

Role of the advanced glycation end products receptor in Crohn's disease inflammation

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and helped in drafting the manuscript; Falcone C helped in the conception of the study and in drafting the manuscript; Ardizzone S was clinically responsible for the A series patients, and critically revised the article for important intellectual content; Fociani P helped in collecting the histological samples of the A series patients, evaluated all histological preparations in a blind manner, and critically revised the article for important intellectual content; Danelli P carried out all surgical procedures of the A series patients, and critically revised the article for important intellectual content; Corazza GR critically revised the article for important intellectual content; all the authors reviewed and approved the final version to be published.

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Abstract

AIM: To investigate the level of mucosal expression and the involvement of the receptor for the advanced glycation end products (RAGE) in delayed apoptosis and tumor necrosis factor (TNF)- α production in Crohn's disease (CD).

METHODS: Surgical and endoscopic specimens from both inflamed and non-inflamed areas of the ileum and/or colon were collected from 20 and 14 adult CD patients, respectively, and used for the assessment of RAGE expression by means of immunohistochemistry and western blotting analysis. Normal tissues from 21

control subjects were used for comparison. The same polyclonal anti-human RAGE antibody (R and D System) was used in all experimental conditions. RAGE staining was quantized by a score including both the amount of positive cells and intensity of immunoreactivity; cellular pattern was also described. The effects of RAGE blocking on apoptotic rate and TNF- α production were investigated on immune cells freshly isolated from CD mucosa and incubated both with and without the muramyl dipeptide used as antigenic stimulus. Statistical analysis was performed *via* the test for trend, with regression models to account for intra-patient correlations. A 2-sided $P < 0.05$ was considered significant.

RESULTS: In inflamed areas, RAGE expression in both the epithelial and lamina propria compartments was higher than control tissues ($P = 0.001$ and 0.021 , respectively), and a cluster of positive cells were usually found in proximity of ulcerative lesions. Similar results were obtained in the lamina propria compartment of non-inflamed areas ($P = 0.025$). The pattern of staining was membranous and granular cytosolic at the epithelial level, while in the lamina propria it was diffuse cytosolic. When evaluating the amount of protein expression by immunoblotting, a significant increase of both surface area and band intensity ($P < 0.0001$ for both) was observed in CD inflamed areas compared to control tissue, while in non-inflamed areas a significant increase was found only for band intensity ($P < 0.005$). Moreover, a significantly lower expression in non-inflamed areas in comparison with inflamed areas was found for both surface area and band intensity ($P < 0.0006$ for both). Finally, RAGE blocking largely affects both the apoptotic rate of mucosal cells (towards an increase in both non-inflamed and inflamed areas of $P < 0.001$ and < 0.0001 , respectively) and TNF- α secretion (towards a decrease in both non-inflamed and inflamed areas of $P < 0.05$ and < 0.01 , respectively), mainly in the presence of antigenic stimulation.

CONCLUSION: RAGE is up-regulated in CD, especially in inflamed areas, and it appears to play a role in the mechanisms involved in chronic inflammation.

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Key words: Apoptosis; Crohn's disease; Chronic inflammation; Immunohistochemistry; Receptor for advanced glycation end products; Tumor necrosis factor- α

Core tip: Receptor for the advanced glycation end products (RAGE) is a multiligand transmembrane receptor whose activation sustains chronic inflammation. The inhibition of RAGE-ligand interaction has proved successful in an experimental model of Crohn's disease (CD). Our work shows an up-regulation of RAGE expression in both inflamed and non-inflamed mucosa of CD patients in comparison to healthy tissue from control subjects. Moreover, RAGE blocking significantly affects both the apoptotic rate and tumor necrosis factor- α secre-

tion of mucosal immune cells, which are considered to be leading mechanisms in the chronic inflammation of CD. These findings pave the way for a possible use of RAGE blocking agents as a new therapeutic tool in this disabling condition.

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INTRODUCTION

Dysregulation of immune tolerance towards components of the intestinal microbiota plays a crucial role in the pathogenesis of Crohn's disease (CD)^[1]. Recently, the receptor for the advanced glycation end products (RAGE) has received a great deal of attention as a key molecule involved in tissue damage occurring in diabetes^[2], atherosclerosis^[3], neurodegeneration^[4], cancer, and inflammation^[5]. It belongs to the immunoglobulin superfamily and is expressed at low levels by a wide range of differentiated adult cells, including immune cells, but at high levels when activated by its ligands^[6]. Remarkably, thanks to its ability to recognize a three-dimensional structure rather than a specific amino acid sequence, it binds a broad repertoire of molecules^[6]; other than the advanced glycation end products, it is also engaged by amyloid- β peptides, high-mobility group (HMG) B1 proteins, and S100/calgranulins^[6]. Since the latter act as damage-associated molecular pattern molecules^[7], RAGE is now considered a pattern-recognition receptor^[8]. It seems very likely that the interaction of RAGE with both S100/calgranulins and HMGB1-proteins plays a proximal role in the inflammatory cascade by triggering the intracellular synthesis of nuclear factor (NF)- κ B which, in turn, promotes the transcription of several pro-inflammatory and pro-fibrotic cytokines, such as tumor necrosis factor (TNF)- α and transforming growth factor- β , respectively^[9]. Most importantly, RAGE is also able to bind the β 2-integrin Mac-1, thus determining the recruitment and migration of leucocytes to the site of inflammation^[10,11].

As far as CD is concerned, an up-regulation of both RAGE ligands and its mediators has already been found^[12,13], with a prominent role played by TNF- α , since blocking with monoclonal antibodies results in both induction and maintenance of remission^[14]. In addition, the inhibition of the RAGE/HMGB1 pathway in experimental models of colitis led to a significant improvement in both clinical and histological features, with a parallel reduction of the levels of pro-inflammatory cytokines^[15]. The lack of definitive information about a possible role

of this receptor in human inflammatory bowel disease has prompted us to study RAGE expression in the intestine of CD patients through both immunohistochemistry and western blotting assays, and to investigate its functional role in affecting the apoptotic rate and TNF- α production of mucosal immune cells, which are key mechanisms in causing chronic inflammation and tissue damage^[16].

MATERIALS AND METHODS

Immunohistochemistry

The immunohistochemical study was performed on archival material from the Pathology Department of the Luigi Sacco Hospital (Milan, Italy), obtained from 20 patients who had undergone surgical procedures of small and/or large bowel resection and/or stricturoplasty for CD (indicated hereafter as A series). Their demographic and clinical features, including body mass index (BMI), disease duration, location, and phenotype according to Montreal classification^[17], the CD activity index (CDAI)^[18], and the drug therapy followed, all determined at the time of surgery, are shown in Table 1. In all patients, the diagnosis of CD had been established according to widely accepted criteria^[19] and confirmed at pathology. Specimens from both macroscopically diseased and non-diseased areas were obtained from each patient. Specifically, in order to prevent confounding results due to the proximity to diseased areas, the samples from normal tissue were collected from non-diseased zones at least 50 cm away from the resected area. In addition, surgical specimens from 9 subjects (M/F: 4/5, median age: 65 \pm 8.32 years, range: 63-86 years; BMI: 21.8 \pm 1.2 kg/m², range: 18.1-24.8 kg/m²) who underwent right hemicolectomy for neoplasms, were selected from macroscopically healthy mucosa of both ileum and colon at more than 20 cm from the lesion, and used as controls. Each patient and control signed the informed consent, and all tissues were studied in an anonymous manner in accordance with the recommendations of the local Bio-Ethics Committee.

