Expression of Macrophage MARCO Receptor Induces Formation of Dendritic Plasma Membrane Processes*

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MARCO is a novel macrophage-specific receptor structurally related to macrophage class A scavenger receptors. It is constitutively expressed in macrophages of the marginal zone of the spleen and in lymph nodes and is up-regulated in other tissues during systemic bacterial infections. In this study, we show that ectopic expression of MARCO in cell lines such as Chinese hamster ovary, HeLa, NIH3T3, and 293 induces dramatic cell shape changes. Typically these changes include formation of large lamellipodia-like structures and of long dendritic processes. The morphological changes are accompanied by disassembly of actin stress fibers and often also by complete loss of focal adhesions. The MARCO-induced changes are dependent on cell adhesion and are inhibited, but not completely abolished, when the cells are plated on fibronectin-coated surfaces. Similarly, a dominant-negative mutant of the Rho family GTPase Rac1 partially inhibited the morphogenic effects of MARCO in Chinese hamster ovary cells, whereas a dominant-negative form of a related protein, Cdc42, did not. Expression studies with a variety of truncated MARCO forms indicated that the proximal segment of the cysteine-rich domain V is important for the morphoregulatory activity. The results indicate that expression of MARCO has a direct effect in generating the phenotype of activated macrophages necessary for the trapping and removal of pathogens and other foreign substances.

Macrophages are monocyte-derived cells that upon activation acquire phagocytic activity for pathogens, capability for antigen presentation to lymphocytes, and the ability to contribute to tissue repair by removing dead and damaged tissue (1, 2). Resting macrophages are fusiform or stellate in shape, whereas activated macrophages have extensive pseudopodia as well as short microvilli and lamellipodia (1). The protrusions are apparently important for phagocytosis, as they extend around the pathogens prior to their engulfment.

MARCO† is a macrophage receptor (3) constitutively expressed in a subpopulation of macrophages in the marginal zone of the spleen and in the medullary cord of lymph nodes, i.e. regions where macrophages are actively engaged in the removal of pathogens and other foreign substances from the blood and lymph fluid. This has implied a direct role for MARCO in the removal of pathogens. This assumption is supported by studies showing that COS, CHO, and fibroblast cells, which normally do not bind bacteria, acquire binding activity for both Gram-positive and Gram-negative bacteria following transfection and expression of MARCO (3). Furthermore, it has been shown that during systemic bacterial infections, expression of MARCO is induced in macrophages located in other tissues, such as liver and lung (4). Together, these data strongly indicate a role for MARCO in the host defense against microorganisms.

MARCO is structurally related to the macrophage class A scavenger receptors (MSR-A) (5, 6). However, despite structural similarities, the pattern of expression is different as MSR-A receptors are constitutively expressed by macrophages in all tissues. All these receptors are homotrimeric proteins with a short cytoplasmic domain, a single transmembrane domain, and a large extracellular part. The extracellular portion of MSR-A has both a triple helical coiled coil and collagenous structures, whereas MARCO contains only a long collagenous triple helix. Additionally, both MARCO and MSR-AI have a C-terminal cysteine-rich domain composed of about 100 amino acid residues, whereas MSR-AII lacks this domain completely (3, 5–7). The bacteria-binding region of MARCO has been localized to this C-terminal region (7). MSR-A has been shown to bind a wide variety of ligands, such as modified low density lipoprotein and bacterial surface components (8–11). Studies with MSR-A knock-out mice have shown that this receptor contributes to the formation of atherosclerotic lesions and plays a role in host defense against pathogens (12, 13), suggesting that the binding activities of MSR-A shown in vitro have physiological significance. In addition, MSR-A has been implicated in macrophage adhesion (14).

