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# Shedding of CD163, a Novel Regulatory Mechanism for a Member of the Scavenger Receptor Cysteine-Rich Family

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**The glucocorticoid-inducible transmembrane protein CD163 is a member of the scavenger receptor cysteine-rich (SRCR) family which is expressed exclusively on human monocytes and macrophages. The expression of the protein is significantly downregulated in response to phorbol 12-myristate 13-acetate (PMA) by a yet unknown mechanism. We now demonstrate that PMA induces shedding of a soluble form of CD163 rather than internalization, revealing a novel regulatory mechanism for a member of the SRCR family. Bisindolylmaleimide I was shown to inhibit phorbol ester-induced shedding, thus implying an involvement of protein kinase C (PKC). Furthermore, cleavage could be prevented by protease inhibitors. Therefore, we suggest that PMA-induced activation of PKC leads to protease-mediated shedding of CD163. These results indicate a specific release mechanism of soluble CD163 by human monocytes which could play an important role in modulating inflammatory processes.** © 1999 Academic Press

The CD163 antigen is a member of the scavenger receptor cysteine-rich (SRCR) superfamily (1). Although a growing number of new members of this protein family has been recently identified, little is known about the regulation and function of these proteins. All known members of the type B SRCR-domain-containing proteins are expressed on the surface of cells associated with the immune system (2). One group B SRCR family member, Sp$\alpha$, has no transmembrane or cytoplasmic domain and is secreted (3). The biochemical functions of SRCR-domains have not been determined with certainty; however, it is assumed that most of these domains are involved in binding to other cell-surface or extracellular molecules. The SRCR-proteins have been proposed to play a role in host defense (4), but their function has not yet been defined conclusively (5–7).

The 130-kDa transmembrane CD163 glycoprotein is restricted to human monocytes and macrophages. The protein expression is markedly induced by glucocorticoids *in vitro* and *in vivo* (8). In addition, it is regularly found in acute and chronic inflammatory lesions. During experimental gingivitis and under allergic contact eczema CD163-positive macrophages appeared preferentially in the late inflammatory phase (9, 10). Thus, this population was considered to have some function in down-regulating the inflammatory process. This was supported by the observation that the population of CD163-positive macrophages secrete proteins with anti-inflammatory properties (11).

Recently, it has been found that treatment of CD163-positive monocytes with PMA resulted in a rapid decrease of antigen density, as assessed by immunofluorescence flow cytometry using the specific CD163-recognizing monoclonal antibodies RM3/1 and Ki-M8 (12). However, the mechanism of this downregulation remained to be established. In the present study we demonstrate that human monocytes release a soluble form of CD163 following PMA treatment, a process which was strongly inhibited by protease inhibitors. We discuss a PKC-regulated shedding as the physiological mechanism of generation of soluble CD163.

## MATERIALS AND METHODS

*Reagents.* Fluticasone-17-propionate (FP) was a generous gift from Glaxo Wellcome (Greenford, UK). Phorbol 12-myristate 13-acetate was from Sigma (Deisenhofen, Germany) and complete mini protease inhibitor cocktail tablets with or without EDTA were supplied by Boehringer-Mannheim (Mannheim, Germany). Bisindolylmaleimide I (BIM) was purchased from Calbiochem (Bad Soden, Germany).

*Antibodies.* Monoclonal antibody Ki-M8 against CD163 was purchased from BACHEM Biochemica (Heidelberg, Germany). The monoclonal RM3/1 antibody was previously generated and characterized by our group (10). Mouse isotype control $IgG_1$ and FITC-conjugated goat anti-mouse IgG were from Dianova (Hamburg, Ger-

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Abbreviations used: SRCR, scavenger receptor cysteine-rich; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; FP, Fluticasone-17-propionate; BIM, bisindolylmaleimide.

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many). Horseradish peroxidase-conjugated goat anti-mouse IgG was from DAKO Diagnostika (Hamburg, Germany).