After tissue sampling during surgery to avoid autolysis artifacts, all specimens were immediately fixed in 40 g/L formaldehyde and embedded in paraffin. Serial 4- μ m-thick sections were cut from the blocks, mounted on electrostatically charged slides (DIAPATH Super Frost Plus[®], Menzel-Gläser, Braunschweig, Germany) and dried overnight. After dewaxing and rehydration, the sections were processed for both traditional histology and immunohistochemistry. In addition, samples from both colonic adenomatous polyps^[20] and normal lung tissue^[21] were used as positive controls to test both the appropriate pre-treatment and primary antibody dilution. After 0.88 mol/L hydrogen peroxide was applied for 5 min, sections were treated with Tris/EDTA buffer (Target Retrieval Solution, DAKO, Carpinteria, CA, United States) at 1:10 dilution and pH 9.0, and then incubated in a microwave oven in citrate buffer (10 mmol/L, pH 7.0) for three cycles of 5 min each at 650 W, to unmask the antigen. Afterwards,

Table 1 Demographic and clinical features of Crohn's disease patients *n* (%)

Characteristics	A series (<i>n</i> = 20)	B series (<i>n</i> = 14)
Sex		
Male	12	6
Female	8	8
Age (yr)		
Median (range)	41 (25-61)	40 (21-68)
BMI (kg/m ²)		
Median (range)	25.5 (20-30)	20.7 (17.2-21.9)
Disease duration (mo)		
Median (range)	75 (1-485)	64 (24-360)
Disease location ¹		
L1	14	5
L2	1	2
L3	4	7
L4	None	None
L1-L4	1	None
Disease behavior ¹		
B1	None	6
B2	11	2
B2-B3	8	6
B3	1	None
CDAI		
Median (range)	250 (151-537)	244 (141-296)
Current therapies		
Patients <i>n</i>		
Mesalazine	8 (40)	3 (21.43)
Steroids	2 (10)	3 (21.43)
IS	3 (15)	3 (21.43)
IS + mesalazine	1 (5)	2 (14.29)
Steroids + mesalazine	1 (5)	1 (7.14)
Infliximab	4 (20)	2 (14.29)
IS + infliximab	1 (5)	0

¹Montreal classification^[17]. BMI: Body mass index; CDAI: Crohn's disease activity index; IS: Immunosuppressors.

the slides were incubated with CAS-Block solution (Invitrogen Corporation, Carlsbad, CA, United States) for 10 min, as this treatment had been already proved successful in avoiding unspecific ligands^[22]. The primary antibody we used was the polyclonal anti-human RAGE antibody (R and D System, Minneapolis, MN, United States) at 1:1000 dilution overnight at 4 °C^[22,23]. Finally, sections were soaked in a stop-wash buffer, rinsed in phosphate-buffered saline, and then incubated with the *Avidin Biotin Complex* (LSAB2 System, HRP, DAKO), followed by the usual reactions to allow color development (Liquid DAB+ Substrate Chromogen System, DAKO) and counterstaining with Harris hematoxylin (Sigma-Aldrich, St. Louis, MO, United States). For the negative control, a goat anti-human IgG1 isotype (R and D System) was used as a primary antibody^[23], while in order to determine the origin of lamina propria RAGE⁺ cells, seriate sections were processed with the following monoclonal antibodies: anti-CD3 (clone PSI, Novocastra Laboratories, Newcastle, United Kingdom), -CD20 (clone L26, DAKO), -CD138 (Clone B-A38, IQ Products, Houston, TX, United States), -CD68 (clone PG-M1, DAKO), and smooth muscle actin (clone 1A4, DAKO), according to the manufacturers' instructions, whilst neutrophils were identified

through morphology. Finally, in order to quantize RAGE expression, the analysis was split into two compartments, epithelial layer and lamina propria, and all sections were examined by two blinded pathologists under high-power field microscopes (HPFs) (*Nikon ECLIPSE E800*) at constant magnification ($\times 400$). As regards the epithelial layer, the number of positive cells was calculated by using a differential count of at least 500 cells and the results were expressed as a percentage, while the intensity of the staining was estimated by applying an ordinal scale (0-3), where "3" indicated strong staining, "2" moderate staining, "1" weak staining, and "0" no staining. Thereafter, both results were combined together in a score as follows: grade I when less than 10% of epithelial cells were positive and/or had weak immunoreactivity, grade II when 10%-50% of epithelial cells were positive and/or had moderate immunoreactivity, and grade III when more than 50% of epithelial cells were positive and/or had strong immunoreactivity. In the lamina propria compartment, the quantization was performed by applying differential counts of positive cells on 10 HPF per slide, and the results were displayed as the mean number of positive cells. The same ordinal scale and grading score of the epithelial compartment (see above) for quantization of staining intensity and whole expression, respectively, were used. Finally, the pattern of cellular staining was reported as cytosolic (granular or diffuse) and/or membranous.

Western blot analysis

Two snap-frozen perendoscopic specimens from both macroscopically diseased and non-diseased areas of the colonic mucosa were collected from 14 CD outpatients (indicated hereafter as B series), whose demographic and clinical features are shown in Table 1, and 12 control patients suffering from irritable bowel syndrome (M/F: 3/9; median age: 49 years, range: 21-72 years; BMI: 23.35 kg/m², range: 18.6-25.6 kg/m²), all admitted to the Center for the Study and Cure of Inflammatory Bowel Disease at the Fondazione IRCCS Policlinico (Pavia, Italy). The CD patients were taking mesalazine, steroids, azathioprine, or biologics, as single or combined therapy (Table 1), whilst the control patients were drug-free. Each patient gave written informed consent, and approval from the Bio-Ethics Committee was obtained (protocol number: 20110002492).

Mucosal samples were lysed in 200 μ L ice-cold lysis buffer (53.8 μ L HEPES pH 7.9 0.93 mol/L, 10 μ L EDTA pH 8.0 0.5 mol/L, 300 μ L KCl 1 mol/L, 100 μ L Nonidet P-40, 4385 μ L distilled water, 50 μ L DTT 0.1 mol/L, 50 μ L PMSF 0.1 mol, 20 μ L aprotinin, 10 μ L leupeptin, 50 μ L NaVO₄ 0.1 mol/L, 10 μ L NaF 0.5 mol/L), sonicated (Sonifier 150, BRANSON, St. Louis, MO, United States), and incubated for 90 min in the dark. After centrifugation, the supernatants were harvested and the protein concentration was determined using the *Bio-Rad Protein Assay* (BIORAD, Hercules, CA, United States). A total of 50 μ g of protein from each sample, plus the loading buffer, were boiled and then

loaded onto 0.347 mol/L sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Invitrogen-Life Technologies, Carlsbad, CA, United States) and run for 90 min. Proteins were then transferred to nitrocellulose sheets by means of the *PowerEase® 500* electrophoresis instrument (Invitrogen), and non-specific binding was blocked with 50 g/L non-fat milk powder in TBS 100 mL/L solution. Afterwards, the membranes were incubated with the primary antibody (polyclonal anti-RAGE immunoglobulin G 1:2000 dilution, R and D System) overnight on a rocker (Stuart SSL4, Fisher Scientific, Illkirch Cedex, France). After washing, the secondary antibody (polyclonal rabbit anti-goat immunoglobulin G-HRP 1:2000 dilution, DAKO) was applied, and the reaction was developed using a chemiluminescence kit (ECL Plus, GE Healthcare, Buc Cedex, France). Finally, the membranes were aligned with the film (GE Healthcare) in the developing liquid (Kodak, Rochester, New York, NY, United States). Negative control blots were obtained by omitting the primary antibody. Finally, blots were stripped and analyzed for β -actin as an internal control, using a rabbit anti-human polyclonal anti- β -actin antibody 1:5000 dilution (Abcam, Cambridge, United Kingdom). Films were acquired using VersaDoc 3000 (BIORAD), and the bands measured in terms of both surface area and intensity by the QuantityOne software (BIORAD) and normalized for β -actin values.

