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Stimulation of Macrophages by Mucins through a Macrophage Scavenger Receptor[1]

Mizue Inoue, Hironori Fujii, Hiromi Kaseyama, Ikuo Yamashina, and Hiroshi Nakada[2]
Department of Biotechnology, Faculty of Engineering, Kyoto Sangyo University, Kita-ku, Kyoto 603-8555, Japan

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We found that phorbol ester-primed THP-1 cells (a human monocyte cell line), which express a scavenger receptor, were stimulated by mucins through the macrophage scavenger receptor, resulting in enhanced secretion of IL-1β. The activity was abolished by treatment of the mucins with sialidase, indicating that sialic acid is involved in binding. ^{125}I-Labeled ovine submaxillary mucin could bind to COS 7 cells transfected with cDNA encoding the scavenger receptor. Binding was inhibited by mucins, fucoidan, and polyinosinic acid but not by polycytidylic acid, this being consistent with the characteristics of the scavenger receptor. When phorbol ester-primed THP-1 cells were cocultured with colon cancer cells producing mucins, IL-1β secreted from the THP-1 cells increased significantly. Adhesion between colon cancer cells and a scavenger receptor transfectant was observed, and binding was inhibited partly by mucins and ligands for the scavenger receptor.

Scavenger receptors were first discovered because of their ability to bind to and internalize acetyl low density lipoprotein (LDL) (1). Type I and II class A macrophage scavenger receptors are trimeric integral membrane glycoproteins that exhibit unusual ligand binding properties (2, 3). High affinity ligands include some modified LDLs, maleylated bovine serum albumin, certain polyribonucleotides, and some bacterial lipopolysaccharides (4, 5). Some polysaccharides such as dextran sulfate and fucoidan are also recognized by these receptors, while heparin, chondroitin sulfate A and C, polysialic acid and glycoproteins such as α1-acid glycoprotein and fetuin are not. It appears likely that these receptors recognize a certain anionic charge distribution on a ligand molecule. In this regard, mucins, highly O-glycosylated glycoproteins, seem to be potent candidates for the ligand activity because of the numerous anionic charges on their molecules. In fact, all the mucins we examined could stimulate macrophages effectively through a scavenger receptor.

Abbreviations used: LDL, low-density lipoprotein; BSA, bovine serum albumin; PBS, phosphate-buffered saline; BSM, bovine submaxillary mucin; OSM, ovine submaxillary mucin; poly I, polyinosinic acid; poly C, polycytidylic acid.

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[2] To whom correspondence should be addressed. Fax: 81-75-705-1888. E-mail: hnakada@cc.kyoto-su.ac.jp.

MATERIALS AND METHODS

Cells and materials. A human monocyte cell line, THP-1 cells, and human colon cancer cell lines, LS 180 and CaCo 2 cells, were obtained from the American Type Culture Collection. THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. LS 180 and CaCo 2 cells were cultured in Eagle's MEM medium and MEM–Earle medium supplemented with 10% fetal calf serum, respectively. Ovine submaxillary mucin (OSM) was purified as described previously (6) and labeled with ^{125}I-NaI by the chloramine T method (7). Bovine submaxillary mucin (BSM) was obtained from Roche Diagnostics, Mannheim.

Stimulation of phorbol ester-primed THP-1 cells by mucins. THP-1 cells were induced to differentiate into macrophage-like cells by treatment with 100 nM phorbol 12-myristate 13-acetate for two days according to Hara *et al.* (8). After washing with RPMI 1640 medium containing 0.2% bovine serum albumin (BSA), the cells were cultured for 1 day in the same medium and then washed again. Then, the cells were cultured for another day in the presence of OSM and BSM (30 μg protein/ml). Each culture supernatant was collected and assayed for IL-1β by ELISA. Asialomucins were prepared by treatment of the mucins with immobilized sialidase (*Clostoridium perfringens*) at 37°C for 20 h. After dialysis against phosphate-buffered saline (PBS), the effects of the asialomucins were examined as described above.

Phorbol ester-primed THP-1 cells (3×10^5 cells) were cocultured with LS 180 or CaCo 2 cells (1×10^5 cells) for 2 days. The culture supernatants were also assayed for IL-1β by ELISA.

