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Autocrine pathways involving S100A8 and/or S100A9 that are postulated to regulate the immunological functions of macrophages in rats

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ABSTRACT

The development of ulcerative colitis (UC) is closely associated with abnormally functioning macrophages. Rat S100A8 (r-S100A8) and r-S100A9 (S100 proteins) is abundantly expressed in immune cells of myeloid origin, macrophages; however, it remains unclear why r-S100A9 is dominantly expressed in the macrophages of UC rats (UCR). The purpose of this study was to verify the immunological roles of S100 proteins in UCR. We observed the distribution of S100 protein-positive macrophages in the large colons of UCR using a fluorescent immunological staining method, so that S100 protein-positive macrophages were restricted to the rectal tissues of the UCR, and that the mRNA levels of r-S100A8 and r-S100A9 were up-regulated by stimulation with recombinant rat S100A8 (rr-S100A8) alone and rr-S100A9 alone, respectively. When the changes in the mRNA levels of r-S100A8 and r-S100A9 in macrophages were examined in *in vitro* study by PCR and real-time PCR, the mRNA levels of anti-inflammatory and inflammatory cytokines increased selectively after stimulation with rr-S100A8 alone and rr-S100A9 alone, respectively. These results suggest that autocrine signal transduction pathways involving S100 proteins regulate the immunological functions of macrophages to maintain homeostasis in the gastrointestinal tract. This may be depended on expression balance of S100 proteins in macrophages. It is strongly suggested that in UCR the immune functions of macrophages are regulated in a complex manner by r-S100A8 and/or r-S100A9 through undefined autocrine pathways on the cells.

1. Introduction

Inflammatory bowel disease (IBD), which is characterized by recurrent inflammation (sometimes severe) of the gastrointestinal tract, is one of the intractable diseases recognized by the Japanese Ministry of Welfare. Ulcerative colitis (UC) is a type of IBD; however, despite intensive investigation of the condition for three decades, it remains unclear why UC generally affects the rectal region of the large colon. Several hypotheses regarding the development of IBD have been reported; however, none of them have been definitively proven [1]. A lack of regulation of the innate and adaptive immune responses in such serious diseases is probably an important risk factor for the development of IBD. IBD is reportedly caused by abnormalities of the genes involved in innate immunity; i.e., those associated with the recognition and/or processing of bacterial components, such as NOD2/CARD15, IRGM and ATG16L1 [2]. The latter reports suggested that abnormal innate-immune antigen-presenting cell (e.g., macrophages) responses to commensal bacteria are important indicators of the early stages of IBD including UC. In addition, immune cell abnormalities might also provide useful information for assessing the status of IBD patients during follow-up examinations.

Macrophages play a pivotal role in the innate immune response, in which immune cells recognize bacteria and incorporate them or their components in order to maintain intestinal homeostasis by negatively regulating excessive immune responses evoked by commensal bacteria [3–5]. Thus, abnormal intestinal macrophage responses to commensal bacteria might result in chronic intestinal inflammation because macrophages in the intestinal mucosa are heavily involved in maintaining intestinal homeostasis and

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protecting the host from attack by commensal bacteria and/or their components. It was recently reported that macrophage-targeting treatment with tacrolimus ameliorated colonic inflammation in experimental colitis models [6,7]. The pharmacological effects of tacrolimus on T-cells and its mechanism of action have recently been clarified [8,9]. Moreover, tacrolimus reportedly down-regulates the NF-κB pathway and induces apoptosis in activated T cells by activating caspase 3 [10,11].

On the other hand, S100A8 and S100A9 (S100 proteins), which are calcium-binding proteins belonging to the S100 family, are abundantly expressed in immune cells of myeloid origin, such as macrophages and neutrophils, during acute inflammation [12]. S100 proteins are heavily involved in many inflammatory diseases, such as chronic rheumatoid arthritis [12], IBD and acute hepatitis [13,14], and play a pivotal role inside and/or outside of immune cells because they account for more than 40% of the total intracellular protein content of immune cells, particularly neutrophils [15]. However, it remains unclear whether S100 proteins are suppressors or activators of macrophages in rats with dextran sulfate sodium (DSS)-induced UC.

In the present study, we investigated whether peritoneal macrophages are activated by S100A8 or S100A9 *in vitro*, and hence, whether S100 proteins are important risk factors in rats with experimentally induced UC. Finally, we discussed the relationship between S100 proteins expression in immune cells of myeloid origin and the onset of UC.

