Brief Definitive Report

CD36 Gene Transfer Confers Capacity for Phagocytosis of Cells Undergoing Apoptosis

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Summary

Phagocyte recognition and ingestion of intact cells undergoing apoptosis are key events in this generally important program of cell death. Insufficient phagocyte capacity for apoptotic cells can result in failure to clear dying cells before membrane integrity is lost, resulting in leakage of noxious cell contents and severe tissue damage. However, no means has been available to increase phagocytic clearance of apoptotic cells. We now report that transfection of the macrophage adhesion molecule CD36 into human Bowes melanoma cells specifically conferred greatly increased capacity to ingest apoptotic neutrophils, lymphocytes, and fibroblasts, comparable to that exhibited by macrophages. Furthermore, when CD36 was transfected into another cell type with limited capacity to take up apoptotic bodies, the monkey COS-7 cell, similar effects were observed. Therefore, CD36 gene transfer can confer "professional" capacity to ingest apoptotic cells upon "amateur" phagocytes.

Cell clearance by apoptosis protects surrounding tissues from damage due to uncontrolled leakage of noxious contents from dying cells (1–6). Intact apoptotic cells can be taken up in vivo by neighboring cells, often of the same type, acting as "amateur" phagocytes. Where the load of apoptotic cells is high, as in the thymus (7) or inflamed site (3, 8), this job is done by "professional" phagocytes, macrophages, which can clear large numbers of apoptotic cells. Nevertheless, this normally efficient clearance system can fail (8, 9). A dramatic example was recently afforded by intraperitoneal administration of antibody to Fas, which triggered rapid and widespread hepatocyte apoptosis (9). However, presumably because of insufficient local capacity to clear an unphysiologically massive load of apoptotic cells, hepatocytes disintegrated and coagulative necrosis of the liver and death rapidly ensued (9). This is an important observation, because there is a growing interest in the possibility that selective triggering of apoptosis might be useful in deleting dangerous cells from the body, such as tumor cells or auto-reactive lymphocytes (10). Unless local phagocytic capacity is able to cope with a massive increase in load of apoptotic cells, such putative treatments could have dire effects on surrounding tissues. Consequently, we sought a means to confer increased capacity for uptake of apoptotic cells.

Mechanisms by which phagocytes recognize cells undergoing apoptosis are incompletely understood (6). However, mAb inhibition experiments suggested that human monocyte-derived macrophages (Mø) can employ the adhesion receptor CD36 (11, 12) in high capacity phagocytosis of apoptotic neutrophils and lymphocytes (4, 5). The data suggest that Mø CD36 cooperates with Mø $\alpha_v\beta_3$ vitronectin receptor integrin to bind the adhesive glycoprotein thrombospondin (TSP), which then acts as a "molecular bridge" binding the Mø to the apoptotic cell (4, 5). However, a role for CD36 in phagocytosis had not been demonstrated directly. In this study we investigated whether CD36 gene transfer can confer phagocytic capacity for cells undergoing apoptosis.

Materials and Methods

Phagocytes Used in the Study. Bowes melanoma cells (gift of Dr. D. B. Rifkin, New York University Medical Center) were stably transfected as previously described (12) with pMV7 neomycin resistance vector (control) or vector incorporating CD36cDNA and selected in medium with G418 (250 μg/ml). By flow cytometry (13, and not shown) >80% of CD36+ Bowes cells expressed CD36 at levels comparable to Mø.

COS-7 cells (from American Type Culture Collection, Rockville, MD) were transiently transfected with cdm8 vector bearing either CD36 cDNA or FcR1(p135) cDNA by standard DEAE dextran-mediated transfection, as described (12, 14). Comparable levels of CD36 and FcR 1 expression were obtained. With regard to CD36, these were ~10-fold lower than the high levels of CD36 expression achieved by stable transfection of Bowes cells (not shown). In common with Bowes cells, COS-7 cells expressed epitopes bound by 23C6 $\alpha_v\beta_3$ mAb.