In our studies on MARCO function we observed that expression of this protein in many different cell lines resulted in dramatic cell shape changes. Typically, these changes include formation of large lamellipodia-like structures, as well as extensive plasma membrane processes. By expressing various truncated forms of MARCO, we show that a segment in the C-terminal cysteine-rich domain is crucial for this activity. The MARCO-induced morphological changes are accompanied by rearrangement of the actin cytoskeleton. Furthermore, they are inhibited, but not completely abolished, when CHO cells are plated on fibronectin-coated surface or when MARCO is co-expressed with a dominant-negative form of the small containing 419, 436, and 441 residues, respectively, from the N-terminal end.

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† The abbreviations used are: MARCO, macrophage receptor with collagenous structure; CHO, Chinese hamster ovary cells; MSR, macrophage scavenger receptor; FCS, fetal calf serum; ECM, extracellular matrix; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate, M-419, M-436, M-441, recombinant MARCO receptor variants.
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Expression of MARCO induces formation of cellular processes and rearrangement of the actin cytoskeleton—An interesting change in cell shape was noticed when cells not normally expressing MARCO were transfected with a plasmid encoding the full-length protein. When cells were grown overnight on glass coverslips in complete medium and examined by immunofluorescence microscopy about 40 h after transfection, MARCO-expressing cells often had processes with lengths several times the size of the cell body. Cell lines studied included CHO, HeLa, NIH3T3, and 293 cells. There was some variation in the morphological effects of MARCO between the cell lines so that the processes were longest in 293 cells and that the other cell lines mentioned above often also had large lamellipodium-type structures (from which the long processes extended). Expression of MARCO in CHO, HeLa, NIH3T3, and 293 cells is shown in Fig. 1 (A, C, and D) and also in Figs. 6 and 7. In contrast, COS7 cells responded quite differently to MARCO transfection. In these cells, MARCO expression led to formation of short spine needle-like protrusions (not shown).

Process formation was not dependent on the transfection method, since a similar effect was observed when MARCO-
expressing DNA was introduced either by calcium-phosphate method or by retroviral infection (not shown). To test if the process formation was due to overexpression of a membrane protein, we expressed the H1 subunit of the human asialoglycoprotein receptor which is, similar to MARCO, a type II membrane protein. Expression of this protein did not induce any morphological changes (not shown), demonstrating that cell shape changes are not simply a consequence of expression of a cell membrane protein. In further studies, it turned out that MARCO-expressing cells could be identified in cell populations simply by staining for actin filaments (Fig. 1B). Compared with untransfected cells, MARCO-expressing cells stained only weakly by the filamentous actin-staining rhodamine-conjugated phalloidin. Furthermore, filamentous actin appeared to be organized differently in these two populations of cells. Examination of cells by a higher magnification revealed that untransfected cells had an organized actin cytoskeleton with numerous long stress fibers (Fig. 2). In contrast, the MARCO-expressing cells almost completely lacked stress fibers. Instead, there were fine actin filaments extending from the cell membrane into the processes, although the phalloidin staining was very weak in these processes (not shown). The processes were frequently heavily branched, and examination of the branching pattern revealed that these processes were “protrusive filopodia” instead of trailing tails, being left behind by migratory cells.

In order to study the early phases of the process formation, we focused on transiently transfected CHO cells, since these cells did not aggregate when held overnight in suspension. Cells were taken into suspension 24 h after transfection and were then plated 12–16 h later on FCS-coated coverslips. When cells were fixed 5 min after plating, all of them were round, and the MARCO-expressing ones were morphologically indistinguishable from untransfected cells (not shown). However, when cells were fixed 30 min after plating, untransfected cells were still fairly round, but most of the MARCO-expressing cells were already well spread (Fig. 3A). In addition, there were lamellipodia-like protrusions extending from the periphery of some of the MARCO-expressing cells (Fig. 3B). Examination of cells 1 h after plating revealed that most of the MARCO-expressing cells had protrusions (Fig. 3C). Furthermore, many of the cells sent out thin dendritic processes. After a few hours the processes could be several times longer than the cell body (3 h after plating in Fig. 3D). It is of interest to note that the cell spreading is fairly symmetrical and that forming lamellipodia appear to extend randomly in different directions. However, soon thereafter MARCO-expressing cells acquired polarity so that the long processes extended only from one pole of the cell.

