Rearrangements of Actin Cytoskeleton during Infection with Escherichia coli O157 in Macrophages

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ABSTRACT. The lamina propria of the large intestine is rich in macrophages, and they might be one of the first lines of the host defense in enterohemorrhagic Escherichia coli (EHEC) O157:H7 infection. Although macrophages were infected with them, they can survive the EHEC O157 infection. We examined the structural rearrangements of the actin cytoskeleton during the microbial infection process. Macrophage actin filaments were rearranged in the following sequence; 1) disappearance of the actin filament bundles in the cytoplasm, 2) accumulation of actin filaments under the cell surface, and 3) construction of actin networks underlying the endosome membrane. Before infection, actin filaments were distributed under the cell surface and in bundles located in the macrophage cytoplasm. Within 2 min, infection caused a rapid and marked loss of the actin filament bundles that had run parallel to the long axis of the cell. Concomitant with the loss, actin filaments became more markedly distributed under the cell surface. In the formation of the endosome, new networks of actin filaments were constructed below the phagosome membrane. The networks contained a large amount of actin as well as a fodrin-like immunoreactivity. The thickness of the networks reached about 400 nm under the phagosome membrane. The actin networks disappeared again after the bacterial digestion. The results of this study showed that actin filaments undergo three major rearrangements of the actin filaments during the infection in macrophages, and suggested that the third rearrangement is mediated by actin-binding proteins, such as a fodrin-like molecules. These morphological changes in macrophages were not clear after infection with other strains of Escherichia coli.

Key words: actin/Escherichia coli O157/macrophages/a fodrin

In 1996, there were several large outbreaks of food poisoning caused by the enterohemorrhagic Escherichia coli (EHEC) O157:H7 in Japan. More than 8,400 people were infected with this strain of bacteria, and 11 of the infections were fatal (56). EHEC O157 is estimated to cause more than 20,000 infections and as many as 250 deaths each year in the United States alone (4). When the intestinal epithelium is damaged and microorganisms penetrate the basement membrane, macrophages may become a very important part of the host defense system in the body. Numerous macrophages are present in the intestine lamina propria (15, 26, 28, 37). They usually perform the functions of phagocytosis, digestion, and destruction of pathogens, but they also have antigen-presenting activity and secrete cytokines (15, 38). At the bacterial density used in the present experiment, infection induced macrophage activation (57, 59) but not macrophage cell death (17, 33). The present experiment aimed to clarify some of the macrophage functions that protect macrophages from cell death after infection, focusing on their cytoskeleton.

The cytoskeleton consists of actin microfilaments, microtubules, and intermediate filaments, and is involved in many activities in living cells. One portion of the cytoskeleton, actin, is the most abundant protein in most cells, often constituting 5% or more of their total protein composition (5, 30, 47). While actin is distributed throughout the cytoplasm, most cells possess an especially dense network of actin filaments and actin-binding proteins beneath the cell membrane. The actin networks give mechanical strength to the cell surface.
and enable the cell to change its shape and to move (7, 21).

Changes in the extent of actin polymerization appear to be an important and regulated aspect of the functions of cells. F actin exists in equilibrium with the globular G form, but the equilibrium constant for the polymerization-depolymerization reaction can be affected by various proteins that bind selectively to G or F actin (30, 47). Actin-binding proteins, such as spectrin and ankyrin, were first discovered as prominent components of the membrane-associated cytoskeleton of mammalian erythroid cells (1). Fodrin is highly homologous to spectrin, and they are both categorized into the spectrin family (35, 41). These proteins help to form the membrane-associated cytoskeleton in a wide variety of cells, and are generally located just under the plasma membrane and participate in binding actin filaments to the cell membrane (1, 41). Ankyrin is a spectrin- and fodrin-binding protein that has been identified in many cells (45), and can link them to an integral membrane protein. Possibly, as in erythrocytes, the actin-spectrin (or fodrin)-ankyrin cytoskeleton may play a key role in immobilizing certain integral proteins into localized patches in the membrane in many cells (2, 12, 35), including macrophages (22, 44).

