Intracellular Localization of CD14 Protein in Intestinal Macrophages

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Abstract. *Background: Our research is focused on intestinal macrophages, which are believed to contribute to the maintenance of intestinal homeostasis. In addition, intestinal macrophages are unique in that there is an impairment of expression of tumor necrosis factor (TNF) from lipopolysaccharide (LPS). This characteristic can be attributed to the lack or poor level of expression of toll-like receptor 4 (TLR4) or CD14 on the membrane of intestinal macrophages. We therefore decided to identify where CD14 was localized in intestinal macrophages. Materials and Methods: The endoplasmic reticulum and Golgi apparatus were double stained and the intracellular localization in the intestinal macrophages was observed using a confocal laser microscope. Results: CD14 of peritoneal macrophages was expressed both in the endoplasmic reticulum and Golgi apparatus. By contrast, intestinal macrophages expressed very little CD14 on the cellular membrane. CD14 was present in the endoplasmic reticulum of intestinal macrophages, but was rare in the Golgi apparatus. Conclusion: The lack of expression of CD14 on the cell membrane of intestinal macrophages may be caused by transport interference from the endoplasmic reticulum to the Golgi apparatus.*

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Macrophages play important roles in host immunity defense with such functions as recognition of invading pathogens, phagocytosis and secretion of reactive oxygen species or cytokines. Macrophages as tissue macrophages show unique biological characteristics depending on the tissues or organs where they exist (1). Macrophages in general recognize foreign substances *via* pattern recognition receptors (PRR). For example, lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria and is the strongest activator of macrophages. It is recognized by toll-like receptor 4 (TLR4) (2). Other adaptor molecules such as MD-2 or CD14 are known to be required to recognize LPS by TLR4 (3). LPS can transmit intracellular signals *via* MyD88 or tumor necrosis factor receptor-associated factor 6 (TRAF-6) following association with the molecules described above, followed by the activation of nuclear factor-κB. This then leads to the secretion of tumor necrosis factor (TNF) or interleukin-6 (IL-6), for example.

The gastrointestinal tract is a unique organ where orally ingested foods exist as a type of foreign substance and where numerous enterobacteria are colonized. It has been demonstrated that the gastrointestinal tract has well developed immune functions and retains many immunity cells such as T or B lymphocytes and macrophages (4, 5). Of these cells, the intestinal macrophages were reported to

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be contributing to the maintenance of intestinal homeostasis in addition to phagocytosis of pathogens that had invaded the intestinal mucosa or intestinal epithelial cells. However, intestinal macrophages are unique in that expression of TNF after exposure to LPS is impaired (unlike other tissue macrophages) (6). This characteristic can be attributed to the lack or poor level of expression of TLR4 or CD14 on the membranes of intestinal macrophages (7-9).

Along these lines, Austin *et al*. reported that infiltrating CD14-positive intestinal macrophages were relevant to the onset of inflammatory bowel diseases. This is because intestinal macrophages that had infiltrated into the intestinal mucosa (following removal of intestinal epithelial cells) expressed CD14 and showed LPS responsiveness (10). In addition, they reported that CD14-positive macrophages existed in intestinal mucosa from patients suffering from inflammatory bowel diseases and that the macrophages secreted TNF (11-13). These results suggest that intestinal macrophages can acquire LPS responsibility if CD14 is expressed on their membranes. On the other hand, Nakata *et al*. showed that the expression level of mRNA such as *CD14*, *TLR4* and *MD2*, relevant to signal transduction of LPS by intestinal macrophages, was almost the same as that expressed by other tissue macrophages (6). They also reported that CD14 protein was produced by intestinal macrophages and, through the use of flow cytometric analyses, it was observed to exist intracellularly, not on the cellular membrane (6). From these results, they concluded that intestinal macrophages have the potential to express CD14 on their membranes. They also succeeded in inducing LPS responsiveness in intestinal macrophages by their co-cultivation with immunoglobulin. These results suggest that intestinal macrophages, under certain physiological conditions, may take part in the onset of inflammatory bowel diseases by expressing various cytokines through an LPS receptor such as CD14. Therefore, elucidation of the mechanisms by which LPS receptors are induced on cellular membranes of intestinal macrophages could bring new insight into the etiology of inflammatory bowel disease. Even though it was possible to recover the expression of CD14 with immunoglobulin, there has been little additional information that could help explain this mechanism.

Based on the information summarized above, we decided that a first step in learning the mechanism concerning LPS unresponsiveness by intestinal macrophages was to identify where CD14 was localized in intestinal macrophages. This was studied mainly by use of a confocal laser microscope.