Expression of recombinant scavenger receptor type I in COS 7 cells. The entire coding region of human macrophage scavenger receptor type I was amplified by means of polymerase chain reaction using a 5′ phosphorylated primer (pAGTGGATAAATCAGTGCTGC), which contains the (+) strand sequence of nucleotides −41 to −22 of the receptor cDNA, and a 3′ primer (GAATTGGAGCCAATTACTGG), which contains the (−) strand sequence of nucleotides 1661–1680. The amplified DNA was inserted into the mammalian expression vector, pCR 3.1 Uni (Invitrogen), to yield the plasmid, pCR 3.1 Uni

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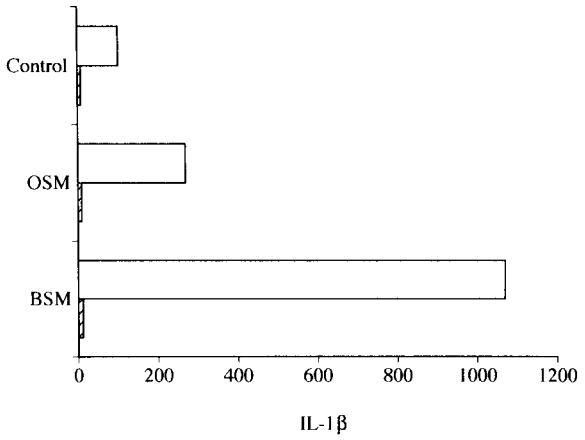

FIG. 1. Effects of mucins on IL-1β secretion from phorbol ester-primed THP-1 cells. THP-1 cells treated (open bars) or nontreated (hatched bars) with phorbol ester as described under Materials and Methods were cultured for 24 h in the presence of BSM and OSM (30 μg protein/ml). Each culture supernatant was collected and assayed for IL-1β by ELISA. The data shown are the mean levels of duplicate experiments and are values relative to those for control cells treated with phorbol ester taken as 100.

SCR. This plasmid was introduced into TOP10F′ cells and amplified according to the manufacturer's protocol. On the first day, COS 7 cells were seeded at 3×10^4 cells per 24-well plate or 1×10^4 cells per spot on multispot glass slides (7×8 mm) in DMEM containing 10% fetal calf serum, penicillin and streptomycin. On day 1, the cells were transfected with plasmid pCR 3.1 Uni SCR or a control plasmid by the Lipofection method. On day 2, the medium was changed to DMEM containing 0.2% BSA. On day 3, the resultant transfectants were used for experiments.

Binding of ^{125}I-labeled OSM to scavenger receptor transfectants. ^{125}I-Labeled OSM was added to COS 7 cells (24-well plate) transfected with scavenger receptor cDNA, followed by incubation at 4°C

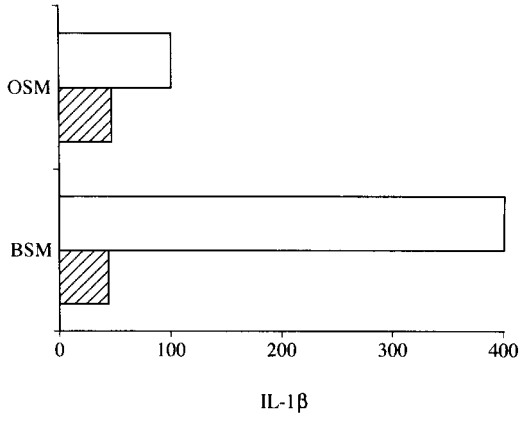

FIG. 2. Effects of asialomucins on IL-1β secretion from phorbol ester-primed THP-1 cells. Phorbol ester-primed THP-1 cells were cultured for 24 h in the presence of OSM and BSM treated (hatched bars) or non-treated (open bars) with sialidase. Each culture supernatant was collected and assayed for IL-1β by ELISA. The data shown are the mean levels of duplicate experiments and are values relative to those for the cells incubated with OSM taken as 100.