The MARCO-induced morphological effects were efficiently inhibited when cells were plated on FCS-coated coverslips for 45 min in the presence of 2 μM cytochalasin D (not shown), demonstrating the crucial role of actin polymerization in this process (at a 2-h time point cytochalasin D was found to have a toxic effect). On the other hand, 10 mM butanedione monoxime, a known myosin inhibitor, did not have significant effects during the initial cell spreading, whereas it markedly inhibited the process formation (not shown). In this experiment, cells were assayed 45 min and 2 h after plating on FCS-coated coverslips.

Segment of Domain V Is Important for the Morphoregulatory Activity of MARCO—In order to identify specific regions of the MARCO polypeptide required for the morphological effects, different truncated forms of MARCO were expressed in CHO cells (Fig. 4). First, we tested truncations M-436 and M-441, which almost completely lacked the C-terminal cysteine-rich domain V and contained, respectively, only the first 16 and 21 residues of this domain. Both forms efficiently promoted the morphological change, demonstrating that most of the domain V is not needed for this activity. We next produced a version of MARCO that lacks the C-terminal half of the collagenous domain (version Bst, lacking residues 299–419), and the morphological change of cells was observed upon expression of this protein, too. Since this indicated that the C-terminal part of the collagenous domain is not needed for the morphological effects, we next tested a form of MARCO, whose extracellular part is composed of the first 4 residues of the spacer domain, the last 65 residues of the collagenous domain, and an intact domain V (version Eco). Somewhat surprisingly, this variant also promoted process formation, albeit not as efficiently as the other truncated forms. Taken together, the only region of the extracellular domain that is common for all these effective truncated versions is a segment of domain V before its first cysteine residue (residues 420–436). Therefore, we predicted that expression of the form M-419, which does not contain any segments of domain V, would not result in the change of the cell shape. Indeed, this was the case, indicating that the segment of...
domain V encompassing its first 15–20 residues is important for this activity. We tried to confirm this result by expressing a version of MARCO in which only this region is deleted (version Del 422–433). However, this protein did not appear on the cell surface (see below) but was apparently retained in the secretory pathway.

Since the truncation M-419 did not promote any morphological changes, we wanted to ensure that it was still expressed on the cell surface (see below) but was apparently retained in the secretory pathway.

Since the truncation M-419 did not promote any morphological changes, we wanted to ensure that it was still expressed on the cell surface. In order to examine this, cells were biotinylated with a membrane-impermeable reagent sulfo-NHS-biotin, after which biotinylated proteins were precipitated by streptavidin-agarose and analyzed by Western blotting using an antibody recognizing the cytoplasmic domain of the MARCO protein. This experiment demonstrated that the form M-419 and the full-length MARCO were expressed at equal levels on the cell surface (not shown) and that some of the morphologically active truncations, such as M-436, are not as abundantly expressed on the cell surface (Fig. 5A). Moreover, along with the results obtained in the transfection studies with a plasmid encoding the H1 subunit of the human asialoglycoprotein receptor (see above), these results indicate that the morphological change is not a nonspecific effect, due only to overexpression of a cell-surface protein. In accordance with this conclusion, we have observed that cells change their morphology also when expression of MARCO is driven from a weak promoter, the minimal cytomegalovirus promoter (not shown). To confirm that only cell-surface proteins were labeled by sulfo-NHS-biotin, we wanted to verify that paxillin, a component of focal adhesions at the inner site of the plasma membrane, was not precipitated by streptavidin-agarose. This was the case, even though an anti-paxillin antibody gave a strong signal with the total cell lysate (Fig. 5B). Similarly, one of the MARCO truncations, Del 422–433, was not precipitated by streptavidin-agarose, although it was abundant in the total cell lysate. Thus,
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3 1 2 3 4 1 2 3 4