Apoptotic Cells Used in the Study. In all cases, apoptotic cells were obtained by well-established protocols, had viability >95%

 J. Exp. Med. • 0022-1007/95/05/1857/06 $2.00
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by trypan blue dye exclusion and typical light microscopical features of apoptosis.

Neutrophils used were prepared from normal human peripheral blood and "aged" in culture in 24 h so as to undergo apoptosis, exactly as described (3).

Lymphocytes used were a B-cell line derived from MUTU-BL Burkitt Lymphoma Group 1 cells (gift of J. Hickman, University of Manchester, UK; refrence 15), which were triggered to undergo apoptosis by culture in serum-free RPMI medium for 48 h; nonapoptotic cells were harvested from cultures in RPMI plus 10% FCS.

Fibroblasts used were SV40-transformed murine 3T3 fibroblasts (gift of D. Ucker, Medical Biology Institute, La Jolla, CA; reference 16), and were triggered to undergo apoptosis by culture in serum-free RPMI medium for 24 h.

Phagocytosis Assays. Bowes cells were subcultured in DME medium plus 10% FCS as adherent monolayers in 96-well tissue culture plates, washed, and then interacted by well-established methods (3–5, 17) for 3 h with 10^5 per well apoptotic cells in the same medium. Noningested apoptotic cells were then washed away with cold saline. In some experiments, monolayers were fixed with 2% glutaraldehyde and then stained for myeloperoxidase to reveal ingested apoptotic neutrophils, as described (16). However, for quantitation of phagocytosis, the monolayer was trypsinized at the end of the interaction and a cytocentrifuge preparation made from each well, which was fixed and stained with Haemalum to reveal ingested apoptotic cells; in neutrophil experiments the cytopreps were also counterstained with myeloperoxidase. The proportion of Bowes cells ingesting apoptotic cells in each cytoprep was then determined by microscopical counting of 300 Bowes cells per slide. In some experiments, data were also presented as the number of ingested PMNs per 100 Bowes cells. In further experiments, at the end of a phagocytosis assay, trypsinized Bowes CD36+ cells were prepared for EM by standard methods.

COS-7 cells were grown in 24-well plates in RPMI 1640 medium plus 10% FCS, and then interacted with apoptotic cells by identical methods to those used for Bowes cells. COS-7 cells were assessed 48 h after transfection.

Inhibitors were included directly in the interaction medium (at the concentrations given below or in the figure legend).

Antibodies Used in Phagocytosis Assays. The mAbs used have all been employed before in studies of phagocyte recognition of apoptotic cells (4, 5, 18), and no mAb inhibited human Mø phagocytosis of opsonized erythrocytes in control experiments. The CD36 mAb (OKM5; IgG1; reference 19) and β_3 mAb (15.4.2; IgG1; gift of M. Ginsburg, Scripps Research Institute, La Jolla, CA; reference 20) were employed at 50 μg/ml. mAb to Thy1.1 (OX7; used as irrelevant control IgG1; from Serotec, Banbury, UK), the $\alpha_v\beta_3$ mAb (23C6; IgG1; gift of M. Horton, St. Bartholomew's Hospital, London, UK; reference 21), the thrombospondin mAb (C6.7; IgG1; gift of V. Dixit; reference 22), a second CD36 mAb (SMø; IgM; gift of Dr. N. Hogg, Imperial Cancer Research Fund; reference 23), and a CD15 mAb (mAb 28; IgM control for SMø; gift of Dr. N. Hogg, reference 23) were used at 1:50 dilution of ascites.

Other Inhibitors. Phospho-L-serine (Sigma Immunochemicals, St. Louis, MO) was used at 1 mM (24, 25). Soluble CD36 was prepared as described (13, 26), and human fibronectin was from Calbiochem (Cambridge Bioscience, UK).