Functional assay

Six fresh additional perendoscopic mucosal biopsies from both macroscopically diseased and non-diseased colonic areas and peripheral blood samples were collected from seven out of the 14 CD outpatients. After digestion with collagenase A 1 mg/mL (Sigma-Aldrich) in complete medium (X-VIVO-15 plus 50 mL/LHS, penicillin/streptomycin 10000 U/10 mg/mL, gentamicin 2.5 mL/L, amphotericin B 4 mL/L, all by Lonza Group Ltd, Basel, CH) for 90 min, the cellular suspension was passed through a cell filter strainer, 40 μ m (BD Falcon™, Franklin Lakes, NJ, United States), centrifuged, and washed twice. The lamina propria mononuclear cells (LPMC) thus obtained were plated overnight in 48-well flat bottom tissue culture plates (Sarstedt, Newton, NC, United States) at 1.0×10^6 cells/well in complete culture medium plus interleukin-2 40 U/mL (Chiron, Emeryville, CA, United States). After washing, 0.5×10^6 LPMC for each well were incubated in the presence of the anti-RAGE antibody or IgG1 isotype antibody (R and D System) as a negative control, at 50 and 100 μ g/mL for 4 h, and then stimulated overnight in the presence or absence of muramyl dipeptide (MDP) (10 μ g/mL, Sigma-Aldrich, St. Louis, MO, United States) plus 1.5×10^6 autologous irradiated (3000 rads) peripheral blood mononuclear cells used as feeding cells. Finally, the supernatants were collected and stored at -80 °C for evaluation of TNF- α levels by enzyme-linked immunosorbent assay (R and D System), while LPMC were harvested for the detection of the apoptotic rate by flow cytometry (BD FACSCanto, FACS Diva software, BD

Table 2 Receptor for the advanced glycation end products expression in Crohn's disease diseased areas *vs* control tissues *n* (%)

Score	Patients	Controls	<i>P</i> value
Grading (epithelium)			0.001
I	2 (20)	8 (80)	
II	6 (86)	1 (14)	
III	6 (100)	0 (0)	
Grading (lamina propria)			0.021
I	7 (44)	9 (56)	
II	2 (100)	0 (0)	
III	4 (100)	0 (0)	
Intensity (epithelium)			0.480
0	2 (67)	1 (33)	
1	3 (43)	4 (57)	
2	9 (69)	4 (31)	
Intensity (lamina propria)			0.260
0	4 (50)	4 (50)	
1	4 (50)	4 (50)	
2	5 (83)	1 (17)	
Epithelial positive cells	% (median: 25 th -75 th)	5 (5-5)	0.008
	55 (20-70)		
Lamina propria positive cells	<i>n</i> /10 hpf (median: 25 th -75 th)	1 (0-2)	0.030
	5 (1-15)		

Biosciences, San Jose, CA, United States) by using the Annexin V/Propidium Iodide kit (*APOTEST™-FITC*, DAKO).

Statistical analysis

Continuous variables were described as median and 25th-75th percentiles, while categorical variables were expressed as counts and percentages. Comparison between data from CD patients and control subjects was performed by using the test for trend and the Mann-Whitney *U* test, as appropriate. Data among CD patients were compared using a general linear or a general ordinal logistic model (according to the type of data), with calculation of the Huber-White robust SE for intra-patient correlation. The Spearman rank correlation test was applied to measure the association between continuous variables. A 2-sided *P* value ≤ 0.05 was considered to be statistically significant. Stata 12.1 (StataCorp LP, College Station, TX, United States) was used for computation.

RESULTS

RAGE expression in diseased areas

In both the epithelial and lamina propria compartments, the grading of RAGE expression in CD was significantly higher than in control tissues (test for trend: *P* = 0.001 and 0.021, respectively; Table 2, Figure 1A and B). When splitting the analysis for the two variables considered, there appeared to be no difference in intensity between the groups in either compartment (Table 2, Figure 1C and D), whilst the amount of positive cells, either at epithelial (Figure 2) or lamina propria level (Figure 3), was significantly higher in CD than in control tissues (test for trend: *P* = 0.008 and 0.030, respectively; Table 2, Figure 1E and F). Remarkably, in CD specimens, most of the strongly

positive cells were found in close proximity to ulcers at epithelial level (Figure 4), while in the lamina propria they were sparsely distributed (Figure 3A and B). Moreover, in all CD samples, the pattern of RAGE expression at epithelial level was both membranous and granular cytosolic (Figure 2B, thin and thick red arrows, respectively), while in the lamina propria it was diffuse cytosolic (Figure 3A and B, black arrows). As regards the type of lamina propria positive cells, the vast majority were plasma cells (median 3, 25th-75th: 1-10) (Figure 5A) and monocytes/macrophages (median 3, 25th-75th: 2-12) (Figure 5B), with only a few scattered T lymphocytes and subepithelial myofibroblasts being stained, whilst neutrophils did not display RAGE expression (data not shown). When evaluating the amount of protein expression at mucosal level by immunoblotting, a significant increase of RAGE expression for both surface area and band intensity (*P* < 0.0001 for both) was observed in CD diseased areas with respect to control tissue (Figure 6). Finally, as regards the clinical features, no correlation was found between the grading of RAGE expression with BMI, CDAI score, or the duration, localization, or behavior of the disease, nor with the drug therapy followed (data not shown).

RAGE expression in non-diseased areas

In both the epithelium and lamina propria compartments, the grading of RAGE expression in CD tissues was generally higher than in control tissues (Figure 1), though statistical significance was achieved only in the lamina propria compartment (test for trend: *P* = 0.025, Table 3). Similarly, no difference between these two groups was found either in intensity (Figure 1) or in the number of positive cells (Figure 1 and Table 3). Within the CD patient group, both grading and amount of RAGE⁺ cells tended to be higher in the diseased areas, though significant variations were never reached (Figure 1 and Table 4). As far as the level of RAGE expression was concerned, a significant increase in non-diseased areas of CD patients in comparison with control tissues was observed only for band intensity at Western blot analysis (*P* < 0.005, Figure 6). Moreover, a significantly lower expression in non-diseased areas in comparison with diseased areas was found for both surface area and band intensity (*P* < 0.0006 for both, Figure 6). Finally, a positive correlation between the grading of RAGE expression at epithelial level and BMI was found in the CD group (median 25.5 ± 3.2, range: 20-30, *P* = 0.0116, *r* = 0.55, Figure 7), whilst no correlation was found with the CDAI score, behavior, localization, duration of the disease, nor with current therapies (data not shown).