The present experiment showed three steps of rearrangement of actin filaments in macrophages during infection with EHEC O157, and that these rearrangements were related to the presence of actin-binding proteins such as α fodrin-like molecules.

Materials and Methods

Isolation of EHEC O157:H7

The EHEC O157:H7 used in the present experiments was isolated from the feces of patients. Sorbitol-MacConkey agar plates (Becton Dickinson Labware, Franklin Lekes, NJ, USA) were used to isolate the bacteria (25), which were grown aerobically at 37°C in tryptic soy broth (TSB) (Difco, Detroit, MI, USA). The bacterial serotype was determined by using specific antibodies against EHEC antigens (Denka Seiken, Tokyo, Japan). Two different strains of Escherichia coli, DH1 and O126:H27, which are non-pathogenic and non-Shiga toxin (ST)-secreting bacteria, respectively, were used as controls.

Macrophage cell culture

Female Wistar rats weighing about 200 g were intraperitoneal injected with 2 ml of 3% thioglycollate medium (Wako Pure Chem., Osaka, Japan) that had been stored for 2 months, and 4 days later the peritoneal exudate along with cells was harvested according to methods described previously (57). The cells were suspended in Dulbecco’s modified Eagle’s medium (DMEM) (Flow Laboratories, Rickmanworth, UK) supplemented with 10% fetal bovine serum (FBS) (Gibco, Madison, WI, USA) but not with antibiotics. The number of cells in the suspension was counted in a hemocytometer, adjusted to a density of 5 × 10⁶ cells per ml, and then 1.0 ml of the suspension was placed on a glass slide in a 9-cm Petri dish (Flow Laboratories). After 3 hours of incubation at 37°C under a 5% CO₂ atmosphere, each glass slide was washed three times with fresh DMEM to remove non-adherent cells. The adherent cell population consisted of more than 95% macrophages, as shown by specific staining with anti-rat macrophage antiserum (Inter-Cell Technologies, Hopewell, NJ, USA). The macrophages showed about 80% confluence after 2 days of incubation, at which point the culture medium was replaced with infection medium (DMEM lacking serum and antibiotics). After 24 hours, for an additional incubation, the macrophage culture was used for the following experiments.

Infection with EHEC O157

The wild bacteria isolated from the fecal samples were grown in TSB, then washed three times in order to remove toxins. After the final wash, the pellet was resuspended in DMEM to give a final concentration of 2 × 10⁹ bacteria per ml. To synchronize the infection process, 1 ml of the bacterial suspension was added to each glass slide, and the slide was centrifuged at 700 g for one min. Preliminary experiments confirmed that the centrifugation caused no changes in the cell shape and the distributions of α fodrin and actin in macrophages. After washing well, the fresh culture medium was added, and the macrophage cell culture was returned to the incubator under a 5% CO₂ atmosphere. Giemsa staining of macrophages showed that about 90% of them contained bacteria 15 min after infection. Cell viability was measured by the previous methods (48).