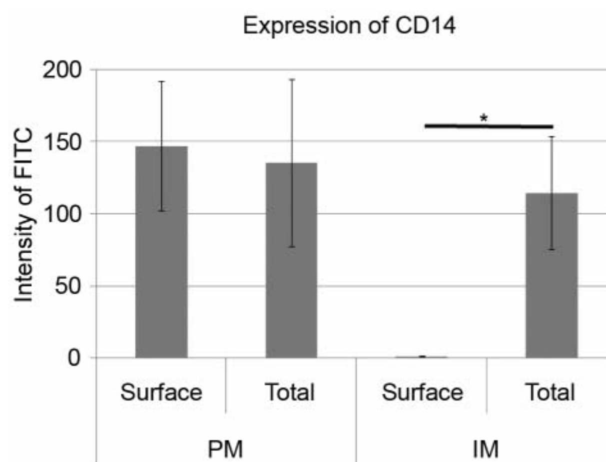

Figure 1. *Peritoneal macrophages (PM) and intestinal macrophages (IM) were fixed, stained directly (Surface), permeabilized and stained (Total) with FITC-conjugated anti-mouse CD14. To measure intracellular (total) CD14, these cells were pre-treated with permeability agent (saponin). They were analyzed by confocal laser microscopy [Ar laser (488nm), resolution level was gain 100]. This figure shows the illumination score of FITC (CD14) per cell in a 12-bit display screen image. The data represent the means ±SD (n=7). For the IM there was a significant difference (*p<0.01) between the surface and total CD14 expression.*

Materials and Methods

Isolation of macrophages. Intestinal macrophages were isolated by the methods of Nakata *et al*. (6). Male C3H/HeN mice (SLC, Japan) were housed in cages in a humidity-controlled animal facility [temperature 22˚C, 12-hour light/dark cycles, chow diets (5L37)]. Mice were anesthetized with Nembutal (10 μl/10 g). Colons were resected from the mice (10 animals per pool) and were opened longitudinally, rinsed in phosphate-buffered saline (PBS) and incubated at 37˚C for 30 min in PBS containing 1 mM dithiothreitol (DTT) and 1 mM ethylenediaminetetraacetic acid, disodium salt (EDTA) to remove mucus and epithelial cells. Tissues were then minced and incubated for 45 min in RPMI-1640 (Sigma, USA) containing 4 mg/ml dispase (Roche, Germany), 400 U/ml collagenase (Wako Chemical, Japan), 200 U/ml deoxyribonuclease (Sigma, USA), 100 μg/ml ampicillin, 50 μg/ml gentamicin and 10% heat-inactivated fetal-calf serum. The digest was filtered with a stainless mesh and then centrifuged through discontinuous Percoll (45% and 20%); the resultant preparation of mononuclear cells was collected from the Percoll-medium. Intestinal macrophages were isolated with an elutriation system (1,960 rpm, 19 ml/min, 18˚C) and the isolated cells were used for experiments.

Intestinal macrophages (5×10^5 cells poured into wells) were kept overnight at 37˚C in a 5% CO_2 incubator immediately prior to use. To collect peritoneal macrophages, 2 ml of 4.05% thioglycolate (Nissui Seiyaku, Tokyo, Japan) medium was injected peritoneally into C3H/HeN mice (SLC, Japan). After 4 days, mice were anesthetized with Nembutal (10 μl/10 g), cells were recovered with an intraperitoneal injection of physiological saline and incubated overnight.

Immunostaining. Intestinal macrophages and peritoneal macrophages were identified by fluorescein isothiocyanate (FITC)-conjugated anti-mouse F4/80 (Serotec, Japan). Cells were washed twice with 1 ml of 3% bovine serum albumin (BSA)/PBS. To stain the endoplasmic reticulum cells, they were incubated with ER Tracker Red (Molecular Probes, Eugen, OR, USA) for 15 min at

37˚C. To stain surface antigens, cells were incubated with FITC-conjugated anti-mouse CD14 (BD PharMingen, San Diego, CA, USA) for 15 min at room temperature. To stain intracellular CD14 and Golgi apparatus, cells were treated with Intra Prep Permeabilization Reagent (Beckman Coulter, USA) and incubated with the FITC-conjugated anti-mouse CD14 and wheat-germ agglutinin, tetramethylrhodamine conjugate (Molecular Probes) for 15 min at room temperature. Control antibody (Abs) included FITC-labeled irrelevant rat anti-mouse Ab of the same isotype used or the same concentration as the other Abs. Cells were analysed by EPICS Altra (Beckman Coulter) or confocal laser scanning microscope (Nikon, Japan) equipped with Ar/HeNe laser.

