Identification of a Domain (155–183) on CD36 Implicated in the Phagocytosis of Apoptotic Neutrophils*

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CD36 is a 88-kDa multifunctional adhesive glycoprotein expressed by platelets, monocytes, microvascular endothelial cells, mammary epithelial cells, erythroblasts, and several tumor cell lines (1). It has been identified as one of the receptors for collagen type I (2) and thrombospondin (TSP) (3, 4). Moreover, CD36 mediates the cytoadhesion of Plasmodium falciparum-infected erythrocytes to brain post capillary venular endothelium, a factor that contributes to the pathogenicity of the P. falciparum malaria (5). Recently CD36 has been shown to act as a receptor for oxidized low density lipoproteins, and thus, it may be implicated in the evolution of atherosclerosis (6). This adhesion molecule has also been reported to act as a signaling molecule capable of mediating an oxidative burst in monocytes (7) and activated platelets (8) by a process that may be associated with tyrosine phosphorylation (9). Recent observations show that E-selectin, expressed by activated endothelial or transfected L cells, is capable of significantly increase the number of CD36 molecules expressed by monocytes (10).

Clearance of apoptotic or senescent neutrophils (PMN) by macrophages is a crucial process in events such as hemostasis, wound healing, and tissue regeneration. Indeed, efficient macrophage phagocytosis of senescent neutrophils undergoing constitutive apoptosis is likely to be critical for successful resolution of inflammatory responses (11). To prevent damage to surrounding tissues, PMN undergo spontaneously apoptosis or programmed cell death (12), which leads to recognition and phagocytosis by macrophages (13). CD36, together with the $\alpha_v\beta_3$ integrin, has been identified as one of the adhesion molecules on the surface of macropages implicated in the clearance of PMN (14). Apoptotic T cells, following the resolution of viral infections, are also recognized by these two adhesion molecules (15). Although increasing evidence supports the role of CD36 in phagocytosis of apoptotic cells, very little is known concerning the structural domain on the CD36 molecule implicated in this recognition process. The epitope for the mAbs that blocked phagocytosis of apoptotic PMN (14, 16) was recently located in our laboratory to a region on CD36 encompassing amino acids 155–183 (17). In this study, human-murine chimeric CD36 constructs together with anti-CD36 mAbs and peptides derived from this region were used to show the functional role of the 155–183 domain.

MATERIALS AND METHODS

The cDNA coding for human and mouse CD36 were generously given by Dr Brian Seed (5) and Dr Gerda Endemann (6), respectively. LipofectAMINE reagent was from Life Technologies, Inc. Restriction endonucleases were from New England Biolabs (Beverly, MA) or Life Technologies, Inc. The COS-7 cell line was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, Wiltshire, UK). Culture media and supplements were from Life Technologies, Inc. Other reagents were from different sources and were of the highest purity available.

Monoclonal Antibodies—Anti-CD36 monoclonal antibodies 10/5 and 13/10, were produced in our laboratory using for immunization a CD36-expressing recombinant vaccinia virus (18). These antibodies were further characterized by immunoprecipitation. Western blots, and fluorescence-activated cell sorting analysis of human platelets, mAbs 10/5 and 13/10 were found to be IgG2a and IgG2b, respectively, as deter...
tide, except that its sequence was scrambled. Stock solutions (14 mM) of control peptide for region 155–169 was identical to the authentic peptide (mouse IgG1, mouse IgG2a, and LYP20) or total mouse IgG was used as negative control. Error bars are S.E. ** significantly different from the phagocytosis by hCD36-transfected cells at p < 0.001 as analyzed by the Student’s t test.

### FIG. 1. Binding of anti-CD36 monoclonal antibodies to COS cells expressing human wild type or human-mouse CD36 chimeric constructs [hmh (155-183) and hhm (155-183)].

The data are presented as the percentage of cells 48 h after transfection binding to monoclonal antibodies 10/5 (open bars) and 13/10 (filled bars). Error bars are S.E. The data are from five independent experiments.

### FIG. 2. Monoclonal antibodies directed against CD36, with epitopes located on domain 155-183, inhibit phagocytosis of apoptotic PMN. mAbs were used at a concentration of 50 μg/ml. The data are from three independent experiments. For each individual experiment, an immunoglobulin of the same isotype as the tested mAb (mouse IgG1, mouse IgG2a, and LYP20) or total mouse IgG was used as negative control. Error bars are S.E. ** significantly different from the phagocytosis by hCD36-transfected cells at p < 0.001 as analyzed by the Student’s t test.

#### COS Cell Transfection

COS cells were transfected with mAbs and flow cytometry with a FACScan® using a LysisI software. Cells—Neutrophils (~98% pure, Wright-Giemsa) were isolated from fresh, citrated blood from healthy donors, by dextran sedimentation and platelet-poor plasma Percoll density gradient centrifugation, aged for 24 h at 37 °C, 5% CO2 in Iscove’s modified Dulbecco’s medium containing 10% autologous platelet-rich plasma-derived serum. Apoptosis was assessed microscopically by counting 200 COS cells/slide. The proportion of COS cells ingesting aPMN was assessed microscopically by counting 200 COS cells/slide.