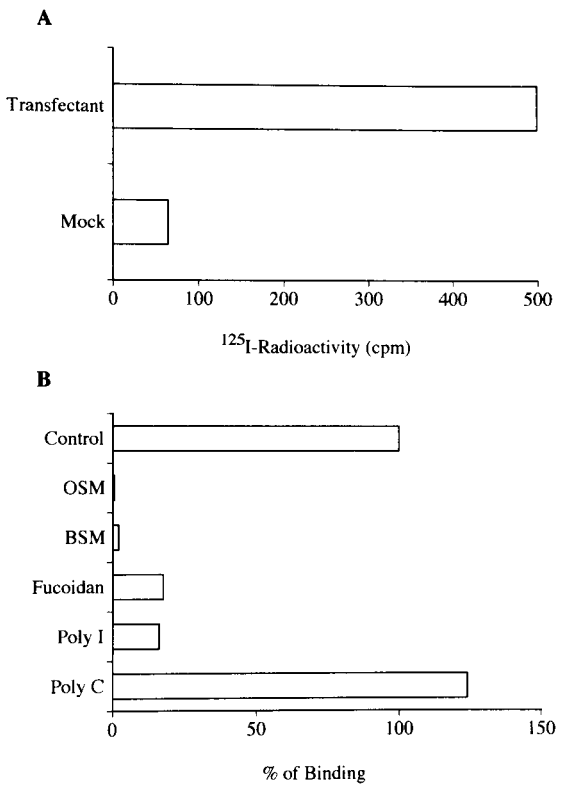

FIG. 3. Binding of ^{125}I-labeled OSM to scavenger receptor transfectants. (A) The scavenger receptor and mock transfectants were incubated with ^{125}I-labeled OSM (10 ng/ml) at 4°C for 2 h and the bound radioactivity was determined as described under Materials and Methods. (B) The scavenger receptor transfectants were incubated with ^{125}I-labeled OSM (10 ng/ml) at 4°C for 2 h in the presence of various inhibitors. The inhibitors used were BSM, OSM (30 μg protein/ml), fucoidan, poly I and poly C (100 μg/ml).

for 2 h. After washing five times with DMEM containing 0.2% BSA, the bound radioactivity was determined. The scavenger receptor transfectants were also incubated with ^{125}I-labeled OSM (10 ng/ml) at 4°C for 2 h in the presence of various inhibitors. The inhibitors used were BSM, OSM (30 μg protein/ml), fucoidan, polyinosinic acid (poly I), and polycytidylic acid (poly C) (100 μg/ml).

Adhesion of colon cancer cells to scavenger receptor transfectants. COS 7 cells (1×10^4 cells) were placed on spots on multispot glass slides (7×8 mm) and then transfected as described above. CaCo 2 and LS 180 cells were labeled by incubation with 5 μM 2,7-bis(carboxyethyl)-carboxyfluorescein tetra(acetoxymetyl) ester at 37°C for 1 h and then washed five times with MEM containing 0.2% BSA. Cells suspended in PBS (3×10^4 cells) were added to each spot on the glass slides, followed by incubation at 37°C for 30 min. Each spot was washed five times with PBS and then the adhered cells were sonicated in 0.6 ml of PBS containing 1% NP-40. After centrifugation at 10,000*g* for 20 min, the fluorescence intensity of the supernatant was determined at 530 nm with an excitation wavelength of 490 nm.

RESULTS AND DISCUSSION

Enhanced secretion of IL-1β from phorbol ester-primed THP-1 cells on treatment with mucins. It has been reported that phorbol ester-primed THP-1 cells

express a scavenger receptor, and that the binding of ligands such as fucoidan and acetyl-LDL results in enhanced secretion of IL-1β and TNFα (9). As shown in Fig. 1, OSM and BSM enhanced the secretion of IL-1β. No detectable secretion of IL-1β was observed for non-primed THP-1 cells irrespective of the presence or absence of mucins. These mucins also significantly enhanced the secretion of TNFα (data not shown). Due to the sensitivity of monocytes to trace amounts of lipopolysaccharides, it is critical whether or not the mucins are contaminated by lipopolysaccharides. The enhanced secretion of IL-1β caused by mucins was not abolished by polymixin B, which is an antibiotic capable of inhibiting IL-1 induction mediated by lipopolysaccharides, indicating that this stimulatory effect was not due to contamination by lipopolysaccharides (data not shown). Since it has been reported that anionic charges on a ligand molecule are essential for the ligand activity, we examined the effect of sialidase treatment. As shown in Fig. 2, treatment of the mucins with sialidase abolished the ability to enhance the secretion of IL-1β, indicating that sialic acid is involved in the binding.

Binding of ^{125}I-labeled OSM to scavenger receptor transfectants. An expression plasmid containing cDNA encoding the scavenger receptor was expressed in COS 7 cells. We examined whether or not the recombinant receptor is expressed on the cell surface and is functional. 1,1′-Dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine-conjugated acetyl-LDL (DiI-acetyl-LDL) was taken up by the scavenger receptor transfectants but not by the mock transfectants (data not shown). The binding of ^{125}I-labeled OSM was assessed by incubating the transfectants with ^{125}I-labeled OSM (Fig. 3A). ^{125}I-Labeled OSM was bound to the scavenger receptor transfectants much more than to the mock transfectants. To further clarify the binding properties of OSM, the ligand activity was estimated in the presence of various materials, including ligands of the macrophage scavenger receptor. Fucoidan and poly I, but