2. Materials and methods

2.1. Ethics statement

All experiments were performed according to the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society (Tokyo, Japan) and the ethical committee of Tenri Health Care University.

2.2. Materials

DSS was obtained from ENSUIKO Sugar Refining Co. Ltd. (Tokyo, Japan), anti-CD68 monoclonal antibody (ED1) was purchased from Santa Cruz Biotechnology, Inc. Streptavidin (STA)-Texas Red; STA-fluorescein 5-isothiocyanate (FITC); and horse anti-mouse IgG-horse radish peroxidase, -FITC, and -Texas Red conjugates were obtained from Vector Inc. (Burlingame, CA). FITC and Texas-Red labeling kits were acquired from Pierce Biotechnology Inc. (Rockford, IL). Anti-β-actin monoclonal antibody was purchased from Sigma–Aldrich. Anti-rat S100A8 monoclonal antibodies (mAb15E9 and mAb10D11) and anti-rat S100A9 ones (mAb2H6 and mAb8H6) previously prepared were used [15]. Tacrolimus was kindly provided by Astellas Pharmacology Inc. (Tokyo, Japan). All other reagents were obtained from Nacalai Tesque or Wakenyaku Co. Ltd. (Kyoto, Japan).

2.3. Protocols for the animal experiments

2.3.1. Induction of experimental ulcerative colitis in rats using DSS

Japanese Wistar rats (male, 9-week old, 220–250 g/rat) were used in this study. They were kept in captivity for about one week prior to the initiation of the animal experiments and were allowed to free access of animal food and water. Five percent DSS/distilled water (DW) was orally administered to the rats (the UCR group, $n = 25$) for 7 days to induce experimental UC, and it was subsequently replaced with DW for the next three days [3,16]. In another group (the TMR group, $n = 25$), the rats were subjected to the same protocol as the UCR; however, they were also orally administered tacrolimus [3 mg/0.1 ml dimethyl sulfoxide (DMSO)/rat] throughout the 10-day study period. Rats that were orally administered DMSO alone (0.1 ml) every day during the study period were used as a negative control ($n = 5$).

2.3.2. Sample preparation for the protein assay

For the protein assay, the three tissue segments (rectum, middle colon, proximal colon) from each rat were independently homogenized in 50 mmol/l sterilized phosphate buffer solution (pH 7.4) containing 0.9% NaCl using a homogenizer and then centrifuged at 12,000×*g* for 10 min at 4 °C. The resultant supernatants were transferred into 1.5 ml polycarbonate tubes and stored at −80 °C until use.

2.3.3. Isolation of peritoneal macrophages from a rat

The peritoneal macrophages of a Wistar rat were isolated as described by Yoshino et al. [3].

2.4. Methods

2.4.1. Semi-quantitative PCR and real-time PCR

The semi-quantitative polymerase chain reaction (PCR) and real-time PCR (RT-PCR) were carried out as described by Yoshino et al. [3]. The primers used are listed in Table 1.

2.4.2. Preparation and purification of recombinant rat S100A8 and S100A9

The recombinant rat S100A8 (rr-S100A8) and rr-S100A9 were produced and purified according to the method of Ikemoto et al. [13–15]. Single band for rr-S100A8 or rr-S100A9 was detected by SDS–PAGE in the presence and absence of 2-mercaptoethanol (2-ME) according to the method of Towbin et al. [17], and the two recombinant proteins were frozen and stored at −80 °C until use.

2.4.3. SDS–PAGE and Western blotting

To detect the r-S100A8 and r-S100A9 protein bands, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting were carried out as described previously [17]. rr-S100A8 and rr-S100A9 were used as positive controls in these experiments, and β-actin as an internal control.

2.4.4. Fluorescent immunochemical staining

Fluorescent immunohistochemical staining was carried out as described previously [15]. Finally, the fluorescent intensity from the Texas Red, FITC or DAPI was assessed using a fluorescent microscope.