FIG. 5. Analyses of proteins in transiently transfected CHO cells. A, amounts of the different MARCO forms on the cell surface. Cell-surface proteins were biotinylated on ice followed by the precipitation with streptavidin-agarose. The precipitated proteins were analyzed by Western blotting using an anti-MARCO antibody recognizing the cytoplasmic domain (monoclonal antibody 1.18). CHO cells were transfected with expression vectors encoding forms Del422–433 (lane 1), M-419 (lane 2), M-436 (lane 3), and M-441 (lane 4). B, two of the samples analyzed in A, M-419 and M-436, were also analyzed for the presence of paxillin in the streptavidin precipitate. Lane 1, total cell lysate; lane 2, total cell lysate from cells expressing the form M-419 (1/100 portion of the total lysate); lane 3, total cell lysate from cells expressing the form M-436 (1/100 portion). Lanes 3 and 4 represent streptavidin precipitates and show that paxillin was not precipitated by streptavidin-agarose, indicating that only cell-surface proteins were biotinylated during the course of the experiment. One-quarter of the precipitated proteins were loaded in the gel.

3 1 2 3 4 1 2 3 4

FIG. 6. Effects of MARCO expression on focal adhesion assembly. CHO (A and B) and HeLa cells (C and D) transiently expressing MARCO were double-stained for MARCO (A and C) and endogenous paxillin (B and D). A and B and C and D, respectively, represent the same field. Cells were examined 40 h post-transfection, after growing them for 16 h on glass coverslips. Focal adhesions do not follow cell margins in the MARCO-expressing cells. Note that CHO cells (B) not expressing MARCO have distinct paxillin-positive focal adhesions (arrows). It is not shown here, but often there are no focal adhesions in those MARCO-expressing cells that have very long extensions, or, as shown in B, if there are focal adhesions, they are not necessarily well formed.

Effects of Different ECM Substra on the Formation of the Long Plasma Membrane Processes—To examine the effects of different substrata on the MARCO-induced cell shape changes, cells were plated on coverslips precoated with FCS or with purified ECM components laminin-1, vitronectin, and fibronectin. As a comparison, cells were plated on uncoated glass. Transiently transfected CHO cells kept in suspension overnight were plated in serum-free medium, and cells were fixed and stained for MARCO and actin filaments 3 h later. This staining showed that extensions on the MARCO-expressing cells were longest when cells were plated on uncoated glass and that plating on different ECM components did not prevent formation of the extensions. However, they were clearly much shorter on the fibronectin-coated surface (not shown). On that surface, both untransfected and MARCO-expressing cells spread extensively.