Results

Transfection of Bowes Cells with CD36 Confers Capacity for Phagocytosis of Apoptotic Neutrophils. The human Bowes mela-

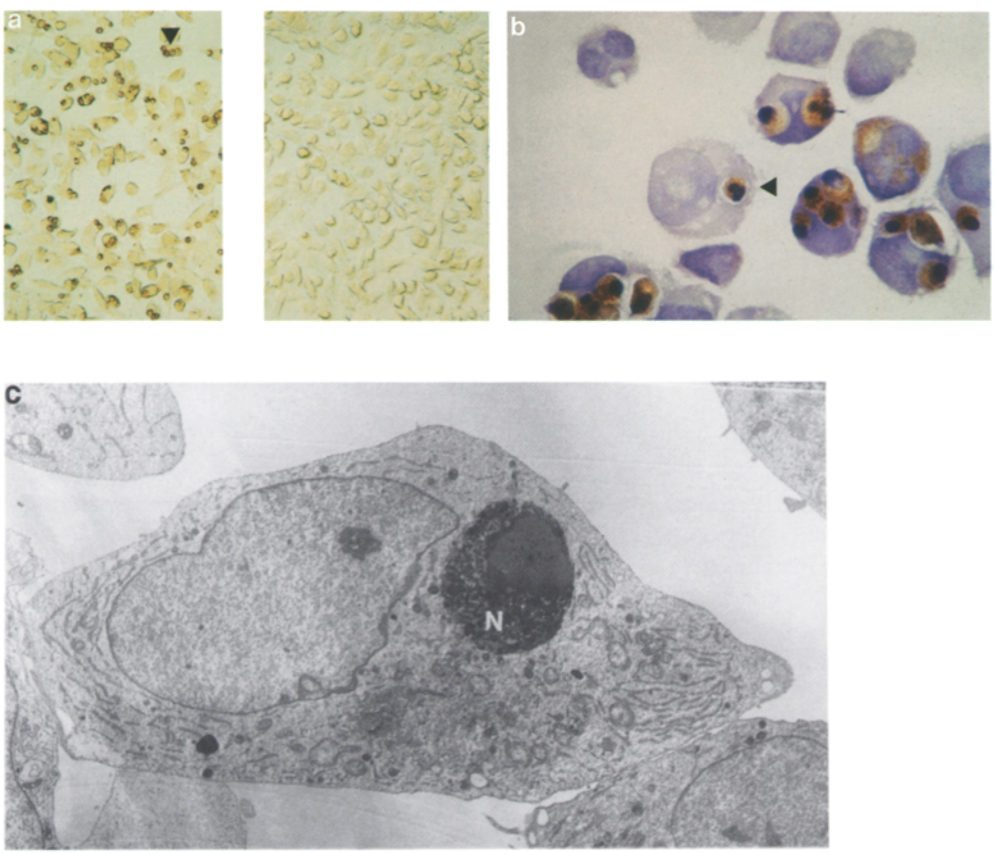

Figure 1. Enhanced phagocytosis of apoptotic neutrophils by Bowes melanoma cells stably transfected with CD36 (examples arrowed). (*a*) Appearance of washed monolayers of CD36+ (*left*) and vector-only control (*right*) Bowes cells at end of 3 h interaction with apoptotic neutrophils; ×200. Note much greater number of myeloperoxidase-positive, brown-staining apoptotic neutrophils bound by CD36+ cells; nuclei are not stained by this technique. (*b*) Cytocentrifuge preparation of CD36+ Bowes cells trypsinized after interaction, demonstrating phagocytosis of apoptotic neutrophils; ×1000. Note condensed chromatin of ingested cells. (*c*) Electron micrograph (×2000) of CD36+ Bowes cell demonstrating uptake of neutrophil with typical features of apoptosis (*N*).