Statistical analysis. Data are expressed as means (±S.D.) Statistical analyses were performed using Student's *t*-test. Differences were considered as significant at a *p*-value of <0.01.

Results

Intracellular expression of CD14 by intestinal macrophages. We confirmed the expression of CD14 by intestinal macrophages, both on cellular membranes and in the cells, quantitatively by confocal laser microscopy. As a positive control, peritoneal macrophages were used, as CD14 is known to be expressed on their membranes and they show LPS responsiveness. As shown in Figure 1, the results for peritoneal macrophages showed no difference between the expression of CD14 in the cellular membrane and in the cells. By contrast, in intestinal macrophages, there was little expression of CD14 on cellular membranes. The difference between the expression on the cellular membrane and within the cells was statistically significant. There was no significant difference between the expression of CD14 in the peritoneal macrophages and intestinal macrophages. From these results, the lack of expression on cellular membranes and intracellular localization of CD14 by intestinal macrophages was quantitatively confirmed.

Analyses of the intracellular localization of CD14 in intestinal macrophages. From the above experiments, intracellular expression of CD14 in intestinal macrophages was confirmed. To determine which step was involved in the impairment of intracellular protein transportation, we analyzed the main cellular compartments in which CD14 is localized. For observation, double staining of CD14 was carried out for both the endoplasmic reticulum and Golgi apparatus.

As shown in Figure 2, the expression pattern of CD14 by peritoneal macrophages and intestinal macrophages reflected that shown in Figure 1. From the analyses of the double-staining experiments, in peritoneal macrophages, the intracellular CD14 apparently exists both in the endoplasmic reticulum and in the Golgi apparatus. By contrast, in intestinal macrophages, CD14 was present in the endoplasmic reticulum but was rarely found in the Golgi apparatus.

The co-localization of CD14 both in the endoplasmic reticulum and in the Golgi apparatus was measured in both peritoneal and intestinal macrophages. As shown in Figure 3, in intestinal macrophages, the amount of CD14 in the endoplasmic reticulum was significantly higher than that in the Golgi apparatus. Taken together, the intracellular localization of CD14 in intestinal macrophages was shown to be limited to the endoplasmic reticulum.

Discussion

To clarify the mechanism of hyporesponsiveness to LPS by intestinal macrophages, in this study, we measured the intracellular localization of CD14 in both the endoplasmic reticulum and Golgi apparatus. These structures were double stained and viewed with a confocal laser microscope. It was demonstrated that there was no difference in the cellular expression of CD14 between peritoneal macrophages and intestinal macrophages. Moreover, the lack of expression on cellular membranes and the intracellular localization of CD14 by intestinal macrophages was quantitatively confirmed as shown in Figure 1.

In addition, the localization of the intracellular CD14 protein in organelles was then examined with the same technique. The results of fluorescence-microscopy of localized CD14 protein in the organelles is shown in Figure 2. Person's co-localization coefficient (14) of CD14 protein in endoplasmic reticulum and Golgi apparatus are shown in Figure 3. These figures clearly demonstrate that intracellular CD14 protein of intestinal macrophages was present in the endoplasmic reticulum, but not in the Golgi apparatus. By contrast, CD14 of the peritoneal macrophages was expressed in both the endoplasmic reticulum and the Golgi apparatus.

It is well known that proteins synthesized in the endoplasmic reticulum are transported to the Golgi apparatus to receive modifications, such as glycosilation, and are then transported to the cellular membrane. Although the precise mechanism of protein transportation from the endoplasmic reticulum to the Golgi apparatus is not yet fully known, there is a possibility that transportation to the Golgi apparatus of CD14 of intestinal macrophages (which have been newly synthesized in the endoplasmic reticulum) is suppressed by unknown mechanisms and/or molecules.

There are a few known cases of physiological regulation of protein expression that are controlled by inhibition of transport of the proteins. One example is that of TLR4 of the small intestinal epithelial cell line (mICc12) which exists in the Golgi apparatus but not on the cell surface (15). The mechanism is that TLR4 is anchored to the Golgi apparatus by GP96, a chaperone protein, in particular by its KDEL sequence located in the *C*-terminus (16). From this mechanism report, it is suggested that chaperone proteins may be involved in the mechanisms by which protein expression on cellular membranes are regulated in a suppressed fashion. Therefore, it is possible that there is