2.4.5. Enzyme-linked immunosorbent assay

Two enzyme-linked immunosorbent assays (ELISAs) for r-S100A8 and r-S100A9 were carried out as described previously [13,15]. In this study, mAb2H6 (5 μg/ml) and mAb15E9 (5 μg/ml) were used as primary antibodies, while mAb8H6-HRP (2 μg/ml) and mAb10D11-HRP conjugates (2 μg/ml) were used as secondary antibodies. r-S100A8 and r-S100A9 in the sera and the colon tissues of experimental rats or culture medium were determined by the ELISAs using rr-S100A8 and rr-S100A9 as standard. The concentration of the two proteins in the serum and culture medium was indicated by ng/ml, while that in the colon tissues by ng/g-wet weight of tissue. However, the concentration of r-S100A8/A9 complexes is indicated by absorbance at 492 nm because it was very difficult to practically prepare rr-S100A8/A9 complexes, as standard, using rr-S100A8 and rr-S100A9 in the study.

Table 1
List of primers used.

Gene	Forward	Reverse	Size (bp)
(a) For semi-quantitative PCR			
S100A8	5′-GCAACTGAACTGGAGAAGGC-3′	5′-GACATATCCAAGGGCCCAG-3′	308
S100A9	5′-GCACGAGCTCCTTAGCTTTG-3′	5′-GACTTGGTTGGGCAGATGTT-3′	408
β-Actin	5′-ACCACAGCTGAGAGGGAAAT-3′	5′-AGAGGTCTTTACGGATGTCAAC-3′	277
(b) For real-time PCR			
S100A8	5′-ACTGGAGAAGGCCTTGAGCAAC-3′	5′-ATCCCTGTAGAGGGCATGGTGA-3′	85
S100A9	5′-TCATGGAGGACCTGGACACAAA-3′	5′-GCAGCTTCTCATGACAGGCAAA-3′	99
β-Actin	5′-TGTGTTGTCCCTGTATGCCTCTG-3′	5′-ATAGATGGGCACAGTGTGGGTG-3′	85
IL-1β	5′-CACCTCTCAAGGAGAGCACAGA-3′	5′-ACGGGTTCCATGGTGAAGTC-3′	81
IL-6	5′-ATATGTTCTCAGGGAGATCTTGGAA-3′	5′-GTGCATCATCGCTGTTCATACA-3′	80
IL-10	5′-GCCAAGCCTTGTCAGAAATGA-3′	5′-TTTCTGGGCCATGGTTCCTCT-3′	75
THF-α	5′-GTGATCGGTCCCAACAAGGA-3′	5′-AGGGTCTGGGCCATGGAA-3′	71
TGF-β	5′-ACCTGCAAGACCATCGACATG-3′	5′-CGAGCCTTAGTTTGGACAGGAT-3′	85

2.5. Statistical analysis

Pair-wise comparisons with the controls were performed using parametric tests. Significant differences between groups were identified using the Student's *t*-test (*t*-test of the difference between the two mean values). Values are means ± SD. *P*-values of <0.05 were considered significant.

3. Results

3.1. Localization of macrophages in the large colons of the UCR and the differential expression of r-S100A8 and r-S100A9

We examined whether the intestinal macrophages in the rectal tissues of the UCR and TMR expressed r-S100A8 and/or r-S100A9. Fluorescent immunochemical staining clearly demonstrated that many immune cells had accumulated in the rectal tissues of the UCR by Day 6 of the experiment (Fig. 1A). Interestingly, r-S100A9 was more dominantly expressed in the immune cells in the rectal tissues of the UCR than r-S100A8 (Fig. 1B, panels B2 and B3); however, most macrophages in the rectal tissues of the TMR co-expressed r-S100A8 and r-S100A9, and their expression levels were almost equal in these cells (data not shown). The above observations strongly suggest that the dominant and differential expression of r-S100A9 in macrophages could be an important indicator of early-stage UC.

3.2. Detection of r-S100A8 and r-S100A9 in the large colons of the UCR and TMR

Small amounts of r-S100A8 (Fig. 2A, panel A1) and r-S100A8/A9 (Fig. 2A, panel A3) complex were detected in the rectal and middle colon tissues of the UCR and TMR on Day 6); however, a much greater amount of r-S100A9 was detected in the limited tissue, rectal tissue, of the UCR (Fig. 2A, panel A2). In addition, Western blotting showed that much more r-S100A9 than r-S100A8 was detected in the rectal tissue of the UCR on Day 6 of the experiment (Fig. 2B, panel B1), but this was not the case in the TMR (Fig. 2B, panel B2).

