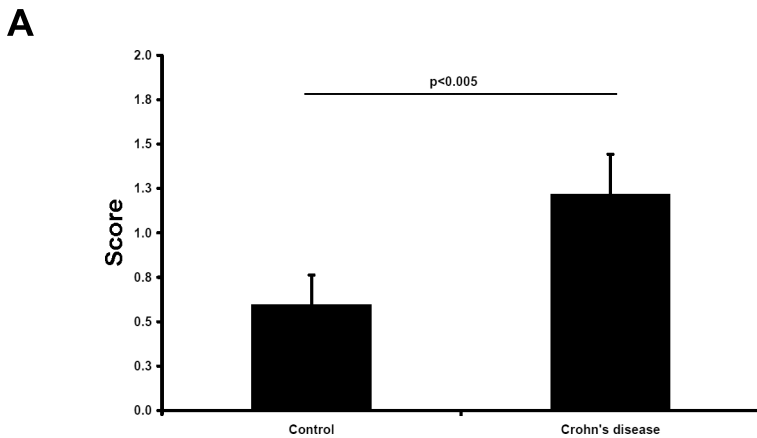


Fig. 3. Expression of RAGE was examined by immunohistochemistry (A). Colon tissue of 14 healthy controls (2 men, 12 women, mean age 49 yr, range 21–65 yr) and 20 patients with CD (6 men, 14 women, mean age 37 yr, range 23–63 yr) was analyzed. RAGE expression was significantly higher in colon samples of patients with CD (C) compared with healthy controls (B). Staining of tissue from patients with CD showed a specific distribution of RAGE expression by infiltrating cells in inflamed areas of the gut (C) compared with healthy controls (B). Data are expressed as means \pm SE.

active disease. Patients' characteristics are given in Table 5. Seventeen patients showed a $-374AA/AT$ genotype ($-374A$ group: 9 men, 8 women), whereas 19 patients showed a $-374TT$ genotype ($-374T$ group: 9 men, 10 women). Ten patients of the $-374T$ group were in complete remission (4 men, 6 women, mean age $35.6 \text{ yr} \pm 3.5 \text{ yr SE}$), and 9 patients of these group showed active disease (5 men, 4 women, mean age $27.9 \text{ yr} \pm 3.8 \text{ yr SE}$). Four patients of the $-374A$ group were in complete remission (2 men, 2 women, mean age $36.1 \pm 5.7 \text{ yr SE}$), and 13 patients of these group showed active disease (7 men, 6 women, mean age $33.7 \text{ yr} \pm 4.2 \text{ yr SE}$). Serum sRAGE levels were significantly higher in patients in complete remission of the $-374A$ group ($1,975 \pm 299 \text{ pg/ml SE}$) compared with patients in complete remission of the $-374T$ group ($1,310 \pm 153 \text{ pg/ml SE}$, $P = 0.049$) (Table 5, Fig. 1). No differences were observed regarding serum levels of the proinflammatory RAGE-ligand S100A12, C-reactive protein, leukocytes, platelets or hemoglobin concentrations between these two groups (data not shown). Patients' characteristics of both groups ($-374A$ and $-374T$ in remission) were comparable regarding medication, age, and sex distribution. Also no differences were observed between patients with active disease of the $-374T$ and $-374A$ subgroups (Table 5).

RAGE expression in human bowel tissue. To analyze whether RAGE expression has any physiological relevance in the gastrointestinal tract, we performed analyses on RAGE expression in healthy and inflamed colon and ileum (Fig. 2). RAGE expression was also examined by immunohistochemistry (Fig. 3). We confirmed constitutive RAGE expression in the epithelium and endothelium. In addition, we can confirm that

RAGE expression increases in inflamed tissue, which is mainly due to infiltrating RAGE-bearing phagocytes.

Consequences of general RAGE underexpression. The $-374T/A$ RAGE polymorphism leading to facilitated RAGE gene transcription may to some degree protect from developing a stricturing subphenotype of CD by augmenting (an otherwise insufficient) innate immune activation due to increased expression of the surface receptor that transmits proinflammatory signals after ligation or by ameliorating inflammation due to increasing levels of sRAGE, which neutralizes proinflammatory ligands. We first tested whether an increase of RAGE expression would lead to enhanced immune activation. If this was the case, then decreased RAGE expression should result in less activity. We addressed this question by employing the murine model of acute DSS colitis, taking advantage of $RAGE^{-/-}$ mice recently generated in our laboratory. Compared with wild-type mice we found no difference in severity of colitis, either clinically or on the tissue or cellular level (Fig. 4, A–C). However, we could show that bone marrow-derived macrophages from $RAGE^{-/-}$ mice could be equally or even better stimulated with S100A12 compared with bone marrow-derived macrophages from RAGE wild-type mice (Fig. 4D).