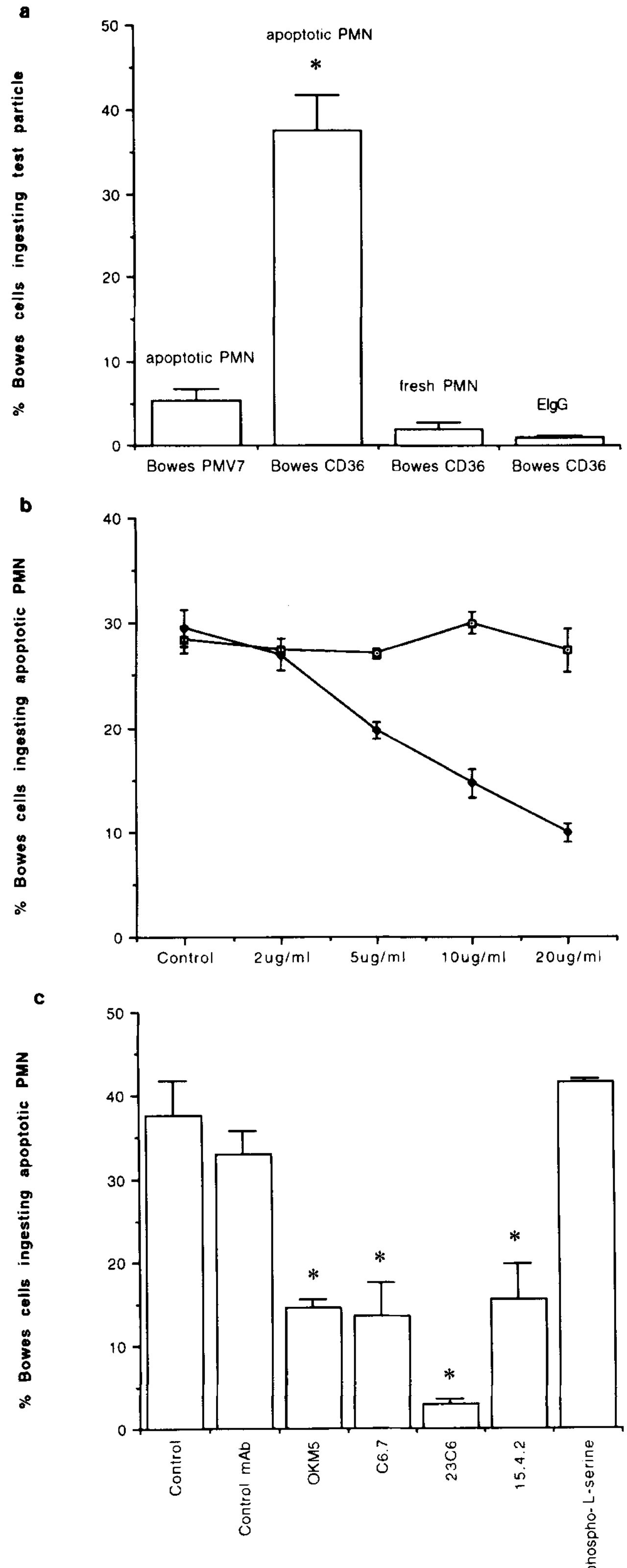

Figure 2. Specificity of CD36+ Bowes melanoma cell phagocytosis of apoptotic neutrophils. (*a*) Negligible phagocytosis of freshly isolated nonapoptotic neutrophils and opsonized erythrocytes by CD36+ Bowes cells, and minimal phagocytosis of apoptotic neutrophils by Bowes cells transfected with pMV7 vector alone. $*p < 0.05$. (*b*) CD36+ Bowes cell phagocytosis of apoptotic neutrophils is inhibited by soluble CD36, but not by fibronectin (*Fn*) control. ⊡, Fn; ●, CD36. (*c*) CD36+ Bowes cell phagocytosis of apoptotic neutrophils is inhibited by mAbs to CD36 (*OKM5*), $\alpha_v\beta_3$ (*23C6*), β_3 (*15.4.2.*), and TSP (*C6.7*). Irrelevant control mAb (OX7) and phospho-L-serine, an inhibitor of macrophage PS receptors, have no effect. $*p < 0.05$.