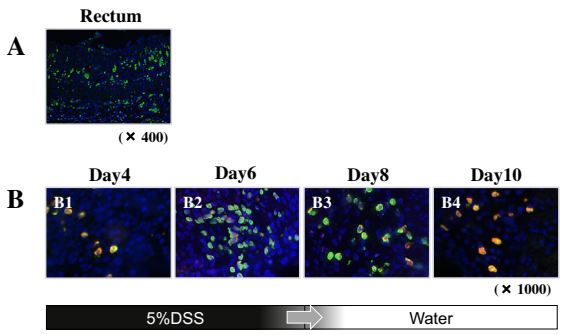

Fig. 1. Fluorescent immunohistochemical staining. (A) CD68 in the rectal tissues of the UCR (panel A) was stained (see Section 2). Green: CD68, blue: nuclei. Magnification: high power field (400×). (B) r-S100A8 and r-S100A9 in the rectal tissues of the UCR (panels B1–B4). Red: r-S100A8, green: r-S100A9, yellow: merged, blue: nuclei. All panels show super high power fields (1000×).

3.3. Changes in the mRNA levels of r-S100A8 and r-S100A9 and several cytokines

As determined by PCR, the mRNA levels of r-S100A8 and r-S100A9 began to significantly increase on Day 4, peaked on Day 6, and then gradually decreased (Fig. 3A, panel A1); however, the mRNA expression of both molecules was almost completely inhibited in the TMR (Fig. 3A, panel A2). In addition, we found that the mRNAs expression levels of r-S100A8 and r-S100A9 in the UCR

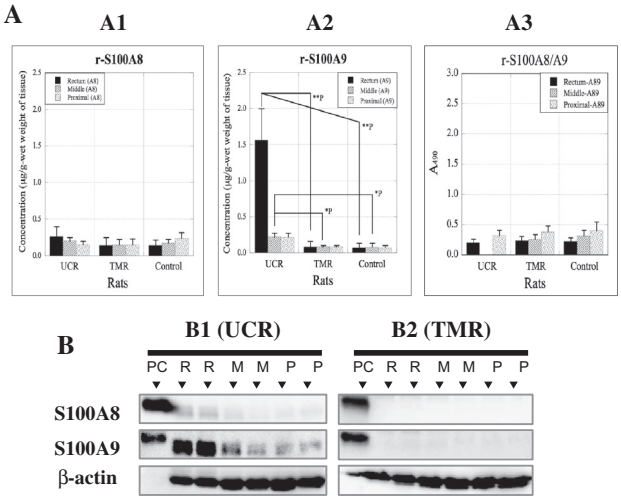

Fig. 2. Expression of r-S100A8 and r-S100A9 in the colon tissues of the UCR and TMR. (A) The large colons of the UCR and TMR were obtained on Day 6 of the experiment and divided into three pieces (R, rectum; M, middle colon; P, proximal colon) in sterilized conditions. The levels of r-S100A8, r-S100A9 and r-S100A8/A9 in each tissue were measured using the ELISAs for them. The vertical and horizontal axes indicate the protein concentration of r-S100A8 or r-S100A9 (ng/ml or ng/g-wet weight of tissue) and the experimental rats used, respectively. The concentration of r-S100A8/A9 complexes is indicated by absorbance at 490 nm (see Section 2). The vertical bars indicate the variation (±SD) in the measured absorbance values in each group (UCR, $n = 5$; TMR, $n = 5$; control rats, $n = 5$). $^{*}P < 0.01$: between the control rats and UCR. $^{**}P < 0.01$: between the UCR and TMR. (B) Western blotting was carried out to detect r-S100A8 and r-S100A9 in the large colons of the UCR, TMR and control rats. PC: positive controls. β-Actin was used as an internal control.