#### Phagocytosis Assays

Effect of Monoclonal Antibodies—Monocyte-derived macrophages were prepared by standard methods from adherent blood mononuclear cells by culture for 8 days in 24-well plates in Iscove’s modified Dulbecco’s medium containing 10% autologous platelet-rich plasma-derived serum, as described (14).

Phagocytosis was assessed microscopically by counting 200 COS cells/slide. The proportion of COS cells ingesting aPMN was assessed microscopically by counting 200 COS cells/slide.

Effects of Monoclonal Antibodies—Monocyte-derived macrophages or transfected COS-7 cells were incubated with mAbs (final concentration, 50 μg/ml) for 30 min at room temperature. Cells were then washed twice with 2 ml of DMEM followed by the addition of 2.5 x 10^6 aPMN/ml. Phagocytosis assay was carried out as above.

Effect of Peptides—The effect of CD36-derived peptides on the phagocytosis of aPMN by monocyte-derived macrophages or hCD36-transfected COS cells was determined using the standard protocol with peptides being added to the aPMN suspension at the concentration indicated prior to its addition to the COS cell monolayer.

Statistical Analysis—Statistical analysis was performed using unpaired Student’s t test.
RESULTS

Expression of Chimeric Constructs of CD36—COS cells constitutively expressing αβ3 as shown by mAb LYP18 (results not shown) were transfected with either human wild type CD36, mouse wildtype CD36 or the chimeric constructs. Transient expression (48 h post transfection) of CD36 on the surface of COS cells was measured through the binding of mAb (10/5 and 13/10) directed against different domains of CD36. COS cells transfected with mCD36 cDNA in which the 155–183 domain is replaced by its human counterpart (chimeric construct hmhm 155–183) had a capacity to phagocytose aPMN (15.4 ± 4.3%) similarly to that observed for the whole IgG (Fig. 3). These results suggest that the inhibitory effect of 10/5 on the phagocytosis of aPMN by hCD36 transfected COS cells (Fig. 2). Similarly, phagocytosis of aPMN by monocytes derived macrophages was inhibited by 10/5 (30.44 ± 9.4%) having the same isotype as 10/5 (IgG2a) or OKM5 (IgG1), did not affect phagocytosis of aPMN (18.57 ± 6%).

Effect of Monoclonal Antibodies on Phagocytosis of Apoptotic Neutrophils by Human CD36-transfected Cells—Transient transfection of COS cells with human CD36 conferred an increased capacity to phagocytose apoptotic neutrophils (18.57 ± 3.77%) compared with the pCDM8 vector without insert (6.05 ± 0.52%, n = 8). COS cells that phagocytosed aPMN expressed CD36 as observed by fluorescent microscopy. Preincubation of hCD36-transfected COS cells with mAbs 10/5 or OKM5, whose epitopes are located within amino acids 155–183 (17), inhibited phagocytosis of aPMN (by 35.9 ± 4.21% and 40.8 ± 7.64%, respectively). However, preincubation of hCD36-transfected cells with the mAb 13/10, whose epitope is present within region 30–76 (17), did not affect phagocytosis. Total mouse IgG, or irrelevant IgG, IgG2a, or an IgG3 (LYP20, an anti-P-selectin) having the same isotype as 10/5 (IgG2a) or OKM5 (IgG1), did not affect phagocytosis of aPMN by hCD36 transfected COS cells (Fig. 2). Similarly, phagocytosis of aPMN by monocytes derived macrophages was inhibited by 10/5 (30.44 ± 9.4%) in the capacity of hCD36 transfected COS cells. Fab fragments of 10/5 but not of 13/10 inhibited phagocytosis (by 39.1 ± 9.9%, n = 3) in a manner similar to that observed with the whole IgG (Fig. 3). These results suggest that the inhibitory effect of 10/5 on the phagocytosis of aPMN is independent of the Fc fragment.

PMN Phagocytosis by Mouse/Human Chimeric Constructs—Wild type human CD36-transfected COS cells show a 3-fold increase (18.57 ± 3.77%, n = 8) in their capacity to internalize and ingest aPMN compared with COS cells transfected with wild type murine CD36 (7.55 ± 2.08%, n = 5) (Fig. 4). Human CD36 cDNA transfection did not elicit phagocytosis of freshly isolated (nonapoptotic) neutrophils (5.89 ± 2.17%, n = 5). Differences in phagocytic capacity of COS cells resulting from wild type human or murine CD36 cDNA transfection were exploited to locate the functional domain on CD36 implicated in the recognition and internalization of aPMN. For that purpose, chimeric constructs of human and murine CD36 were generated and subsequently transfected in COS cells. COS cells transfected with a mCD36 cDNA in which the 155–183 domain was replaced by its human counterpart (chimeric construct hmhm 155–183) had a capacity to phagocytose aPMN (15.4 ± 4.3%, n = 5), which was significantly higher compared with wild type murine CD36 (p < 0.01). In contrast, COS cells...
transfected with a chimeric construct in which the human CD36 domain 155–183 was replaced by its murine counterpart (hmh 155–183 CD36) had a significantly reduced (p, 0.01) capacity to phagocytose apoptotic aPMN (7.79 ± 4.3%, n = 5) similar to mock transfected cells (Fig. 4).