Functional assay

In order to investigate whether RAGE blocking has the ability to favor LPMC apoptosis and to reduce the production of the pro-inflammatory cytokine TNF-α, LPMC from both diseased and non-diseased areas of CD patients were incubated with the RAGE blocking antibody and stimulated with MDP, a component of the bacterial cell-wall peptidoglycan, which is present in most

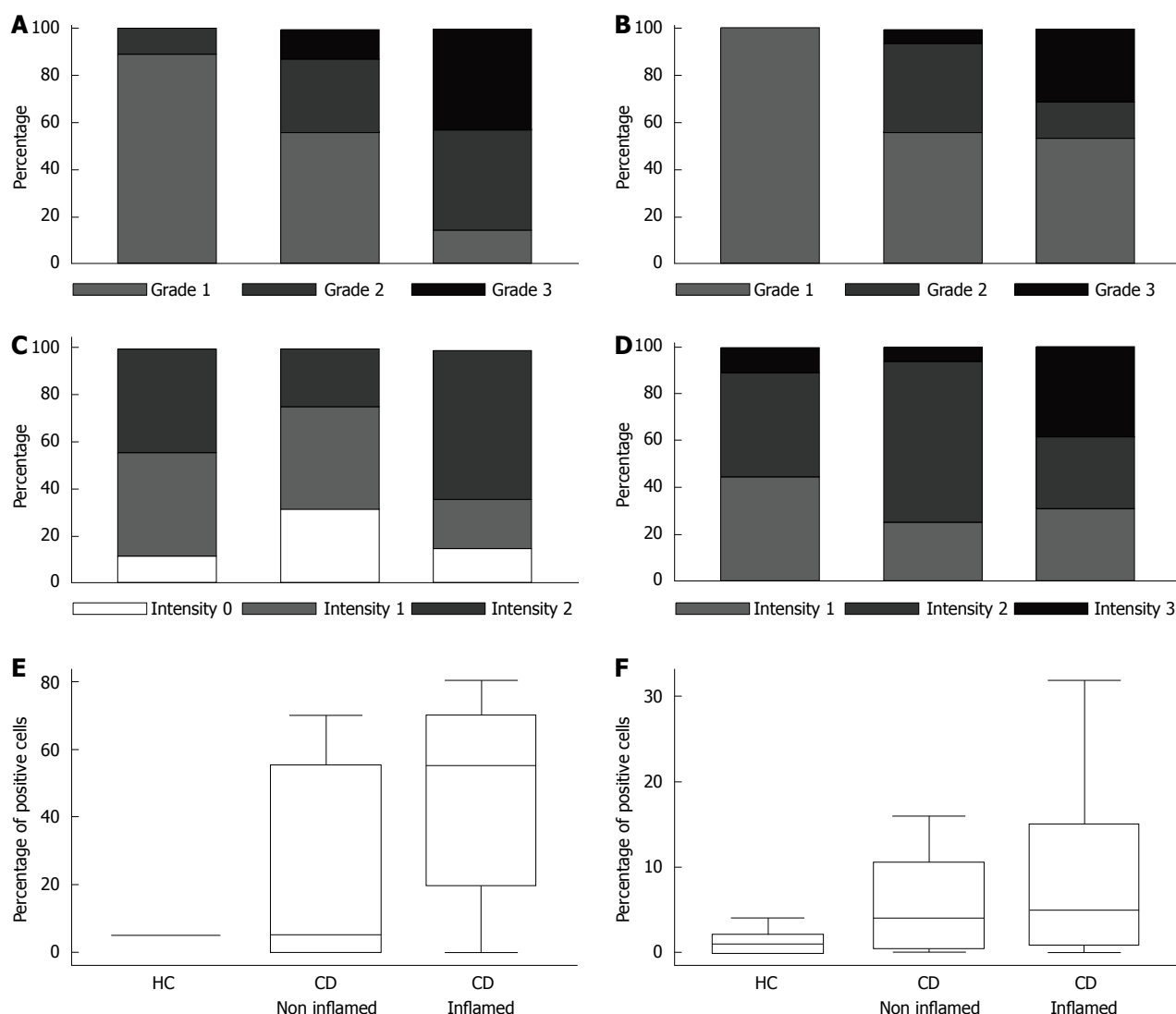


Figure 1 Receptor for the advanced glycation end products expression. The percentage of samples in each category of both grading (A and B) and intensity (C and D) staining, and the number of positive cells (E and F) are reported. The average values of receptor for the advanced glycation end products (RAGE) immunoreactivity detected at the epithelial (A and C) and the lamina propria (B and D) compartments of patients suffering from Crohn's disease (CD) and the control subjects (HC) are given. In CD patients, the averages of assessments between the areas of diseased mucosa and the areas of non-diseased mucosa were also compared. Moreover, the number of positive cells in the epithelial compartment (E) was calculated by a differential count of at least 500 cells and the results expressed as a percentage, while in the lamina propria (F) compartment, it was performed by differential counts of positive cells on 10 high-power microscopic fields per slide ($n/10$ HPF), and the results displayed as the mean number of positive cells. A higher, but not statistically significant, number of RAGE+ cells were observed in both diseased and non-diseased areas of CD patients in comparison with normal tissue of HC. The box-plots in panels E and F show median, 25th, and 75th percentile and extremes.

species of the gut microbiota, and whose ligation with the intracellular receptor NOD2 triggers inflammatory cascade in CD^[24]. As expected, the apoptotic rate of the cell population obtained from non-diseased areas was higher than that found in cells isolated from diseased mucosa, both in the presence and absence of the antigenic stimulus ($P < 0.001$ for both, Figure 8A and B). When incubating the LPMC from non-diseased areas with the lower concentration of the RAGE-blocking antibody, a significant increase in the apoptotic rate in comparison with the basal values was observed both in the absence ($P < 0.01$) and presence of MDP ($P < 0.001$), while the higher dose only slightly increased the percentages of cell death in both cases (Figure 8A and B). Similar effects

were observed on cells from diseased areas only when applying the higher dose of the RAGE blocking agent ($P < 0.0001$ for both), since the lower dose was effective only in the presence of MDP stimulus ($P < 0.005$, Figure 8A and B). As far as TNF- α secretion is concerned, in the absence of antigenic stimulation, a higher basal level was observed in cultures with cells from diseased mucosa ($P < 0.0002$), and this was consistently reduced by the higher dose of the anti-RAGE agent ($P < 0.01$, Figure 8C). Upon antigenic stimulation, both doses of the blocking antibody appeared capable of lowering the level of TNF- α production, both when using cells from diseased ($P < 0.01$ and < 0.005 , respectively) and non-diseased areas ($P < 0.05$ and < 0.01 , respectively, Figure 8D).

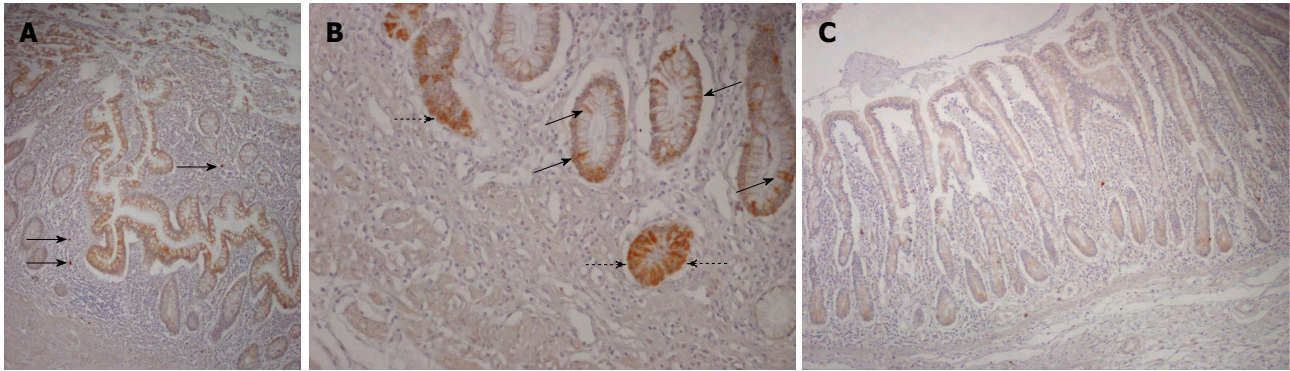


Figure 2 Receptor for the advanced glycation end products staining in Crohn's disease and healthy mucosa. A: Almost all cells of the epithelial compartment at both crypt and surface levels showed positive immunostaining (brown cells) in a representative case of Crohn's disease affected areas, with only a few scattered positive cells in the lamina propria (black arrows); B: The pattern of the cellular staining in the crypt zone of a representative case of Crohn's disease affected area is shown, indicating both membranous and cytosolic distribution; C: A representative case of control mucosa is shown, with weak staining of the epithelial cells and only a few scattered positive cells in the lamina propria (receptor for the advanced glycation end products, immunoperoxidase-hematoxylin; original magnification, $\times 200$).