Antibodies

Polyclonal antibodies against smooth-muscle-type actin (Biomedical Tech. Inc., Stoughton, MA, USA), α actinin (Transformation Res. Inc., Framingham, MA, USA), bacterial outer membrane antigen, O157, (Denka Seiken), and acid phosphatase (Chemicon Int. Inc., Temecula, CA, USA) were purchased. Polyclonal antibodies against smooth-muscle-type actin and myosin were prepared in our laboratory from rabbits repeatedly immunized with purified antigens, actin and myosin of human platelets. Different α fodrin monoclonal antibodies were separately purchased from two companies; Locus Genex Oy. (Helsinki, Finland) and Affinity Res. Pro. (Manhead, Exeter, UK). Monoclonal antibodies against type 1 Shiga toxin (ST-I) and type II ST (ST-II) were obtained from mice immunized with each synthetic peptide composed of the 20-amino acid sequences corresponding to the N- and C-terminal residues of the A subunits of ST-I and ST-II, separately. Commercially available monoclonal antibodies
against ST-I and ST-II were also used (Toxin Tech., Sarasota, FL, USA). Another monoclonal antibody against bacterial outer membrane antigen, O157, was also prepared in our laboratory. Fluorescein isothiocyanate (FITC)-conjugated phalloidin was purchased from Sigma (St. Louis, MO, USA). In the present experiment, the dilutions were 1:10^4-1:10^5 for the polyclonal antibodies and 1-10 ng/ml immunoglobulins (final concentration) for the monoclonal antibodies, and 0.17 mg/ml FITC-conjugated phalloidin.

**Immunofluorescence and electron microscopy**

Macrophages were immersed in 2.5% acrolein in 0.01 M PBS for 10 min at 37°C, and then for 2 hours at 4°C. The purpose of the use of acrolein is to fix cells as soon as possible. After fixation, the samples were washed with PBS, and treated with the blocking solution, Tris-glycine buffer (0.1 M, pH 7.2) containing 1% freshly prepared borohydride (Wako Pure Chem.) and 1% bovine serum albumin (BSA) for 10 min in order to block aldehyde formation. As described previously (50, 51), the samples were treated with 1% normal goat serum (NGS) for 1 hour at room temperature, and then for 24 hours at 4°C with polyclonal and/or monoclonal antibodies appropriately diluted with PBS containing BSA. After washing, the sections were incubated with FITC- and/or Texas Red-conjugated anti-rabbit and/or -mouse goat immunoglobulins (1:500 dilution) for 30 min at room temperature, washed, and mounted in 50% glycerol in PBS (pH 8.6) containing 50 mg/ml 1,4-diazabicyclo-octane (DABCO, Aldrich Chemical Co., Milwaukee, WI, USA) as an anti-bleaching agent. The specimens were observed with a confocal laser microscope (CSU-10, Yokogawa Electric Corporation, Tokyo, Japan).

In order to identify with immunofluorescence observation, we used the same fixative for electron microscopic analysis. The macrophage cell cultures on the glass slides were fixed with 2.5% acrolein in 0.01 M PBS, and post-fixed with 1% osmium tetroxide at 4°C for 1 hour. After fixation, the tissues were dehydrated with a graded ethanol series, embedded in Epon 812, and cured for 3 days at 50°C. For immunoelectron microscopy, thin sections were treated with 3% hydrogen peroxide for 10 min (50, 51). In order to block aldehyde formation, thin sections on grids were floated in Tris-glycine buffer containing 1% freshly prepared borohydride and 1% BSA for 10 min. Thin sections were treated with 1% NGS for 1 hour, and then incubated with the required anti-ST-I or -ST-II immunoglobulin (1 ng/ml) in PBS, pH 7.4, containing 1% BSA. After rinsing with PBS, the sections were incubated for 1 hour with 10-nm colloidal gold-labelled goat anti-mouse IgG immunoglobulin (1:200 dilution) (British Biocell Int., Cardiff, UK), followed by rinsing with PBS and then with distilled water. All sections were stained with uranyl acetate and lead citrate, and then examined with an electron microscope (Hitachi H-800, Hitachi, Japan). The specificity of each immunoreaction was confirmed by a preadsorption test involving the addition of excess STs to the first antibody.

**Immunoblotting**

Cultured macrophages before and after infection with EHEC O157 were scraped with a cell lifter (Costar, Cambridge, MA, USA) and suspended in trichloroacetic acid at 0°C. After centrifugation at 10,000 g at 4°C for 10 min, the pellet was dissolved in an SDS sample buffer containing 2-mercaptoethanol, homogenized with a homogenizer at 0°C, and incubated at 90°C for 10 min. The sample was separated on a 5% SDS-polyacrylamide gel, and electrotransferred to a nitrocellulose sheet (32). The blot was incubated with anti-α fodrin antibody, followed by peroxidase-conjugated goat anti-mouse IgG antibody (Pierce, Rockford, PA, USA), and visualized with a Konica immunostaining kit (Konica, Tokyo, Japan).