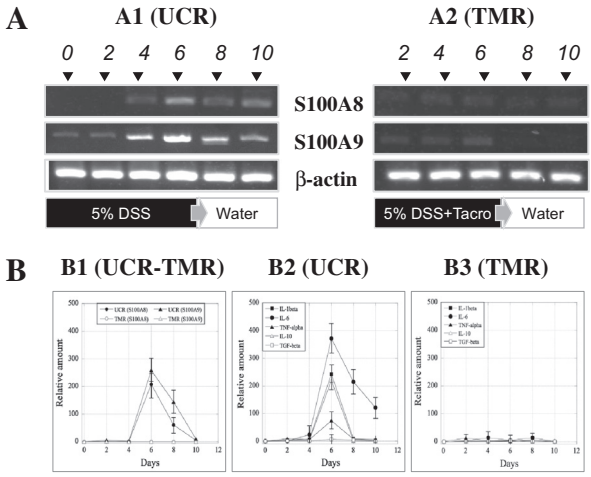

Fig. 3. Changes in the levels of r-S100A8 and r-S100A9 mRNAs in the UCR and TMR. (A) The mRNA levels of r-S100A8 and r-S100A9 in the rectal tissues of the UCR and TMR were determined by PCR. (B) Panel B1 shows the results of RT-PCR concerning the mRNAs of r-S100A8 and r-S100A9. The mRNA levels of inflammatory (TNF-α, IL-6 and IL-1β) and anti-inflammatory (IL-10 and TGF-β) cytokines in the rectal tissues of the UCR and TMR were determined using RT-PCR every 2 days during the experiment. The untreated rats was used as a control. In both methods, β-actin mRNA was used as an internal control. The horizontal and vertical axes indicate the number of days after the administration of 5% DSS alone or 5% DSS plus tacrolimus and the relative amount of the product cDNA detected in the UCR or TMR tissue, respectively, compared with that detected in the control rat tissue. The vertical bars indicate the variation (±SD) in the relative amounts of r-S100A8 and r-S100A9 mRNAs in each group (UCR, $n = 25$; TMR, $n = 25$; control rats, $n = 5$).

during the experiment reached the maximum on Day6 of the experiment, and that the fluctuation pattern of r-S100A8 was almost coincided with that of r-S100A9 (Fig. 3A, panel A3). On the other hand, in the UCR the mRNA expression levels of IL-1β, IL-6 and TNF-α were markedly increased on Day 6 of the experiment (Fig. 3B, panel B1). After peaking on Day 6, the levels of these cytokines gradually decreased. In contrast, a marked but transient increase in the mRNA expression level of IL-10 was seen on Day 6; however, tacrolimus completely inhibited the mRNA expression of these cytokines in the rectal tissue of the TMR (Fig. 3B, panel B2).

3.4. Changes in the levels of S100 proteins and their mRNAs, and several cytokines in macrophages stimulated with rr-S100A8 or rr-S100A9

The concentration of r-S100A8, which was determined by a preparative ELISA [18], increased significantly upon stimulation with rr-S100A8 (10 μg/ml) alone, but that of r-S100A9 hardly increased (Fig. 4, panel B1). Similarly, only the concentration of r-S100A9 significantly increased, when peritoneal macrophages were stimulated with rr-S100A9 (10 μg/ml) alone (Fig. 4, panel B2). Interestingly, the level of r-S100A8/A9 complex apparently increased in the culture medium (Fig. 4, panel B3).

We also determined the mRNA levels of r-S100A8 and r-S100A9 in macrophages using semi-quantitative PCR after stimulating the cells with rr-S100A8 or rr-S100A9. As stimulated with rr-S100A8 (10 μg/ml) alone, the mRNA expression of r-S100A8 was strongly induced, but not almost in r-S100A9 (Fig. 4, panels B1 and B2). In contrast, the mRNA expression of r-S100A9 was discriminatively induced by means of rr-S100A9 (10 μg/ml) alone, but not almost in r-S100A8 (Fig. 4, panels A3 and A4). Furthermore, such unexpected induction depended on the concentrations of rr-S100A8 and rr-S100A9, respectively (Fig. 4A, panels A1–A4)

Upon stimulation with rr-S100A8 (10 μg/ml) alone, significant increases in anti-inflammatory cytokine (IL-10 and TGF-β) mRNA levels were observed 2 h later (Fig. 4B, panel B1). On the contrary,