Overexpression of RAGE ligands and anti-inflammatory sRAGE. Our data further show that there is a clear increase of proinflammatory RAGE ligands such as S100A12, which is released at sites of inflammation, i.e., from affected bowel tissue taken from patients with active CD. However, this increase in proinflammatory S100A12 is not counterbalanced by an increase of anti-inflammatory sRAGE (Fig. 5, A and B). Thus augmented release of neutralizing sRAGE, e.g., in sub-

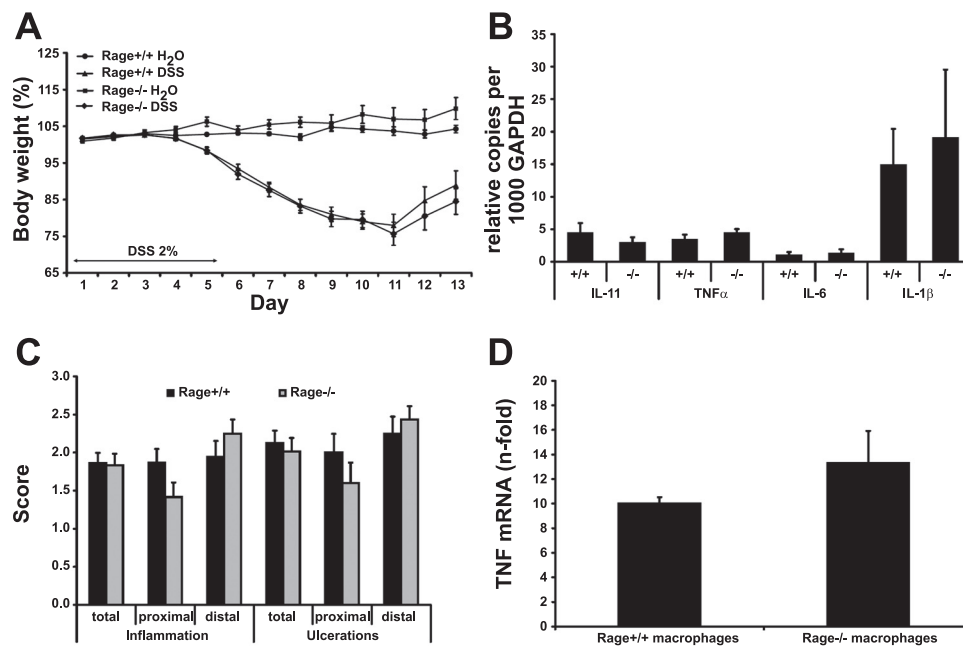


Fig. 4. Acute colitis was induced in RAGE knockout ($-/-$) and RAGE wild-type ($+/+$) mice by treatment with 2% dextran sodium sulfate (DSS) in drinking water for 5 days. DSS-treated animals exhibited weight loss and rectal bleeding similar to previous reports. Weight loss began around *day 4* (A) and became significant compared with untreated control animals by *day 7* (-12.02% of baseline weight, $P < 0.01$). No difference was observed during the course of acute DSS-induced colitis in $RAGE^{-/-}$ and wild-type mice, as assessed by markers of colitis severity, i.e., determination of weight loss (A), expression of proinflammatory mediators like IL-6, IL-11, IL-1 β , and TNF- α assessed by qRT-PCR (B), histopathological evaluation regarding inflammatory infiltrates, ulceration, and crypt damage (C), or colonic shortening (data not shown). Bone marrow-derived cells from $RAGE^{-/-}$ mice could be equally or even better stimulated with S100A12 ($10 \mu\text{g/ml}$) compared with bone marrow-derived cells from RAGE wild-type mice (D). Experimental groups contained 5 mice and the data are expressed as means \pm SE from 5 independent experiments.