**Results**

**Rearrangements of actin filaments after EHEC O157 infection**

Before infection with EHEC O157, fluorescence light microscopy showed long actin-containing bundles in a fixed macrophage stained with anti-actin antibody (Fig. 1A). The distribution of actin filaments in the macrophages was not limited to the bundles; they were also observed along the cell surface membrane. There was a significant tendency for the bundles of actin filaments to run parallel to the long axis of the cell, and the ends of the bundles were inserted into the plasma membrane. These actin bundles were observed in more than 95% of macrophages before infection. A rapid and marked disappearance of the actin bundles occurred within 2 min after addition of EHEC O157 to the macrophage cell culture. Two minutes after infection, the staining of actin filaments was clearly intensified under the macrophage cell surface (Fig. 1B). Within 15 min, the actin filaments bundles were no longer seen in the cytoplasm of about 95% of the macrophages. Concomitant with the loss of the actin filament bundles, many strongly immunopositive vesicles appeared throughout the cytoplasm. From 2 to 15 min after infection, the number of the actin-containing vesicles dramatically increased in the cytoplasm, and after 15 min, the immunoreactivity sometimes formed a circular structure around a vacuolar structure in the cell (Fig. 1C). In this experiment, 24 hours after infection (culture medium was replaced 3 hours after infection), more than 80% of the cells reformed the actin filament bundles again in the cytoplasm in the same manner as observed before infection (Fig. 1D). The use of FITC-conjugated phalloidin confirmed the results of actin distribution in macrophages obtained with anti-actin antibody. No macrophage cell death could be observed.
during 24 hours after the infection in the experiment. These morphological changes in macrophages were not clear after the infection with two different strains of Escherichia coli, DH1 and O126:H27, which are non-pathogenic and non-STs-secreting bacteria, respectively, at the same bacterial density used as the EHEC O157.

**Distribution of a fodrin-like immunoreactivity**

Immunoblotting of the total lysates of rat macrophage cell cultures before and after the EHEC O157 infection revealed positive bands at 240 kDa with anti-α fodrin antibody (Fig. 2). A weak but significant increase in the total amount of α fodrin-like immunoreactivity could be observed in the macrophages cell culture 30 min after infection.

Before infection with EHEC O157, α fodrin-like immunoreactivity was widely distributed throughout the macrophage cytoplasm (Fig. 3A). The distribution of α fodrin-like immunoreactivity did not reveal any filamentous bundles, such as the actin filaments. At 2 min after the EHEC O157 infection, strongly immunopositive vesicles appeared in the cytoplasm, and the number of vesicles markedly increased for the next 15 min. After 15 min, some immunoreactivity arranged along
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Fig. 2. Immunoblotting of rat macrophage cell cultures before (A) and 30 min after (B) infection. The same volume (0.1 μg of protein) of macrophage extracts were loaded on the two lanes. Note the positive single band at 240 kDa with anti-α fodrin antibody (arrow). After the bacterial infection, a weak but significant increase in the total amount of α fodrin-like immunoreactivity is observed. Another band (small arrowhead) is also slightly apparent after infection.

a circular structure appeared in the macrophage cytoplasm (Fig. 3B). Two different antibodies against α fodrin showed identical results.

The number of α fodrin-like immunopositive vesicles before and after infection are shown in Figure 4. The number of vesicles dramatically increased until 15 min after infection, reached a plateau, and then gradually decreased and completely disappeared again by 24 hours after infection.