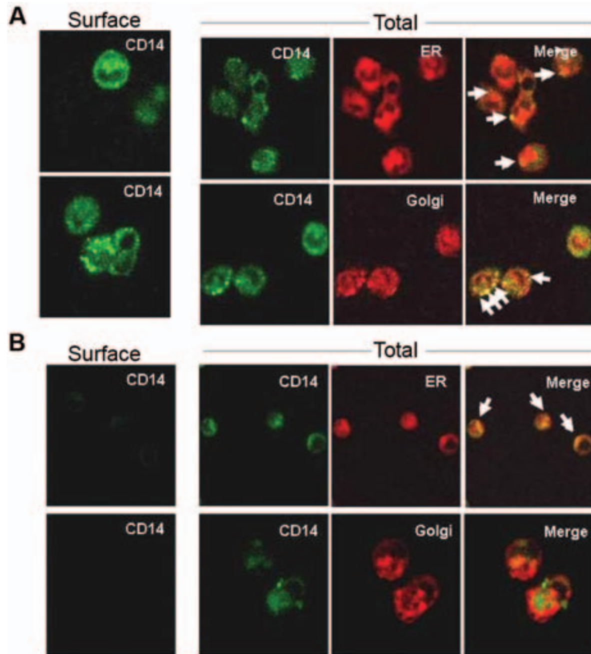

Figure 2. *Peritoneal macrophages (A) and intestinal macrophages (B) were fixed and CD14 (green) was stained with FITC-conjugated anti-mouse CD14 (Surface). To measure intracellular (Total) CD14, the cells were pre-treated with a permeability agent (saponin). Intracellular localization of CD14 was analyzed by ER Tracker Red (red) and wheat-germ agglutinin, tetramethylrhodamine conjugate (red). The arrows indicate overlap of pixels. Images were captured with a TE2000-E (Nikon) confocal microscope equipped with an Ar/HeNe laser.*

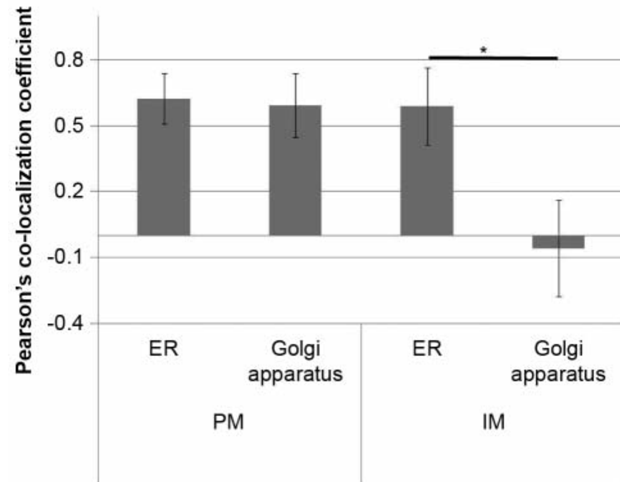

Figure 3. *Pearson's linear correlation coefficient can be used to measure the overlap of the pixels shown in Figure 2. A coefficient between 0 and –1 indicates no co-localization, whereas a value of 1 attests to a perfect co-localization. Average coefficients were 0.63 (ER), 0.59 (Golgi apparatus) in peritoneal macrophages (PM) and 0.59 (ER), –0.06 (Golgi apparatus) in intestinal macrophages (IM), as analyzed by EZ-C1 version 3.5 software (Nikon). The data represent means ±SD (n=5). Significant differences (*p<0.01) were obtained between the ER and Golgi apparatus groups for IM.*

some unknown chaperone protein that impairs the transport of CD14 (which has been synthesized in intestinal macrophages) from the endoplasmic reticulum to the Golgi apparatus.

The cellular dynamics of the CD14 protein in intestinal macrophages may not be incidental, but has some physiological significance. For instance, because the intestine is the organ that absorbs food, it is always in contact with Gram-negative bacteria such as *Escherichia coli* and *Bacteroidaceae* (17). Therefore, intestinal macrophages need to be regulated in a way that avoids superfluous inflammatory reactions. The necessity for expression of CD14 protein in the resident intestinal macrophages is not yet clear. However, quick inflammatory responses are initiated against pathogens marked by immunoglobulin A that have invaded into the lamina propria and require elimination.

The number of patients suffering from inflammatory bowel diseases is increasing. Though the precise mechanisms of the disease are still unknown, it is believed that the etiology involves CD14-positive macrophages infiltrating into the intestinal mucosa (18). These CD14-positive macrophages were generally thought to be recruited from blood monocytes, but our experiments have suggested another possibility. Namely that resident intestinal macrophages possess the capacity to express CD14 on their membranes and this abnormal expression of CD14 can be driven by certain stimuli. The CD14 then triggers inflammatory bowel diseases. The involvement of intestinal macrophages in the onset of inflammatory bowel diseases has not yet been proven with certainty. However, elucidating the mechanisms that determine how intestinal macrophages react to foreign stimuli could provide insight into prevention and/or therapy for inflammatory bowel diseases.

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