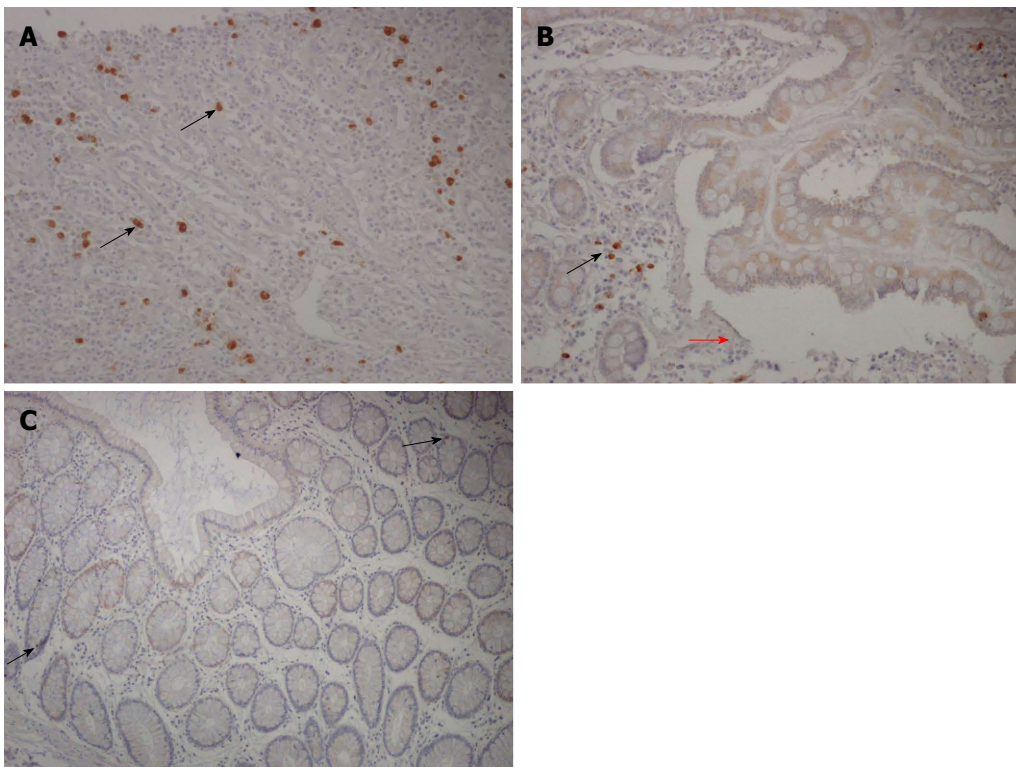


Figure 3 Receptor for the advanced glycation end products staining in Crohn's disease and healthy mucosa lamina propria. A: Many lamina propria mononuclear cells proved receptor for the advanced glycation end products (RAGE)-positive (the brown cells) in a representative case of Crohn's disease affected areas; B: A cluster of RAGE-positive lamina propria mononuclear cells next to an ulceration (red arrow) in a representative case of Crohn's disease affected area showing a cytosolic distribution; C: A representative case of control mucosa is shown, with weak staining of a few lamina propria mononuclear cells (black arrow) (RAGE immunoperoxidase-hematoxylin; original magnification, $\times 200$).

DISCUSSION

Originally identified in diabetic tissues^[25], RAGE was subsequently found to be up-regulated in many pathological conditions^[6] and, in recent years, it also seems to be involved in the pathogenesis of chronic inflammatory diseases^[26], such as rheumatoid arthritis^[27], systemic lupus erythematosus^[28], and multiple sclerosis^[29]. As regards the gastrointestinal tract, RAGE has been found to play a role in chronic gastritis due to *Helicobacter pylori* by favor-

ing its adhesiveness to epithelial cells^[30], as well as in CD, where an increased expression was found in phagocytes infiltrating inflamed areas^[31]. Moreover, the administration of HMGB1 blocking agent has proved to be of benefit in experimental models of Crohn's colitis, through a decrease in the expression of RAGE and related pro-inflammatory cytokines^[15]. On this basis, we first of all investigated RAGE expression by immunohistochemistry on surgical specimens from CD patients where samples of healthy tissue at a certain distance from the workpiece

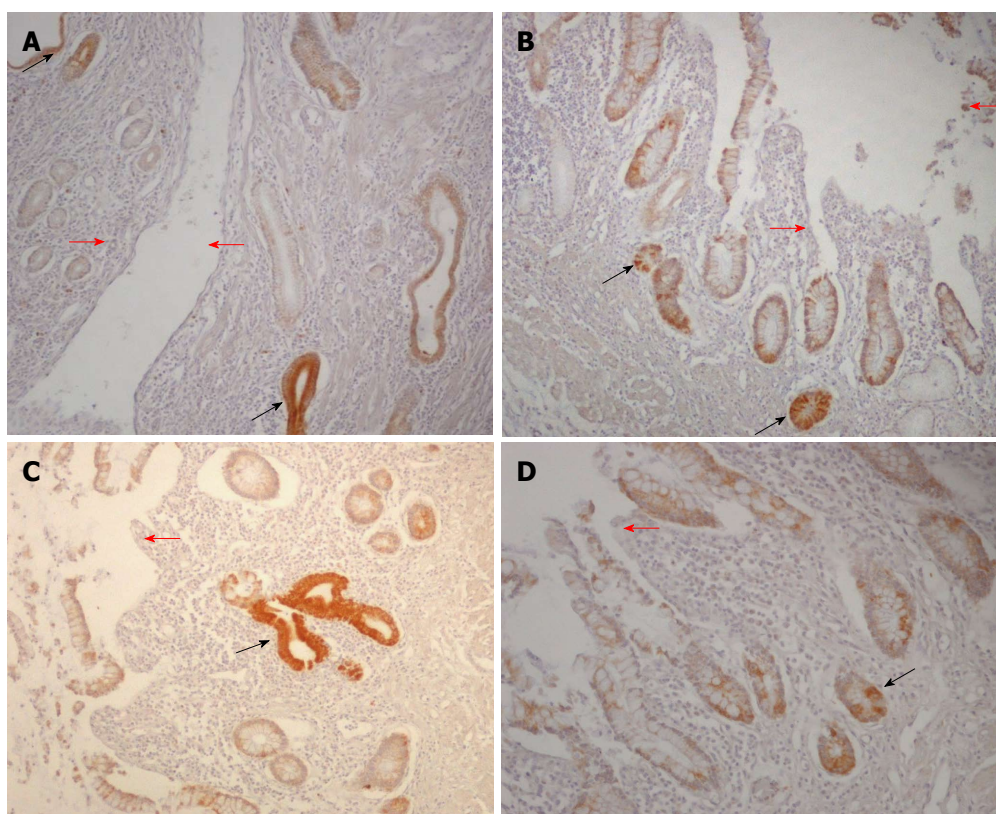


Figure 4 Receptor for the advanced glycation end products staining in Crohn's disease ulcerative areas. A: Almost all cells of the epithelial compartment in close proximity to an ulceration (red arrows) proved receptor for the advanced glycation end products (RAGE)-positive (the brown cells), with the ulcer-associated cell lineage showing the highest immunoreactivity (black arrows); B and C: A detail of the epithelial cells in the crypt compartment at level of an ulceration (red arrows) showing moderate to strong RAGE-positive staining (black arrows); D: A detail of the epithelial cells of the surface compartment next to an ulceration (red arrow) showing strong RAGE immunoreactivity (black arrows) (RAGE immunoperoxidase-hematoxylin; original magnification, $\times 200$).