Double immunofluorescence

Double immunofluorescence of actin and EHEC O157 showed that the actin immunoreactivity under the cell surface may have some relationship to the distribution of the bacteria (Fig. 5A). The bacteria contacted some portions of the plasma membrane, where the actin immunoreactivity was strongly stained. The antibody against the bacterial membrane antigen, O157, detected all bacteria outside and inside the cell until digestion. At 15 min after infection, many vesicles immunopositive to α fodrin could be identified surrounding the incorporated EHEC O157 (Fig. 5B). Some strongly immunoreactive vesicles against α fodrin were arranged along a circular structure in the macrophage cytoplasm. Double immunofluorescence using antibodies against α fodrin and acid phosphatase, showed that the

Fig. 3. Immunofluorescence micrographs of α fodrin in macrophages before (A) and 30 min after (B) infection. (A) Before infection, α fodrin-like immunoreactivity is distributed in the macrophage cytoplasm. No immunopositive bundles as seen with the anti-actin antibody before infection are observed. (B) Thirty minutes after infection, strongly immunopositive vesicles appear in the cytoplasm and some of the immunopositive vesicles arrange along a circular structure (arrows). N: Nucleus. All photographs are at the same magnification (×2,200).

Fig. 4. Total numbers of α fodrin-like immunopositive vesicles in 200 different cells. A plane 2 μm from the bottom of the cell was scanned with a confocal laser microscope. Two hundred different cells were scanned, and the total number of immunopositive vesicles was counted. The immunopositive vesicles appear at 2 min after infection, and the number markedly increases until 15 min, reaches a plateau, and then gradually decreases. At 24 hours after infection, the immunopositive vesicles completely disappear again.
vacuolar structure surrounded by α fodrin-like molecules was lysosomes because it contained lysosomal enzyme (Fig. 5C). Electron microscopy showed that the actin networks were very well developed when underlying the phagosomes containing the EHEC O157 (Fig. 6A, B). The thickness of the actin networks reached about 400 nm under the phagosome membrane. No space could be found between the internalized bacteria and the cytoplasmic phagosome membrane (Fig. 6A, B). Immunoelectron microscopy showed that the immunolabelling of ST-II was confined within the phagosomes and phagolysosomes in macrophages (Fig. 6C). No gold particles were significantly localized in the cytoplasm outside phagosomes and phagolysosomes at the bacterial density used, and all macrophages remained alive for at least 24 hours. The same results were obtained using the antibody specific to ST-I.

Discussion

The results of this study show: (i) that the organization of the actin cytoskeleton was rapidly rearranged following infection with EHEC O157, (ii) that concomitant with the disappearance of actin filament bundles, the actin filaments under the cell surface increased, (iii) that during the formation of the endosome, the underlying actin networks were reconstructed below the endosome membrane, and (iv) that numerous α fodrin-like molecules accumulated in the reconstructed actin networks, probably serving the role of an actin-binding protein.

Before infection, macrophages had many actin filament bundles in the cytoplasm along the long axis of the cell. In a variety of cultured cells, the actin filament bundles often bind to the plasma membrane in a way that allows them to pull on the extracellular matrix or on another cell. In a macrophage, the actin bundles often lie parallel to the plasma membrane and may bind to it at several points along its length. Presumably, these fibers confer strength and rigidity on regions of the plasma membrane in a macrophage. In the present experiment, the actin filament bundles also possessed both non-muscle-type myosin- and α actinin-like immunoreactivities (data not shown). The actin bundles may be identified as "stress fibers." (34). Actin filaments are believed to link together to form a stiff threedimensional network by means of cross-linking proteins, such as α actinin, and they control the assembly of actin filaments, link them together into bundles or networks, and determine their position, length, and other properties (21, 49, 55). Macrophages have unique characteristic features including strong adhesion to glass and plastic (16). The adhesion function is probably caused by the macrophage "stress fibers."