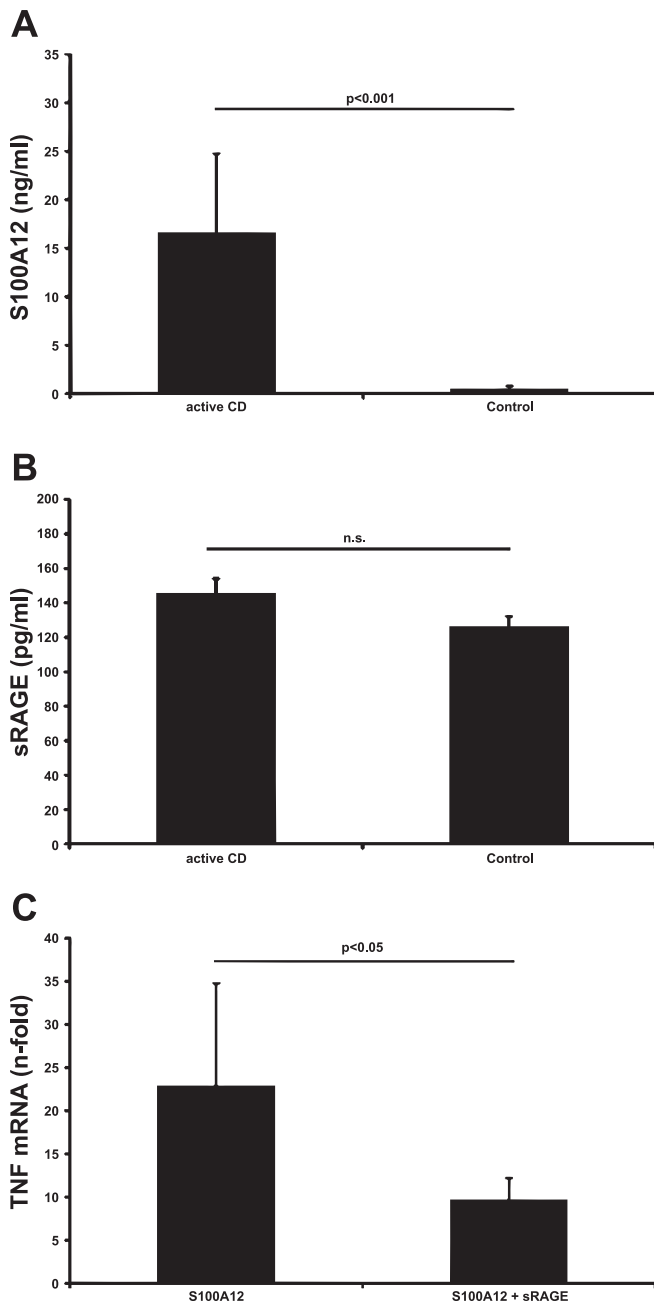


Fig. 5. After 24 h culture the release of S100A12 and sRAGE from intestinal tissue obtained during endoscopy of patients with active CD and healthy controls was analyzed by using a double sandwich ELISA system. Specimens from 8 patients with active CD (mean age 31 yr, range 19–62 yr, male-female ratio 1.7) and 15 healthy controls (mean age 50, range 31–67 yr, male-female ratio 1.5) were obtained during endoscopy. Random colonic biopsies were collected in healthy subjects, and histological examination was normal in all these individuals. We found a strongly increased spontaneous release of the neutrophil activation marker S100A12 in patients with active CD (16.6 ± 8.2 ng/ml SE) compared with healthy controls (0.5 ± 0.3 ng/ml SE; $P < .001$) (A). Elevated sRAGE levels were also found in supernatants from bowel tissue of CD patients having active disease (145.9 ± 9.1 pg/ml SE) compared with noninflamed tissue of healthy controls (125.7 ± 6.4 pg/ml SE) (B). However, this trend was statistically not significant (n.s., $P = 0.169$). Human blood monocytes which were stimulated for 4 h with S100A12 ($10 \mu\text{g/ml}$) showed higher levels of TNF- α expression (n-fold 22.9 ± 11.9) compared with monocytes stimulated for 4 h with S100A12 ($10 \mu\text{g/ml}$) and sRAGE ($40 \mu\text{g/ml}$) (n-fold 9.7 ± 2.6) (C), indicating that sRAGE protects from activation of inflammatory cells by S100A12.

jects with the -374A RAGE promoter genotype as indicated from serum data, may compensate. In line with that, we could confirm that sRAGE protects from activation of inflammatory cells by S100A12 (Fig. 5C).

DISCUSSION

RAGE and its proinflammatory S100 ligands are reported to be enriched in joints of subjects with rheumatoid arthritis and to amplify the immune/inflammatory response (22). In human subjects, a case-control study demonstrated an increased prevalence of the 82S allele in patients with rheumatoid arthritis compared with control subjects. However, other studies also showed a negative association of this SNP with diabetic complications (25, 33). For two promoter SNPs, an enhanced transcriptional activity has been shown, which may lead to increased levels of circulating RAGE. It has been shown that circulating sRAGE acts as a decoy receptor that neutralizes proinflammatory endogenous ligands (18, 24, 37, 48). On the other hand, sRAGE may also promote monocyte survival and differentiation (47). An association of RAGE polymorphisms with inflammatory bowel disease is not known so far. Therefore, we analyzed the most relevant functional RAGE polymorphisms (G82S; -374T/A , -429T/C) in two independent samples of patients suffering from CD.

The G82S polymorphism was not associated with CD. However, the minor allele of this polymorphism is very rare: the allele frequency was below 5% in all study samples with slightly higher allele frequencies in the CD cases compared with the controls. Thus studies in much larger samples will be necessary to evaluate whether the G82S polymorphism has any impact on CD risk or not.