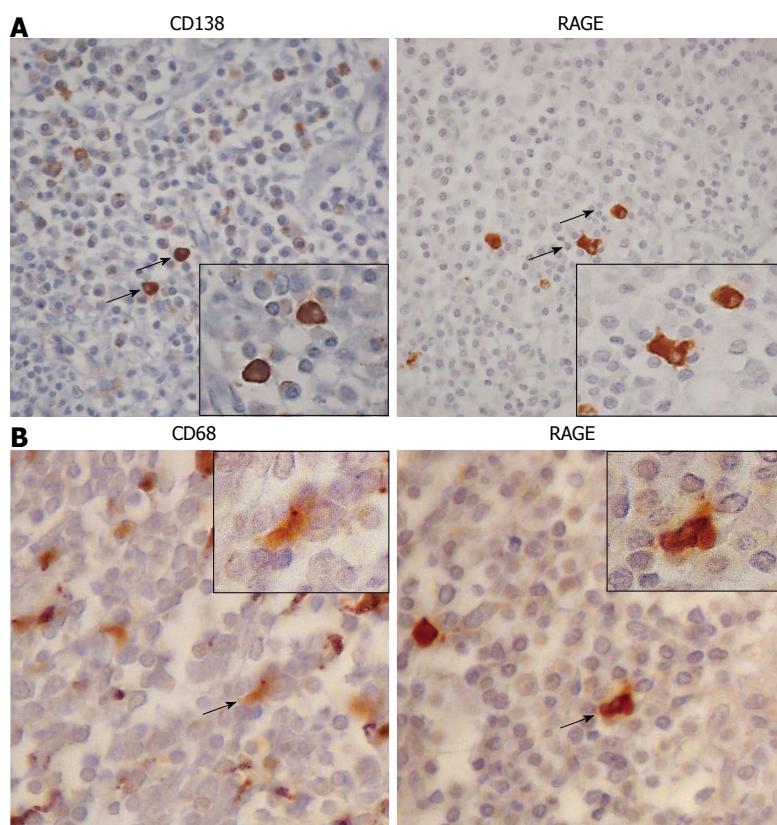


Figure 5 Type of receptor for the advanced glycation end products (+) immune cells. Representative seriate sections of Crohn's disease lamina propria with heavy inflammatory infiltrate, in which the correspondence between the positivity for both CD138 (panel A) indicating the plasma cells, and CD68 (panel B) showing the macrophages, with receptor for the advanced glycation end products (+) cells given (Immunoperoxidase-hematoxylin; original magnification, $\times 200$). The same cellular elements indicated by the black arrows are shown in the boxes at higher magnification (Immunoperoxidase-hematoxylin; original magnification, $\times 400$). RAGE: Receptor for the advanced glycation end products.

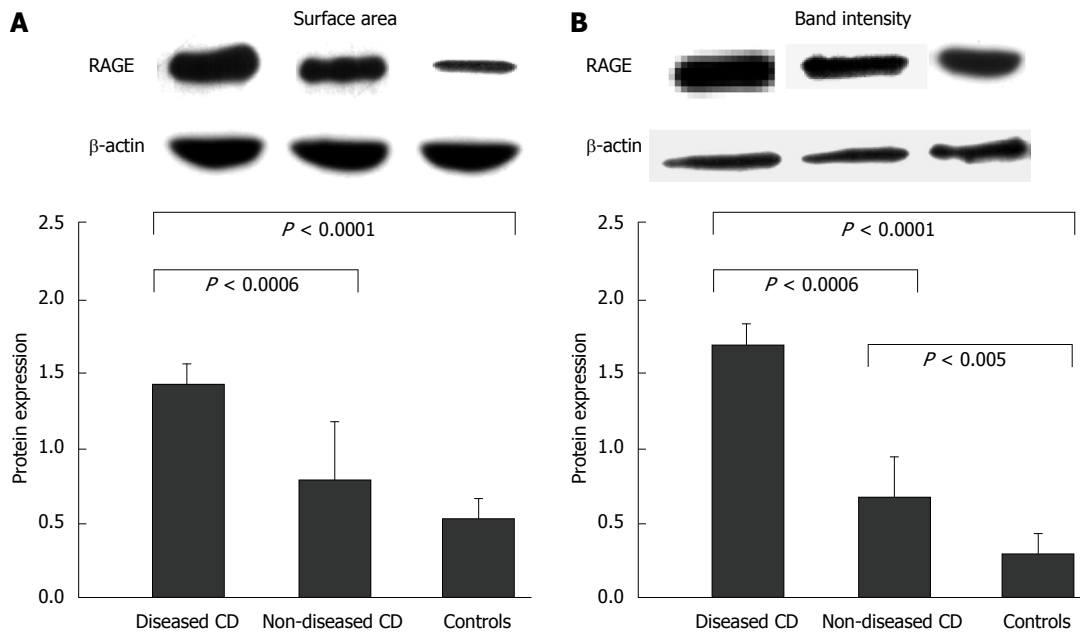


Figure 6 Receptor for the advanced glycation end products expression at mucosal level. Immunoblotting of mucosal samples from both diseased and non-diseased areas of Crohn's disease (CD) patients and from normal areas of control patients with the polyclonal anti-receptor for the advanced glycation end products (RAGE) antibody. The protein levels were measured by scanning densitometry as band area (A) and band intensity (B), expressed as arbitrary units, and normalized towards β -actin levels. In the upper part of both panels, representative cases of band area and intensity with respect to those of β -actin are shown. Specifically, the values for RAGE expression in diseased areas for both band area and band intensity were significantly higher (1.48 ± 0.16 and 1.64 ± 0.14 , respectively) than those found in non-diseased areas (0.71 ± 0.40 and 0.66 ± 0.23 , respectively) and healthy mucosa (0.44 ± 0.17 and 0.27 ± 0.15 , respectively).

Table 3 Receptor for the advanced glycation end products expression in Crohn's disease non-diseased areas vs control tissues *n* (%)

Score	Patients	Controls	<i>P</i> value
Grading (epithelium)			0.091
I	9 (53)	8 (47)	
II	5 (83)	1 (17)	
III	2 (100)	0 (0)	
Grading (lamina propria)			0.025
I	9 (50)	9 (50)	
II	6 (100)	0 (0)	
III	1 (100)	0 (0)	
Intensity (epithelium)			0.210
0	5 (83)	1 (17)	
1	7 (64)	4 (36)	
2	4 (50)	4 (4)	
Intensity (lamina propria)			0.490
0	4 (50)	4 (50)	
1	11 (73)	4 (27)	
2	1 (50)	1 (50)	
Epithelial positive cells	% (median: 25 th -75 th)		0.530
	5 (0-55)	5 (5-5)	
Lamina propria positive cells	<i>n</i> /10 hpf (median: 25 th -75 th)		0.079
	4 (0-10)	1 (0-2)	

Table 4 Receptor for the advanced glycation end products expression in diseased vs non-diseased areas in Crohn's disease patients *n* (%)

Score	Diseased	Non-diseased	<i>P</i> value
Grading (epithelium)			0.17
I	5 (71)	2 (29)	
II	4 (44)	5 (56)	
III	1 (25)	3 (75)	
Grading (lamina propria)			0.53
I	6 (55)	5 (45)	
II	3 (75)	1 (25)	
III	1 (25)	3 (75)	
Intensity (epithelium)			0.37
0	3 (60)	2 (40)	
1	4 (67)	2 (33)	
2	3 (33)	6 (67)	
Intensity (lamina propria)			0.15
0	2 (40)	3 (60)	
1	7 (70)	3 (30)	
2	1 (25)	3 (75)	
Epithelial positive cells	% (median: 25 th -75 th)		0.24
	12.50 (0-50)	40 (20-60)	
Lamina propria positive cells	<i>n</i> /10 hpf (median: 25 th -75 th)		0.24
	4 (1-13)	5 (1-15)	

of intestine were also available. This is why we set out to determine whether the activation of the RAGE pathway occurs only within an inflamed *milieu*, or if it represents a predisposing condition which involves the entire length of the intestine in CD. Our results clearly show an increased grade of RAGE expression, in comparison with control tissues, in the entire bowel of CD patients, albeit predominately in macroscopically damaged areas.