*Cell culture.* Monocytes were isolated from pooled buffy coats by a two step density gradient centrifugation in Ficoll–Hypaque and Percoll (Pharmacia, Freiburg, Germany) as previously described (13). The cells (purity $\geq$ 90%) were cultivated at a density of $2 \times 10^6$ cells/ml in Teflon bags (Heraeus, Hanau, Germany) in McCoy's 5A medium (Biochrom, Berlin, Germany) supplemented with 15% FCS, 2 mM glutamine and antibiotics. To upregulate CD163, monocytes were stimulated with FP ($10^{-8}$ M) for 2 days.

*Preparation of supernatants and cell lysates.* After 2 days of cultivation, monocytes were harvested by centrifugation and resuspended in PBS at a titer of 1 x $10^7$ cells/ml. Aliquots of 1 ml were stimulated with or without PMA ($10^{-8}$ M) for 1 h at 37°C. Where indicated, cells were preincubated with inhibitors for 10 min at 37°C. Cells were pelleted by centrifugation and supernatants were collected. Cell pellets were lysed with 100 $\mu$l cold lysis-buffer (1% octylthioglucopyranoside in TBS, 1 mM $CaCl_2$, complete-mini EDTA-free) on ice, and lysates were precleared by centrifugation at 10.000*g* for 10 min at 4°C.

*Western blot.* Supernatants and cell lysates were subjected to SDS–PAGE under non-reducing conditions in 6% gels in a sample buffer containing 0.1% SDS and transferred to nitrocellulose membranes. Membranes were incubated for 30 min in blocking buffer (TBS, 0.1% Tween 20, 5% skim milk powder) and probed with Ki-M8 antibody (4 ng/ml in blocking buffer) for 1 h. Bound antibody was detected by peroxidase-conjugated goat anti-mouse IgG (1:2000 in blocking buffer) and enhanced chemiluminescence using the ECL kit (Amersham, Freiburg, Germany) according to manufacturer's instructions.

*Flow cytometry.* Expression of CD163 on monocytes was determined by FACScan analysis. Monocytes were permeabilized using the Cytofix/Cytoperm Kit according to the manufacturers' instructions (Pharmingen, Hamburg, Germany). Monocytes were incubated with mAb RM3/1 (3 $\mu$g/ml) for 45 min on ice. Mouse $IgG_1$ was included as an isotype control at the same concentration. The cells were subsequently stained with FITC-labeled goat anti-mouse IgG for 20 min on ice. The fluorescence intensity of $10^4$ cells was measured by FACS analysis (FACscan, Becton–Dickinson, Heidelberg, Germany). The parameters used were 488 nm excitation wavelength, 250 mW and logarithmic amplification. The antigen density of RM3/1 positive cells were obtained from the main fluorescence channel at 510 to 530 nm using Lysis software (Becton–Dickinson).

## RESULTS

*Downregulation of CD163 by PMA.* Treatment of FP stimulated monocytes with PMA results in rapid loss of surface expression of CD163 by a yet unknown mechanism (12). To investigate whether the removal of the protein is accomplished by internalization, FACS-analysis of non-permeabilized in comparison to permeabilized monocytes was performed to determine changes in intracellular CD163 concentration. After stimulation with PMA for 1 h at 37°C the amount of surface bound as well as intracellular CD163 was markedly diminished versus FP stimulated control in non-permeabilized and permeabilized cells, respectively (Fig. 1). This favors a shedding mechanism of the protein rather than an internalization.

*PMA induces shedding of CD163.* Western blot analysis of cell lysates and culture supernatants revealed that PMA-treated monocytes release a soluble