Dominant-Negative Rac1 Inhibits the Morphological Effects of MARCO in CHO Cells—Small GTPases of the Rho family have been shown to control rearrangement of the actin cytoskeleton (17). Specifically, activated Cdc42 promotes formation of filopodia, whereas the activated form of Rac1 induces cell spreading and lamellipodia formation. Since the MARCO-induced cell shape changes somewhat resemble the phenotypic changes caused by activated Cdc42 and Rac1, we investigated whether these GTPases play any role in the MARCO-induced cell spreading/process formation. In order to study this, we co-expressed MARCO and dominant-negative forms of Cdc42 and Rac1 in CHO cells (Fig. 7). Transiently transfected CHO cells were taken into suspension 24 h after transfection and were plated 12 h later on FCS-coated coverslips. Cells were fixed after 30 min, since it was known that the MARCO-expressing cells are already extensively spread at this time point (Fig. 3A). Cells co-expressing MARCO and dominant-negative Cdc42 did not differ morphologically from the MARCO-expressing cells. In contrast, dominant-negative Rac1 markedly inhibited the MARCO-induced cell spreading (not shown). We also examined cells that were plated on coverslips for 16 h. Again, dominant-negative Cdc42 did not have inhibitory activity (Fig. 7B), but Rac1V12N17 clearly inhibited the MARCO-induced morphological changes (Fig. 7, C and D). However, Rac1V12N17 did not completely abolish the MARCO-induced effects. There were a few large MARCO-positive cells but no cells with long processes. The dominant-negative forms of Cdc42 and Rac1 contain a Myc epitope tag at their N termini, and analysis of total cell lysates by Western blotting using an anti-Myc antibody demonstrated that these GTPase mutants were produced at equal
One possible explanation for the different phenotypic effects of the dominant-negative Rac1 and Cdc42 was that MARCO/Rac1V12N17 transfectants expressed MARCO at lower levels than MARCO/Cdc42N17 transfectants. In order to study if this was the case, we analyzed the cell-surface levels of MARCO using the biotinylation/streptavidin-agarose precipitation procedure followed by Western blotting using an anti-MARCO antibody. In three different experiments the MARCO/Rac1V12N17 transfectants were found to have at least as much MARCO on the cell surface as the MARCO/Cdc42 N17 transfectants (Fig. 7E). These results demonstrate that the phenotypic effects of dominant-negative Rac1 are not simply due to an inhibitory effect on the cell-surface expression of MARCO. As in previous studies, we confirmed that the biotinylation reagent labeled only cell-surface proteins by using an anti-paxillin antibody (not shown).

DISCUSSION

In this study, we have shown that expression of the macrophage MARCO receptor results in extensive changes in cell morphology through induction of dendritic plasma membrane processes. Of the five cell lines tested, COS7 cells were the only exception, since in these cells MARCO expression had minor morphological effect by leading to the formation of short pine needle-like protrusions. The reason for this different response is unclear, but it could be related to the strength of adhesion between cells and serum-coated surface. Supporting this notion, we found that processes were longest in 293 cells, which are weakly adhering cells, whereas COS7 cells spread well and adhere tightly to serum-coated glass surface.

Several lines of evidence indicate that the morphological effects are specific effects of the MARCO receptor itself. By using various truncated forms of MARCO, it was shown that...
the part of domain V before its first cysteine residue is needed for this activity. This conclusion is based, for example, on the following two observations. First, a MARCO form completely lacking domain V did not have any effect on cell morphology, although it was expressed at high levels on the cell surface. Second, a form that extends only 16 residues to domain V was found to be as active as the full-length MARCO. In a previous study, we showed that when cells were transfected with expression vectors encoding these two forms, only cells expressing the longer form bind heat-killed bacteria (7). This result is another indication that the region of domain V proximal to the collagenous domain is functionally important in MARCO. Our previous work also suggested that this region is exposed on the MARCO protein since it was very sensitive for proteolysis when expressed as a fusion protein (7).

It remains to be investigated whether the cytoplasmic domain and/or the transmembrane domain are also needed for the morphological activity of MARCO. Thus far, we have tested a truncated form lacking the N-terminal half of the cytoplasmic domain, and the morphological effects are not abolished. The use of chimeric proteins is needed to determine the role of these domains, since MARCO is a type II membrane protein, and the membrane-proximal region, which contains several positively charged residues, is very likely important for a correct topological orientation of MARCO.

MARCO was shown to induce process formation on different surfaces, such as uncoated glass, laminin-1, and vitronectin. In fact, the processes were longest when the cells were plated in serum-free media on uncoated glass. Of the purified ECM components tested, fibronectin was the only protein that clearly had an inhibitory effect on the MARCO-induced process formation. Thus, cell adhesion on fibronectin and MARCO expression seem to have opposite effects on the organization of the actin cytoskeleton. Indeed, cell adhesion on fibronectin has been reported to promote rapid actin stress fiber and focal adhesion formation (18), whereas the present study shows that expression of MARCO promotes dissolution of stress fibers and focal adhesions.