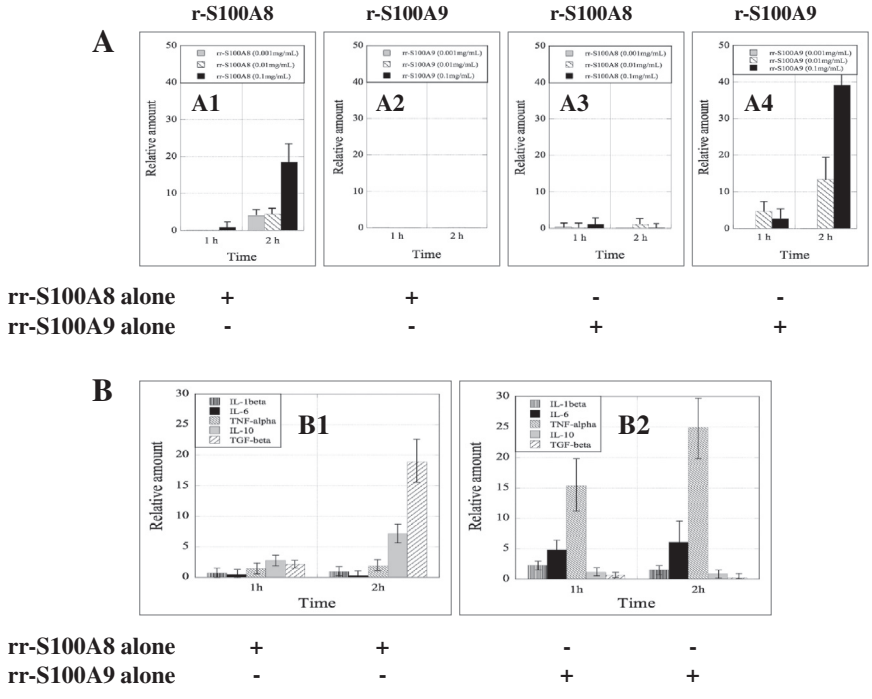

Fig. 4. Changes in the mRNA levels of r-S100A8 and r-S100A9, and anti-inflammatory and inflammatory cytokines in macrophages stimulated with rr-S100A8 or rr-S100A9. (A) The mRNA levels of r-S100A8 and r-S100A9 in macrophages were determined by RT-PCR after the cells had been activated with rr-S100A8 or rr-S100A9 (see Section 2). Macrophages in culture medium were stimulated in the concentration of 1, 10 and 100 (μg/ml) with rr-S100A8 or rr-S100A9 for 1 h or 2 h in 5% CO_2 at 37 °C *in vitro*. Relative r-S100A8 and r-S100A9 cDNA values were calculated based on the amounts of these cDNA detected in the macrophages with no stimulation. (B) The mRNA levels of IL-10, TGF-β, IL-1β, IL-6, TNF-α in macrophages were determined by RT-PCR after the cells had been activated with rr-S100A8 (10 μg/ml) or rr-S100A9 (10 μg/ml) for 1 h or 2 h in 5% CO_2 at 37 °C *in vitro*. Relative these cytokine's cDNA values were calculated based on the amounts of these cDNA detected in the macrophages with no stimulation.

when the cells were stimulated with rr-S100A9 (10 μg/ml) alone, significant increases in the mRNA expression levels of inflammatory cytokines (IL-1β, IL-6, and TNF-α) were observed 1 h later (Fig. 4B, panel B2).

4. Discussion

In this paper, we described the possibility that unknown autocrine signal transduction pathways involving immunological regulators, r-S100A8 and r-S100A9, could operate in macrophages to regulate their immunological functions; i.e., to maintain homeostasis in the gastrointestinal tract, and presented data supporting the utility of both of these proteins as biomarkers of IBD. The value of r-S100A8 and r-S100A9 in macrophages as indicators of UC is based on their origins and immunological properties, as well as the specificity of the monoclonal antibodies for the two proteins.

As shown in Fig. 1A, the histological examination provided us important information regarding the mechanism responsible for the onset of UC. One noteworthy finding was the limited localization of many r-S100A9-positive macrophages, in which r-S100A9 was dominantly expressed, in the rectal tissue of the UCR. It is widely known that in rats experimental UC seems to originate from the rectal tissue near the anus. In view of this fact, our findings coincided with the standard diagnostic criteria for UC used by many physicians. This provides us important and valuable information to explain the true mechanism responsible for the onset of UC, in which mRNAs are used as useful indicator. As suggested in Fig. 3, the mRNA expression of r-S100A8 and r-S100A9 could be induced through signal transduction pathways via undefined receptor(s) in which NF-κB acts as an internal mediator. This may be supported by the previous report that tacrolimus acts as an immunological modulator of IBD and inhibits the phosphorylation of p38, p65 and c-Jun N-terminal kinases, which are nuclear proteins, in peritoneal macrophages from IL-10-KO mice [3].