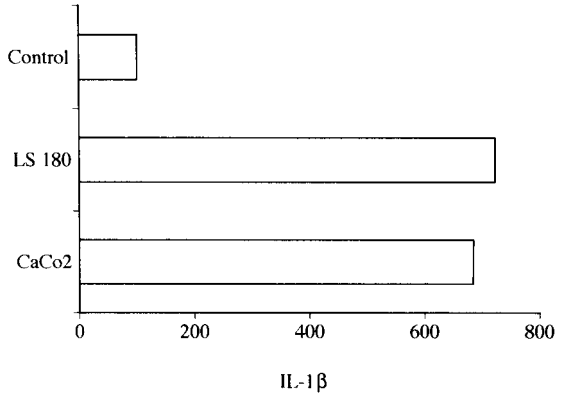

FIG. 4. Secretion of IL-1β from phorbol ester-primed THP-1 cells cocultured with LS 180 and CaCo 2 cells. Phorbol ester-primed THP-1 cells were co-cultured with LS 180 and CaCo 2 cells, or cultured separately (control cells) for 2 days. Culture supernatant was assayed for IL-1β by ELISA. The data shown are the mean levels of duplicate experiments and are values relative to those for control cells taken as 100.

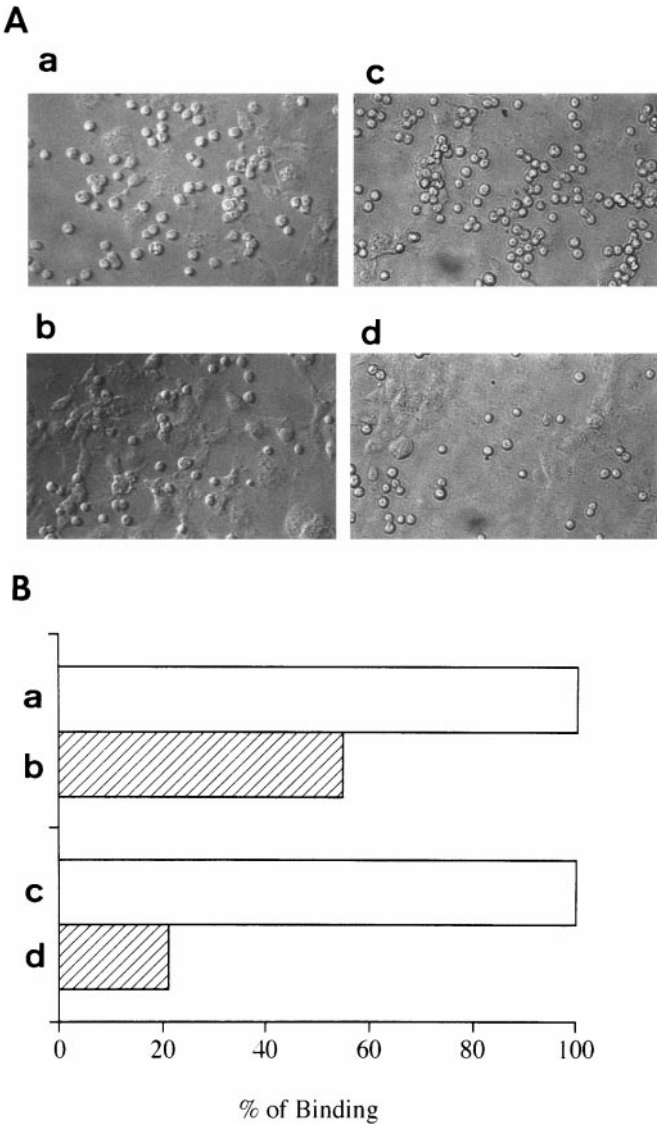

FIG. 5. Adhesion of colon cancer cells to scavenger receptor transfectants. LS 180 (a, b) and CaCo 2 (c, d) cells were labeled with 2,7-bis(carboxyethyl)-carboxyfluorescein tetra(acetoxymethyl) ester and incubated with scavenger receptor and mock transfectants as described under Materials and Methods. (A) Binding of these cells to the scavenger receptor transfectants (a, c) and mock transfectants (b, d) was observed. (B) The fluorescence intensity of the cells adhered to the scavenger receptor transfectants (open bars) and mock transfectants (hatched bars) was estimated as described under Materials and Methods (triplicate determinations). Values are expressed as percentage relative to those for cells adhered to the scavenger receptor transfectants taken as 100%.