It is not yet known whether MARCO binds glass or different ECM proteins. We are currently producing a soluble MARCO for the binding studies. In this context, it is noteworthy that a protein related to MARCO, macrophage scavenger receptor A, has been shown to function as an adhesion receptor, whose ligand is present in fetal calf serum (14). Interestingly, thiglycollate-elicited peritoneal macrophages from MSR-A knock-out mice have also been found to spread on glass slower than wild-type macrophages (13). It is, however, possible that MARCO exerts its effects by interacting with another cell-surface protein expressed on the same cell, as was found to be the case for integrin \(\alpha_6\beta_1\) in embryonic stem cells. In these cells expression of \(\alpha_6\beta_1\) integrin induces changes in the morphology (filopodia formation) and migration without having to engage with its ECM ligand (19). Instead, the \(\alpha_6\beta_1\)-induced motility appears to depend on the association of \(\alpha_6\beta_1\) with CD81, a member of the tetraspan superfAMILY of cell-surface molecules (19).

Dominant-negative Rac1 partially inhibited the MARCO-induced cell shape changes in CHO cells. We believe that this is not due to nonspecific toxic effects since these cells were still capable of functioning normally, at least if judged by the amount of MARCO they produced and transported to the cell surface. The partial inhibition suggests that exogenous dominant-negative Rac1 could not completely inhibit the activity of the endogenous Rac1 or Rac1-related protein. On the other hand, it is known that activated Rac1 causes a phenotype that differs from that induced by MARCO. It promotes cell spreading and formation of lamellipodia but does not induce formation of long plasma membrane processes. Thus, the Rac1 pathway is not the only pathway activated by MARCO. Surprisingly, dominant-negative Rac1 did not have any effect on MARCO-induced cell shape changes in HeLa cells. We do not know the reason for this, but it is conceivable that MARCO activates some Rac1-related protein in these cells and that dominant-negative Rac1 is not able to interfere with the activity of this protein.

Several protein kinases, such as p21(Cdc42/Rac)-activated kinase aPAK, have been shown to play a role either in the formation or dissolution of stress fibers and focal adhesions (18, 20). Also, lipid kinases have been shown to be involved in the actin remodeling. For example, expression of type I phosphatidylinositol-4-phosphate 5-kinase induces formation of pine needle-like protrusions in COS7 cells (21). Interestingly, these protrusions resemble those induced by MARCO expression in COS7 cells. The effects of phosphatidylinositol-4-phosphate 5-kinase on cell morphology are most likely due to elevated levels of phosphatidyl 4,5-biphosphate which is able to uncap actin filament barbed ends, thereby promoting actin polymerization (22). Recent evidence also suggest that the Rho family GTPases are able to regulate phosphatidyl 4,5-biphosphate levels. For example, Rac was shown to induce rapid synthesis of phosphatidyl 4,5-biphosphate in permeabilized platelets (22).

Our efforts to elucidate the signaling pathways activated by MARCO have been partly hampered by the lack of a population of cells uniformly expressing MARCO. CHO transfectants appear to lose most of their MARCO-expressing cells during their propagation. It is possible that the morphological effects of MARCO hinder cell division, and we wish to overcome this problem by the use of an inducible expression system.

MARCO is expressed constitutively only in macrophages in lymph nodes and marginal zone of the spleen (3). The marginal zone macrophages are thought to be important in the trapping and clearance of microorganisms from the bloodstream (23). Our results indicate that MARCO has several functions that contribute to this activity of the marginal zone macrophages. First of all, as shown previously (3, 7), MARCO is able to bind bacteria. Second, the morphoregulatory activity resulting in the formation of large lamellipodia-like structures and long plasma membrane extensions provides an enlarged cell-surface area. This, in turn, facilitates efficient trapping of pathogens by cell-surface receptors.

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