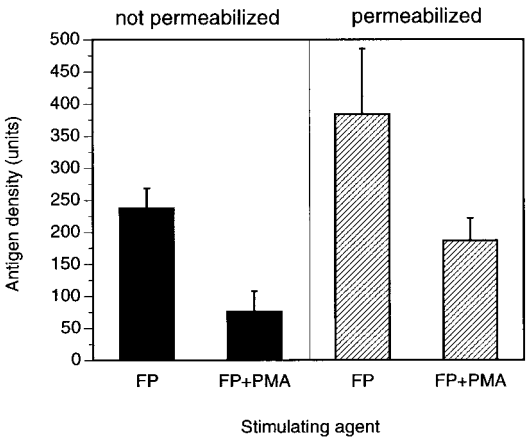


**FIG. 1.** Downregulation of CD163 by PMA. FACS analysis of permeabilized and non-permeabilized human monocytes stimulated with the glucocorticoid FP and PMA. Mean and standard deviation of four samples from independent experiments are given. Cells were immunostained with the specific CD163 recognizing mAb RM3/1.

form of CD163 (Fig. 2). In control monocytes not treated with PMA, CD163 is detected only in the cell lysate (lane 2) whereas the supernatant of the cell lysate shows no positive signal with the Ki-M8 antibody (lane 1). In contrast, after PMA-stimulation, CD163 could be detected in the supernatant of PMA stimulated monocytes (lane 3) while the amount of membrane-bound CD163 was strongly reduced (lane 4).

*Prevention of CD163 shedding by PKC inhibitor.* PMA and other active phorbol esters are known to activate PKC (14). Therefore, we investigated the involvement of PKC in shedding of CD163. PMA reduced cell surface expression of CD163 (Fig. 3, lanes 1 and 2). The effect of PMA was abolished when monocytes were preincubated for 10 min with bisindolylmaleimide I (BIM, 10 nM), a specific inhibitor of PKC (lane 3). The PKC inhibitor alone without PMA treatment has no influence on the release of soluble CD163 (lane 4). Therefore, the PMA-induced release of CD163 suggested a role for protein phosphorylation in the shedding process.

*Protease inhibitors reduce PMA-induced release of soluble CD163.* To investigate whether proteases are involved in the release of soluble CD163, we examined the effect of a protease inhibitor cocktail (complete, 1 tablet/ml) against serine-, cysteine- and metalloproteases on CD163 shedding. Again, PMA reduced cell surface expression of CD163 (Fig. 4, lanes 1 and 2). Pretreatment of monocytes with inhibitor cocktail 10 min before addition of PMA prevented release of membrane-bound CD163 (lane 4), while the protease inhibitor cocktail alone had no effect of CD163 surface expression (lane 3).

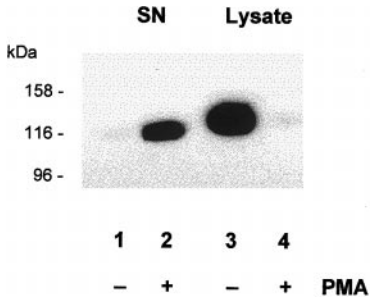


**FIG. 2.** Influence of PMA on shedding of CD163. Monocytes (pretreated 2 days with fluticasone propionate) were not further stimulated (lanes 1 and 3) or stimulated with $10^{-8}$ M PMA for 45 min (lanes 2 and 4). Supernatants (lanes 1 and 2) and cell lysates (lanes 3 and 4) were resolved by 6% SDS–PAGE and analyzed by Western blot with mAb Ki-M8 against CD163.

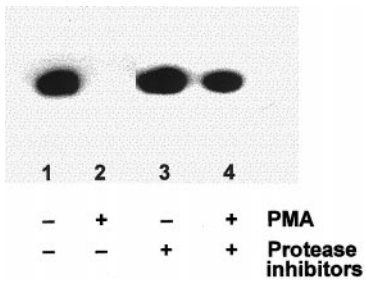


**FIG. 4.** Inhibition of CD163 shedding by a protease inhibitor cocktail. Monocytes were pretreated for 10 min with a protease inhibitor cocktail (lanes 3 and 4) and subsequently stimulated with $10^{-8}$ M PMA (lanes 1 and 2). Cell lysates were analyzed by Western blot with mAb Ki-M8 against CD163.

## DISCUSSION

In this report we have demonstrated the generation of a soluble form of the CD163 transmembrane protein, a member of the SRCR family. CD163 shedding *in vitro* was strongly induced by PMA and a PKC-regulation of the cleavage mechanism was involved in the generation of soluble CD163.