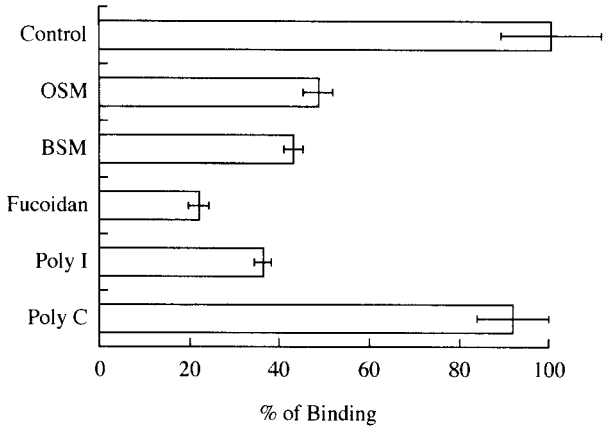

FIG. 6. Adhesion of CaCo 2 cells to scavenger receptor transfectants in the presence of various inhibitors. CaCo 2 cells were labeled and incubated with scavenger receptor transfectants in the presence of various inhibitors as described under Materials and Methods. The fluorescence intensity of the adhered cells was estimated and values are expressed as % relative to those for cells adhered in the absence of inhibitors taken as 100%. The inhibitors used were BSM, OSM (30 μg protein/ml), fucoidan, poly I and poly C (100 μg/ml).

not poly C reduced the binding of ^{125}I-labeled OSM to the scavenger receptor transfectants, as shown in Fig. 3B. These results are consistent with the characteristics of the scavenger receptor.

Enhanced secretion of IL-1β from phorbol ester-primed THP-1 cells by coculture with LS 180 and CaCo 2 cells. Generally, epithelial cancer cells not only secrete mucins but also express mucins on their cell surface. We examined whether or not these mucins stimulate the phorbol ester-primed THP-1 cells. As shown in Fig. 4, these colon cancer cells stimulated the phorbol ester-primed THP-1 cells effectively. Secreted IL-1β increased about seven fold by co-culture with the colon cancer cells. IL-1β secreted from LS 180 and CaCo 2 cells was negligible (data not shown). We further examined whether or not these mucins on the cell surface mediate the adhesion between scavenger receptor transfectants and colon cancer cells. These cancer cells adhered to the scavenger receptor transfectants more than to the mock transfectants, as shown in Fig. 5. To further confirm that the scavenger receptor was responsible for the binding, the adhesion of CaCo 2 cells in the presence of the ligands was investigated. As shown in Fig. 6, BSM, OSM and other known ligands for the receptor significantly inhibited the adhesion. The binding of ^{125}I-labeled OSM or colon cancer cells to the scavenger receptor transfectants was inhibited by mucins, fucoidan and poly I but not by poly C. This difference between poly I and poly C is completely consistent with the results reported previously (2).

It has been claimed that in cancer tissues, cancer cells, inflammatory cells and adjacent vasculature interact with each other, the interaction being mediated by chemical factors. Mucins secreted and/or present on the cell surface could stimulate the secretion of cytokines from macrophages. It has also been reported that IL-1β and TNFα can induce the expression of a vascular endothelial growth factor in human colon, glioma and prostate cancer cells (10–12). Mucins may play a significant role as amplifying factors for macrophages that have infiltrated into epithelial cancer tissues. We detected scavenger receptor mRNA in macrophages that had infiltrated into colon cancer tissues (Nakada *et al.*, unpublished data). IL-1β and TNFα can also induce the expression of various adhesion molecules in endothelial cells, in which the soluble forms of E-selectin and VCAM-1 accelerate angiogenesis (13). Thus, these microenvironmental conditions may facilitate the growth of cancer cells and angiogenesis.

We also performed similar experiments using human mononuclear cells isolated from the buffy coats of healthy human donors and confirmed that the secretion of IL-1β increased on incubation with mucins (Nakada *et al.*, unpublished data). Mucins are synthesized by a variety of secretory epithelial tissues as membrane-bound or secreted forms. It has been reported that mucinous colon cancers can be diagnosed at a more advanced stage, and that mucinous human colon cancer cell lines exhibit greater tumorigenicity and metastatic ability, compared to non-mucinous cell lines (14). The mechanisms responsible for these biological effects are poorly understood. It is possible that the mucins present in epithelial cancer tissues or secreted into the blood-stream may alter the functions of immune effector cells.

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