We found also no association of the 429T/C RAGE polymorphism with CD in the case-control analysis. TDT showed a CD transmission disequilibrium for -429T/C in the German ($P = 0.016$) and US-American ($P = 0.040$) sample. However, the number of transmissions observed in the US sample was rather small and the positive TDT result in the German sample might be due to cotransmission with the 374T/A polymorphism, which is located only 55 bases downstream of the 429T/C polymorphism. The case-control analysis revealed that the 429T/C polymorphism is not associated with CD risk.

In contrast, the 374T/A RAGE polymorphism was associated with CD in the German study samples. The -374A allele was significantly less frequent in German patients with CD compared with controls. There was no association in the US-American sample. Analysis of the association of RAGE polymorphism -374T/A with CD stricturing behavior in CD patients showed a significant association in the German sample ($P = 0.04$), whereas a trend for an association was observed in the US-American sample ($P = 0.07$). Interestingly, the German study sample comprises 64% CD patients that suffer from stricturing disease whereas in the US-American sample most of the patients are not affected by stricturing behavior of CD (12% US-American CD patients with stenoses). These differences in disease phenotype might explain the observed differences in association of the RAGE promoter polymorphism -374T/A with CD.

The -374A variant facilitates RAGE gene transcription, which may augment innate immune function and could potentially alter the course of CD. Higher levels of circulating

sRAGE could also confer protection especially from more stenosing disease by blocking DAMPs that play a role in tissue damage (17). Indeed, CD patients with the $-374T/A$ polymorphism had higher levels of sRAGE during remission of disease, which could contribute to less subclinical disease activity (assessed by CD activity index and C-reactive protein serum concentrations), whereas serum concentrations of the proinflammatory RAGE-ligand S100A12 were comparable between both groups. Therefore we hypothesized that augmented promoter activity leads to elevated levels of sRAGE, which serves as an endogenous antagonist by neutralizing the proinflammatory ligand S100A12 (7, 10, 24, 48, 50). This concept is supported by numerous studies reporting anti-inflammatory effects of sRAGE in animal models of inflammation including IBD (21, 51).

Nevertheless, enhanced RAGE expression may protect from CD, especially inflammation with stricturing behavior, by two mechanisms: 1) More RAGE surface expression augments acute innate immune function that helps to compensate for a reduced host defense in CD (43); and 2) more circulating sRAGE neutralizes proinflammatory ligands, thus serving as a countermechanism against tissue damage and chronic intestinal strictures. To address this question, we performed studies in human tissue, animal models, and cell cultures. Our data point to the fact that elevated levels of sRAGE may indeed have some protective effect on the intestinal inflammation. As protective effects of sRAGE have been shown in IL-10 knockout colitis (21), and on the other hand blockade of RAGE signaling ameliorated inflammation in a model of adoptive T cell transfer colitis (44) by inhibiting infiltrating cells, we conclude that the protective effects of increased RAGE expression will be most likely conferred by a more pronounced sRAGE production that may neutralize proinflammatory ligands. Because of insufficient numbers of subject, we were not able to prove whether patients with the $-374A$ RAGE variant have higher sRAGE expression in intestinal tissue that could serve as a protective factor. This will be left for future investigations.

Our study also shows how important replication studies are for genetic variations with low frequencies or only moderate associations. The association of the $-374T/A$ SNP was significant in the German study sample (OR = 0.708, $P = 0.016$) but not in the US-American sample. The differences between the two samples may be due to the fact that in the German sample 61.1% patients showed a stricturing disease behavior, whereas in the US-American sample only 12.3% had a stricturing phenotype. This is in agreement with the age distribution of the two cohorts. Adult patients with long-standing disease were included in the German sample, whereas only pediatric CD patients with primarily an inflammatory phenotype were included in the US-American sample. Thus, because of differences in the disease subphenotypes among the two included samples, a general association of the $-374T/A$ polymorphism with CD or a modulating effect of the degree of inflammation with respect to formation of stenoses needs to be investigated in larger studies on additional populations.

In conclusion, in the study populations analyzed, the G82S polymorphism was not associated with CD. Whereas the $-429T/A$ promoter SNP showed no clear association as well, the $-374T/A$ RAGE promoter polymorphism was associated with CD, especially in the subgroups of patients with stenosis. The promoter polymorphism $-374T/A$ might be a modulator

of the course of the disease rather than a disease causing variant. We conclude that this genetic variant leading to facilitated RAGE gene transcription may to some degree protect from developing CD with severe stricturing behavior primarily by increasing levels of soluble RAGE, which neutralizes proinflammatory mediators that have a role in chronic intestinal tissue damage.

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GRANTS

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DISCLOSURES

The authors declare no potential competing interests.

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