Specifically, the vast majority of epithelial cells in the diseased areas, both on the surface and in crypt regions, express RAGE with a whole cellular staining, compared to control tissues where only a few cells were positive. It is worth noting that the highest numbers of positive cells were found close to ulcerative lesions. In the non-diseased tissue, a trend towards up-regulation was evident, but does not reach the statistical significance. Also

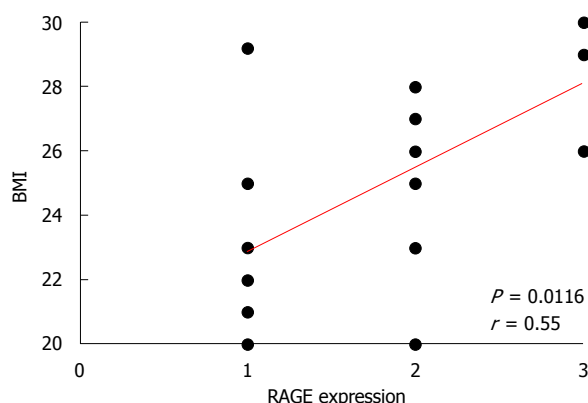


Figure 7 Correlation between receptor for the advanced glycation end products expression and body mass index. A positive correlation between the grading of receptor for the advanced glycation end products (RAGE) expression in the epithelial compartment of non-diseased Crohn's mucosa and body mass index (BMI) was clearly evident.

in the lamina propria, the grade of RAGE⁺ cells was higher in CD diseased areas compared to healthy tissues, although their distribution did not seem to be associated with ulcerative lesions and the pattern of expression was cytosolic. These differences in both distribution and cellular pattern between epithelial and lamina propria cells seem to indicate a separate role of RAGE, with that in the epithelial cells possibly being a response to external injury, while that in the lamina propria cells mostly being involved in magnification of inflammation. Here, the diffuse cytoplasmic staining may be a consequence of the activated status, whereas the granular cytosolic and membranous staining in the epithelial compartment may represent a secretory pathway^[32]. Remarkably, the up-regulation of RAGE in epithelial cells under inflammatory stimuli has been shown to mediate the trans-epithelial migration of neutrophils through its binding with the specific β_2 integrin CD11b/CD18^[33]. It is worth noting that we did not find any extracellular positivity in the stromal area, indicating that there is no storage of RAGE in the extracellular matrix. Furthermore, when splitting the results between the two variables included in the grading system, *i.e.*, the intensity of the staining and the number of positive cells, we found that a significant difference was still evident only for the latter. In the non-diseased tissue of CD patients, a significantly higher grade of RAGE expression was found in the lamina propria compartment compared to control tissue. The reason may lie in the small number of samples and the high variability found mainly in the CD group. The results found at immunohistochemistry were confirmed by the analysis of RAGE expression at protein level by immunoblotting on mucosal samples, where an increase in both diseased and non-diseased areas of CD patients in comparison with healthy tissues from irritable bowel syndrome patients was observed. Moreover, the up-regulation of RAGE expression even in non-inflamed areas suggests that in CD, an unbalanced activation of the RAGE pathway may take place along the entire length of the intestine, reaching the

highest levels in inflamed areas where the concentration of its ligands is higher. In this regard, an up-regulation of both S100A12 and HMGB1 in inflammatory bowel disease has been already shown^[12,13]. In addition, it is recognized that the activation of the RAGE pathway plays a crucial role in regulating apoptosis and autophagy^[34], and in favoring the differentiation of T cells towards a T helper-1 phenotype^[35], both being leading mechanisms in generating tissue damage in CD^[1,36,37]. Therefore, we explored RAGE blocking's ability to affect the delayed apoptosis and TNF- α production of LPMC isolated from diseased and non-diseased mucosa of CD patients, and found that pre-treatment with the anti-RAGE antibody induces a dose-dependent increase of the apoptotic rate, with the effect clearly evident also at a lower dose in the presence of MDP. The latter represents the main bacterial wall component which is recognized by NOD2, an intracellular pattern recognition receptor whose variants are associated with CD^[38]. The binding of MDP to mutated NOD2 is followed by activation of pro-inflammatory pathways mainly regulated by nuclear factor- κ B^[39]. It is interesting to note that the latter molecule is also the transducer of the RAGE pathway upon activation by S100/calgranulins and HMGB1 proteins^[9]. Our further evidence of a significant and dose-dependent decrease of TNF- α production, mainly in those cells from inflamed mucosa following RAGE blocking, fits perfectly into this context. It seems likely, therefore, that a progressive accumulation of RAGE ligands in those tissues primed by genetic and/or environmental factors leads to an increased expression which, in turn, causes magnification rather than dampening of the inflammation^[26,39].

Finally, as far as clinical features are concerned, at variance with the epithelial compartment of the non-diseased areas, where a positive correlation between the grading of RAGE expression and BMI was clearly evident, no correlation was found in diseased areas, probably by virtue of the high concentration of ligands, which *per se* leads to an increased expression that overcomes any systemic regulation^[40]. Also, no correlation with the duration, localization, behavior, activity of the disease, or current therapies was observed in any condition. Notably, in the diseased areas, we did not find any correspondence between the zones of high RAGE⁺ cells density in the epithelial compartment and those in the lamina propria. It is conceivable, therefore, that this might depend on different timing or mechanisms of activation of the RAGE pathway in the two compartments, which deserves further investigation. Also, the limited size of our sample groups, dictated by the need to obtain surgical specimens from macroscopically healthy tissue at a certain distance from the workpiece of intestine, implies that larger studies are needed in order to confirm our evidence.

In conclusion, the evidence we obtained of an increased expression of RAGE on both epithelial and lamina propria cells in CD, mainly in macroscopically injured areas, represents a further step up in the understanding of its pathogenesis, thus paving the way for future thera-