Phagocytosis of aPMN Is Inhibited by Peptides Derived from Human CD36—A peptide derived from amino acids 155–169, at a final concentration of 500 μM, significantly inhibited the phagocytosis of aPMN by hCD36-transfected COS cells and monocyte-derived macrophages (by 46.63 ± 4.19%, n = 3 and 27.178 ± 6.06%, respectively). No effect was observed for the peptide 155–169 in its scrambled form or by the peptide derived from the region 148–164 (Fig. 5).

DISCUSSION

In the present study we have identified, for the first time, an important functional domain on CD36 involved in the phagocytosis of apoptotic neutrophils. The role of this domain in mediating phagocytosis of human apoptotic neutrophils, in tandem with αvβ3, is supported by a number of lines of evidence: 1) COS cells transfected with mCD36 were observed to have a minimal capacity to phagocytose human aPMN compared with hCD36. Inserting the human CD36 155–183 domain into mCD36 imparts to the chimeric CD36 a capacity to phagocytose aPMN. 2) Conversely, inserting the mCD36 155–183 domain in the hCD36 abolishes its phagocytic capacity. 3) Monoclonal antibodies directed against the 155–183 domain but not mAbs directed toward the N-terminal domain block phagocytosis. 4) A peptide derived from hCD36 amino acids 155–169 but not its scrambled form blocks phagocytosis.

A recent study has shown that the functional repertoire of human CD36 includes the capacity to promote, together with αvβ3, phagocytosis of apoptotic cells on COS and Bowes melanoma cells (16). Moreover, it has been reported that the cooperation of CD36 and the αvβ3 integrin is crucial for recognition and phagocytosis of aPMN (22–24). Our results are in line with these findings but also show that mCD36 cDNA does not have the same capacity as hCD36, when transfected in COS cells expressing αvβ3, to phagocytose human apoptotic neutrophils. Indeed, it is of interest to note that human and murine macrophages may use different receptors for the recognition and phagocytosis of aPMN (25). Differences in phagocytosis capacity of COS cells, resulting from wild type human or murine CD36 transfection, together with epitope mapping of blocking mAbs have allowed us to identify a domain (amino acids 155–183) on hCD36 implicated in the recognition and internalization of aPMN.

The 155–183 domain is adjacent to a region encompassing amino acids 139–155 that has been reported to represent a part of the OKM5 epitope and that is implicated in the initial binding to TSP (26). It is important to note that domain 139–155 is identical in both the human and mouse proteins with the exception of residues 146 and 152 (17). Thus, phagocytosis of apoptotic neutrophils is dependent on the 155–183 region, but residues in the adjacent 139–155 sequence may be important as well as observed for mAb binding (17, 26). Strong evidence is available to show that interaction between aPMN- and monocyte-derived macrophages or CD36-transfected cell lines is mediated by TSP (14, 16). According to the two step model of Leung et al. (26), the 139–155 domain might be implicated in the initial binding of TSP that would consequently give rise to a high affinity binding site (amino acids 93–110) on CD36. Exposition of this second site allows further consolidation of the CD36-TSP interaction. Human TSP, in the same way as blocking mAbs, may initially interact with CD36 via a discon-
cious site involving amino acids 139–155 and 155–183 domains. Replacing the 155–183 domain, in human CD36, by its murine homologue may either affect the stability of the TSP-CD36 interaction and/or the transmission of necessary outside-in signals (9) to the macrophage and prevent the recognition and phagocytosis of aPMN. Alternatively, any changes in the sequence of 155–183, from human to murine, may locally affect the conformation of CD36 and consequently alter its capacity of phagocytosis. A peptide (155–169) derived from region 155–183 inhibits, in contrast to its scrambled form or a peptide derived from region 148–164, phagocytosis of aPMN by hCD36-transfected COS cells. Differences in functional reactivity between these two peptides may conceivably be due to one (155–169) but not the other (148–164), acquiring a conformation that is closer to the native state of this region. The work of Asch et al. implicates amino acids 87–99 in the binding of TSP and shows that this binding is linked to the phosphorylation of Thr92 (27). Moreover, the group of Silverstein uses TSP and shows that this binding is linked to the phosphorylation of human CD36, directly implicated in cell-cell interactions.

The domain encompassing amino acids 155–183 plays a critical role in the phagocytosis of aPMN. Moreover, recent data from our laboratory suggest that region 155–183 is a multifunctional domain implicated as well in the cytoadherence of *P. falciparum*-infected erythrocytes to CD36 (29), as a signaling molecule implicated in inducing platelet aggregation and secretion (18) and in oxidized LDL binding (30). The domain 155–183 is coded by a single exon (exon VI) (31), and it is possible that this exon delineates an independent, structural, and functional domain of human CD36, directly implicated in cell-cell interactions.

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