The present study demonstrates for the first time the shedding of a member of the SRCR family. Besides CD163, group B of the SRCR family includes the cell surface proteins CD5 (15) and CD6 (16), predominantly expressed by mammalian T cells and some specialized B cells, WC1 (17, 18), expressed by $\gamma\delta$ T cells in cattle, and Sp$\alpha$ (19), which is found in lymphoid tissue. Sp$\alpha$ is an example for a structurally different protein since it has no transmembrane domain and is supposed to be secreted. For the other group B members of the SRCR family a shedding mechanism has not yet been reported.

In this context, proteolytic modulation of membrane proteins has shown to be an important regulatory mechanism in cell biology. Ectodomain shedding can convert membrane-anchored growth factors into diffusible factors, membrane receptors into competitors of their own ligand (20) or accessories to ligand binding (21), and cell adhesion receptors into products no longer capable of mediating physical interactions with other cells or components of the extracellular matrix (22). These membrane-associated proteins include, but are not limited to, cytokines and growth factor precursors such as Fas ligand and TNF$\alpha$ (23, 24), receptors for cytokines such as IL-6 (25), and adhesion molecules such as L-selectin and ICAM-1 (26, 27).

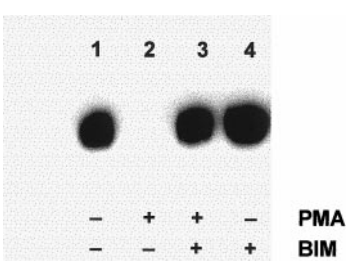


**FIG. 3.** Effect of the PKC inhibitor BIM on the PMA-induced CD163 shedding. Monocytes were pretreated for 10 min with BIM (10 $\mu$M) (lanes 3 and 4) and then incubated with $10^{-8}$ M PMA (lanes 2 and 3). Cell lysates were subjected to Western blot analysis with mAb Ki-M8 against CD163.

No clear similarity can be found between the various membrane proteins that undergo ectodomain shedding; in particular, no sequence similarity can be found within the cleavage sites of these molecules. Considering the wide range of membrane molecules that are known to be shed from leukocytes, it is most likely that a variety of different proteases mediate the shedding of these molecules (28). Recently, generation of a soluble protein form has been suggested to be mediated by "secretases," a group of enzymes with shedding function. Though structural characterization of these group of membrane protein secretases is poor, they are known to share common features such as sensitivity to metalloprotease inhibitors and activation by phorbol esters (29). The release of most soluble cytokine receptors and growth factors is strongly enhanced by PMA (for reviews see 21, 30), a process which could be also demonstrated for the CD163 protein. Since phorbol esters are potent activators of PKC (14) we could consequently establish that the cleavage of CD163 by PMA involves a PKC-dependent mechanism.

A truncated, soluble form of CD163, consisting of the first three scavenger receptor domains consisting on average of 110 residues, followed by 27 residues, has been postulated by Law *et al.* (1). However, this hypothetical soluble form with a calculated molecular mass of approximately 45 kDa has never been detected. The soluble form of CD163 we describe here cannot be distinguished by SDS–PAGE from that of the transmembrane molecule of 130 kDa. This clearly suggests a cleavage proximal from the cell membrane. Therefore,

the ectodomain of this protein appears to be shed by PMA-activated proteases at or near the cell surface as reported for the shedding of many other membrane protein ectodomains (31, 32) and is thus a novel regulatory mechanism for CD163.

The observation reported in this study further calls attention to the biological importance of selective proteolytic cleavage in regulation of cell function. The precise physical function of CD163 remains to be elucidated, but this membrane protein has been implicated in the down-regulation of the inflammatory process. Shedding of CD163 by phorbol esters might serve as an *in vitro* model for an additional insight into the function of CD163 in monocytic cells. The characterization of tissue- or receptor-specific proteases involved, as well as the identification of specific inhibitors, could possibly open up a field of broad biological and pharmacological relevance in the control of inflammatory processes.

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