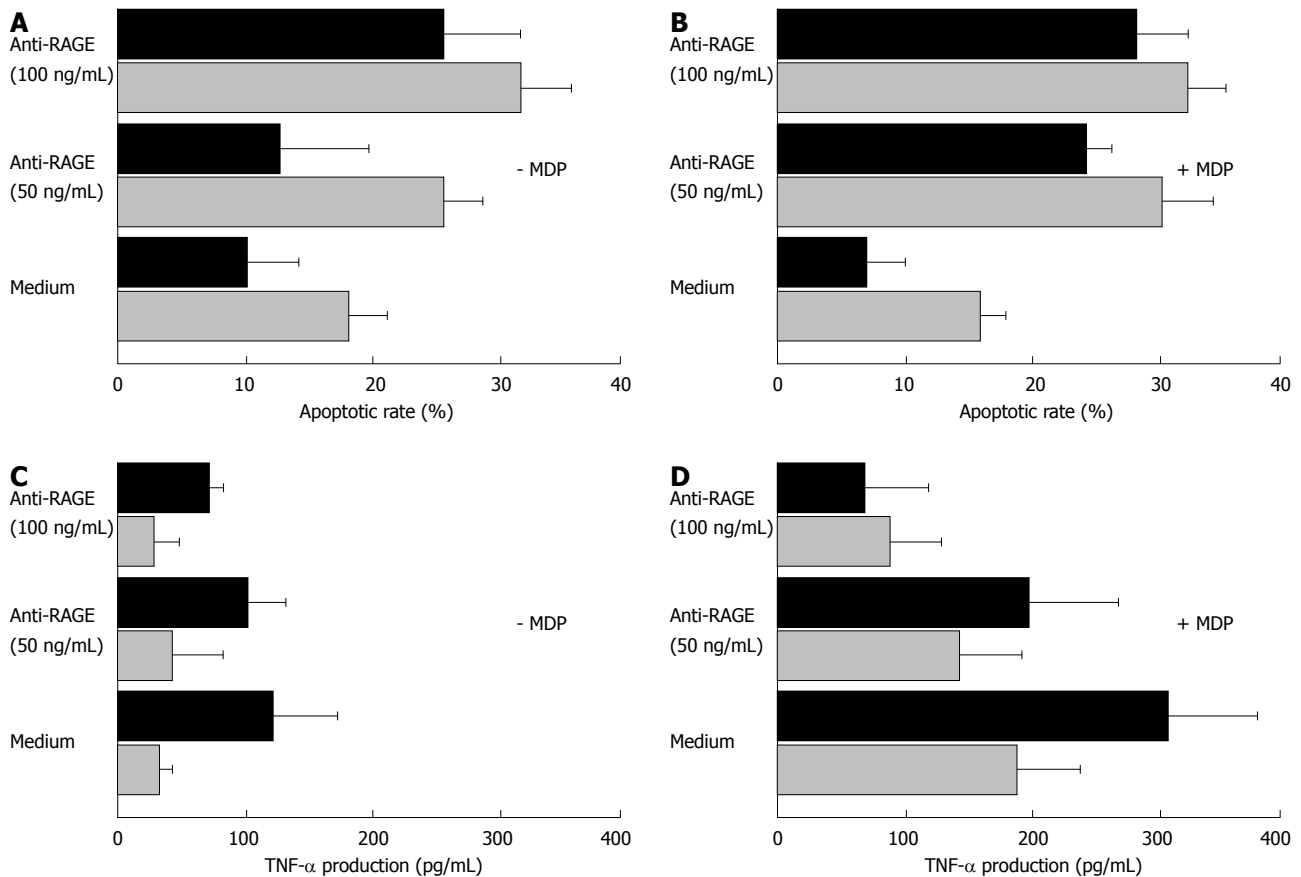


Figure 8 Functional assays. The *in vitro* apoptotic rates of lamina propria mononuclear cells (LPMC) incubated in the absence or presence of two different concentrations of the anti-receptor for the advanced glycation end products (RAGE) blocking antibody, and with or without the muramyl dipeptide (MDP) used as antigenic stimulation are given in panels A and B. The analysis was carried out by flow cytometry, and the mean percentage values \pm SD of at least three experiments for each condition were the following: in the absence of MDP (A), LPMC from non-diseased mucosa (grey bars) showed a spontaneous apoptotic rate of 18.4 ± 3.1 , and a value of 26.9 ± 2.8 and 32.7 ± 4.6 in the presence of 50 and 100 ng/mL concentration of the anti-RAGE blocking antibody, respectively; when using LPMC from diseased mucosa (black bars), a value of spontaneous apoptotic rate of 10.1 ± 4.2 was found, while when incubating with 50 and 100 ng/mL concentration of the anti-RAGE blocking antibody, values of 13.0 ± 6.2 and 26.1 ± 5.5 were found, respectively. In the presence of MDP (B), LPMC from non-diseased mucosa showed a spontaneous apoptotic rate of 15.6 ± 2.1 , and values of 31.9 ± 3.8 and 33.3 ± 2.9 in the presence of 50 and 100 ng/mL concentration of the anti-RAGE blocking antibody, respectively; when using LPMC from diseased mucosa, a value of spontaneous apoptotic rate of 7.8 ± 2.6 was found, while when incubating with 50 and 100 ng/mL concentration of the anti-RAGE blocking antibody, values of 24.1 ± 2.2 and 28.0 ± 4.1 , respectively, were found. The tumor necrosis factor (TNF)- α production of LPMC cultured *in vitro* in the absence or presence of two different concentrations of the anti-RAGE blocking antibody, and with or without the MDP as antigenic stimulation are given in panels C and D. The analysis was carried out by ELISA assay on culture supernatants, and the mean values \pm SD were as follows: in the absence of MDP (C), the cytokine level was 32 ± 14 pg/mL in the cultures with LPMC from non-diseased areas, and 124 ± 48 pg/mL in those with LPMC from diseased areas; when incubating with 50 and 100 ng/mL of the anti-RAGE blocking antibody, values of 41 ± 38 and 27 ± 21 pg/mL for LPMC from non-diseased areas, and of 102 ± 29 and 67 ± 11 pg/mL for LPMC from diseased areas, respectively, were observed. In the presence of MDP (D), the TNF- α level was 184 ± 49 pg/mL in the cultures with LPMC from non-diseased areas, and 307 ± 68 pg/mL in those with LPMC from diseased areas; when incubating with 50 and 100 ng/mL of the anti-RAGE blocking antibody, values of 138 ± 50 and 87 ± 41 pg/mL for LPMC from non-diseased areas, and of 198 ± 68 and 71 ± 47 pg/mL for LPMC from diseased areas, respectively, were observed.

peutic use of RAGE blocking agents. Although it does not seem to be the primary cause, once set in motion, the RAGE pathway may be considered to contribute significantly to chronic inflammation.

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COMMENTS

Background

Crohn's disease (CD) is a disabling, lifelong, inflammatory disease which affects the gastrointestinal tract in a discontinuous manner, and whose lesions depend on a dysregulated immune response towards antigens of the gut microbiota. The receptor for the advanced glycation end products (RAGE) is a multiligand transmembrane receptor, whose activation sustains chronic inflammation.

Research frontiers

The inhibition of RAGE-ligand interaction was proved successful in an experimental model of CD. However, the information available on the role of RAGE in human inflammatory bowel disease is scanty.

Innovations and breakthroughs

In this study, the authors show an up-regulation of RAGE expression in the entire bowel of Crohn's patients, although prevalently in macroscopically affected areas, compared to healthy tissue of control subjects, as evaluated by both immunohistochemistry and Western blot analysis. Moreover, RAGE blocking significantly affects both the apoptotic rate and tumor necrosis factor- α secretion of mucosal immune cells, which are the key mechanisms involved in chronic inflammation and tissue damage.

Applications

This study contributes to their understanding of the pathogenesis of CD and provides a scientific basis for future therapeutic use of RAGE blocking agents.

Terminology

RAGE is a multiligand receptor which belongs to the immunoglobulin superfamily, and is natively present on the surface of monocyte/macrophage lineage cells and vascular cells. It is constitutively expressed during embryonic development, but progressively down-regulated in adulthood, where it is present at low levels in most normal tissues. The exceptions are the skin and lungs, where expression remains high throughout life. An increased expression of RAGE was found in a number of different pathological conditions, including chronic inflammatory diseases, neurodegenerative disorders, ageing, and cancer.

Peer review

The authors investigated the involvement of RAGE in CD and found an enhanced expression in both the macroscopically diseased and non-diseased intestine, together with an attenuated inflammatory response of mucosal immune cells following RAGE blocking. The study is well designed and conducted, and the results are clearly presented. This is an excellent study whose only limitation is the relatively small sample size.

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