We paid attention the marked increase in the IL-6 mRNA in the colon (rectum) of UCR, because as reported by Lee et al., the S100A9 expression may be due to not only macrophages in the colon tissues but also colonic epithelial cells, which are activated with IL-6 through signal transducer and activator of transcription 3, STAT3, on the cells in the UCR [18]. In this viewpoint, our current research is in progress using r-S100A9 small interfering (si) RNA on UCR. The mRNA levels of these molecules in the macrophages from the UCR markedly increased, but not in the cells from the TMR (Fig. 3B), indicates that the cells from the UCR had been activated. Therefore, r-S100A8 and r-S100A9, as well as inflammatory and anti-inflammatory cytokines, might be important proteins not only in the etiology of IBD (including UC) but also in the cell biology of immune cells of myeloid origin, although it remains unclear whether S100 proteins are involved in the causative mechanism of UC.

It is noteworthy that *in vitro* study peritoneal macrophages selectively expressed r-S100A8 and r-S100A9 upon stimulation with rr-S100A8 and rr-S100A9, respectively (Fig. 4A). Although each receptor corresponded to r-S100A8 or r-S100A9 was not definitively identified, there may be each signal transduction pathway identified with r-S100A8 or r-S100A9 in the cells. We examined whether r-S100A8 and r-S100A9 are ligands for undefined receptors on macrophages, and hypothesized that the cells are activated by r-S100A8 or r-S100A9 through an autocrine signal transduction pathway involving such unknown receptors. Consequently, as shown in Fig. 4, the possibility that a limited receptor recognized with r-S100A8 or r-S100A9, but undefined one, exists on macrophages of myeloid origin are strongly suggested. We suppose that macrophages may have each signal transduction pathway of S100A8 or S100A9 to independently induce a corresponding protein, S100A8 or S100A9, to regulate macrophages' function via undefined receptor. Although the true receptors for S100A8 and S100A9 are not still identified, one of which may be a cluster differentiation (CD) 68. Indeed, we recently found that both rr-S100A8 and rr-S100A9 proteins tightly bound to CD 68 in macrophages *in vitro* study (data not shown); however, in the viewpoint of the reactivity of S100 proteins with CD68, rr-S100A8 was significantly different in rr-S100A9. Here, we paid attention to the difference in the molecular weight of rr-S100A8 and rr-S100A9. This difference may be a causative reason for selectively inducing a corresponding protein, S100A8 or S100A9, alone in macrophages, because in the recent *in vitro* study, the reactivity of S100 proteins with CD14 was hardly observed (data not shown). The observation is suggestive of a difference in higher order structure of the two complexes, rr-S100A8-CD68 and rr-S100A9-CD68, and presents a possibility that we can explain the mechanism for different another signal transduction pathway involving S100 proteins via CD68 in macrophages. These observations may also support our hypothesis that autocrine pathway involving S100 proteins via at least CD68 could exist in macrophages. While, less endotoxin derived from *Escherichia coli* cells, if any, did not provide significant effect on the activation of macrophages, because its content is practically very small as described previously [15].

To be noteworthy is that the balance between the protein concentrations of r-S100A8 and r-S100A9 seems to be an important factor in this process, and hence, immunological assessments of this balance could be used to determine the current state of macrophages in the gastrointestinal tract; however, it is unclear why this balance is disrupted in UC. It is suggested that the immunological roles of r-S100A8 and r-S100A9 may be harmonized via representative receptors, such as TLR-4 and RAGE, or other receptors expressed on macrophages, because such receptors recognize plural antigens, such as bacteria, virus and other substances, and that understanding the immunological roles of these S100 proteins, which are clinically useful indicators of UC, is important for elucidating the mechanism responsible for the onset of UC.

In conclusion, it is suggested that in UCR the immunological functions of macrophages are regulated in a complex manner by r-S100A8 and/or r-S100A9 through undefined autocrine pathways on the cells. To prove our concept in detail, further investigations of the signal transduction pathways that operate in macrophages are needed.

Footnotes

IBD, inflammatory bowel disease; UC, ulcerative colitis; DSS, dextran sulfate sodium; STA, streptavidin; FITC, fluorescein 5-isothiocyanate; PCR, polymerase chain reaction; RT-PCR, real-time PCR; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor; NF-κB, nuclear factor-kappa B; CD68, cluster of differentiation 68; TLR, toll-like receptor; RAGE, the receptor for advanced glycation end-products.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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