The networks of actin filaments in the cell cortex disassemble locally, enabling the surface of the cell to move and engulf the foreign microorganism (13, 18, 19). In addition to α-actinin, macrophages have some other actin-binding proteins, which account for some of the actin cross-linking activity (41, 43), and gelsolin confers calcium sensitivity on the actin gelation process (58). Moreover, there may be fascin in a macrophage (42, 46). The distribution of these actin-binding
proteins can account in part for the phenomena of sol-gel transformations of actin observed in macrophages. The factors that determine organization and stability of the macrophage plasma and cytoplasmic membranes are poorly understood. Many studies addressing this question have focused on other cells in which the cytoskeletal proteins, such as spectrin and fodrin, that associate with different regions or domains at the cytoplasmic surface of the membrane (11, 40), help to form the membrane-associated cytoskeleton in a wide variety of cells (8, 29, 40). Spectrins have been extensively studied in erythrocytes, where they play a key role in stabilizing the plasma membrane and generating the typical shape of these cells. Fodrin was initially found in neurons, but it is now considered to be the general tissue isoform of spectrin (35, 41). Interestingly, isoforms of ankyrin-3 are associated with the lysosome membrane in mouse macrophages (20, 44). The lysosome membrane probably contain various integral membrane proteins, one of which may work as a trans-
porter, for example, binding to part of ankyrin and in turn being linked to the spectrin (fodrin)-based actin cytoskeleton (9, 31). In the plasma membrane, many integral proteins, such as Na/K-ATPase (10), voltage-dependent Na channel (54), amiloride-sensitive Na channel (53), H/K-ATPase (52), hyaluronic acid receptor (36), inositol 1,4,5-triphosphate receptor (3), and others (39) are sustained by the actin networks constructed by ankyrin and spectrin/fodrin. Although nothing is known about the relationships between integral proteins of the endosome membrane and the underlying cytoskeletal networks in a macrophage, the actin networks may play a physiological role in maintaining the polarized distribution of the integral proteins in the membrane.

The coexistence of fodrin and ankyrin under the endosome membrane would seem to support this hypothesis of the underlying actin cytoskeleton organization, as in red blood cells (1, 2). One might expect a similar model of actin organization under the macrophage cytoplasmic membrane to that of the plasma membrane. Presumably, the functions of the submembrane actin-fodrin-ankyrin cytoskeleton are: (1) to strengthen the membrane and prevent leakage of the contents into the cytoplasm, and (2) to play a key role in immobilizing certain integral proteins into localized patches in the membrane. If the contents of the endosome leaked, the toxins released by the bacteria would be diffused throughout the cytoplasm, and the protein synthesis of a macrophage would be suppressed, causing macrophage cell death (17, 33). Although the internalized EHEC O157 remained alive for about 15 min and could secrete toxin, the toxin distribution was restricted to within the lumen of the phagosomes and phagolysosomes. The actin-fodrin-ankyrin cytoskeleton tightly held down the endosome membrane. No space could be observed between the membrane and the bacterial cell wall. In this way, the actin networks may provide mechanical support to the membrane and prevent leakage of toxins.

The morphological changes were not clear after the infection with other strains of Escherichia coli, raising the possibility that STs released by the bacteria directly induce the actin rearrangements in macrophages. It has been widely reported that the toxins cause the formation of attaching and effacing lesions in many cells (14, 24, 26, 27). Ismaili et al. (23) reported that the lesioned area in the intestinal microvilli is characterized by accumulation of polymerized actin and α-actinin. Nevertheless, in macrophages, the toxin distribution was confined within phagosomes and phagolysosomes. No gold particles were localized in the cytoplasm outside the lumen at the bacterial density used in the present experiment, and all macrophages remained alive. Furthermore, the addition of ST-1 and ST-II into the macrophage cell culture caused no actin rearrangements shown in the infection process with the EHEC O157 in macrophages. The effects of STs on the actin cytoskeleton remain obscure and additional experiments are needed to solve the problem.

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