noma cell line is known not to express CD36, or to bind TSP (13), but did expression $\alpha_v\beta_3$ when assessed by flow cytometry (data not shown). When stably transfected with pMV7 neomycin resistance vector bearing CD36, there was no detectable change in $\alpha_v\beta_3$ expression versus Bowes cells transfected with vector alone, while CD36 was expressed at a high level comparable to Mø (13). When compared with Bowes cells transfected with vector alone, CD36+ Bowes cells exhibited a dramatically increased capacity to ingest apoptotic neutrophils (Figs. 1 and 2 *a*), which approximated that exhibited by human monocyte-derived macrophages (~40% of which take up apoptotic neutrophils; reference 3). This increase was also apparent when phagocytosis was expressed as the number of apoptotic neutrophils ingested per 100 Bowes cells (5 ± 2.0 for vector alone and 55 ± 10.5 for CD36+ Bowes cells). CD36 transfection did not cause a nonspecific stimulation of phagocytic activity because CD36+ Bowes cells did not ingest freshly isolated, nonapoptotic neutrophils, nor did they take up IgG-opsonized erythrocytes, used as a control particle (Fig. 2 *a*).

Conferred Phagocytosis Mimics the Recognition Mechanism Employed by Human Mø. There was clear evidence that transfected CD36 was functioning in conferred phagocytosis. First, soluble CD36 exerted specific concentration-dependent inhibition of phagocytosis of apoptotic neutrophils by CD36+ Bowes cells (Fig. 2 *b* and Table 1). Second, the IgG1 CD36 mAb OKM5 (which does not bind apoptotic neutrophils), but not an isotype control mAb, inhibited ingestion of apoptotic neutrophils (Fig. 2 *c* and Table 2). In keeping with our previous observations on human Mø, we found that uptake of apoptotic neutrophils was inhibited by $\alpha_v\beta_3$ and TSP mAbs, suggesting that in Bowes cells CD36 also cooperated with these structures as proposed for the Mø (5). Moreover, phagocytosis was not affected by inhibitors of the other major macrophage mechanism for ingestion of apoptotic neutrophils, recognition of exposed phosphatidylserine (PS) by putative PS receptors (PSR; Fig. 2 *c* and Table 2) (24, 25).

CD36 Confers Capacity to Recognize Apoptotic Cells of Other Lineages. We assessed whether transfected CD36 specifically conferred increased capacity to ingest apoptotic cells other than neutrophils. CD36+ Bowes cells took up apoptotic lymphocytes significantly more avidly than Bowes cells transfected with vector alone; nonapoptotic lymphocytes were not recognized by transfectants. Furthermore, specificity of recognition of apoptotic lymphocytes was confirmed by the inhibitory effects of soluble CD36 and mAbs to CD36, TSP and $\alpha_v\beta_3$; PSR inhibitor had no effect. (Fig. 3 *a*). Identical results were obtained with apoptotic fibroblasts (Fig. 3 *b*), demonstrating that CD36 can also confer capacity to phagocytize apoptotic cells of a nonleukocyte lineage.

Table 1. *Soluble CD36 Specifically Inhibits the Phagocytic Capacity of CD36+ Bowes Cells for Apoptotic Neutrophils*

	Number of PMNs ingested per 100 Bowes cells	
Concentration	CD36	Fibronectin
Nil (control)	47 ± 8.0	–
2 μg/ml	27 ± 2.0	47 ± 6.0
5 μg/ml	25 ± 3.0	41 ± 5.5
10 μg/ml	19 ± 0.5*	38 ± 2.5
20 μg/ml	12 ± 0.5*	37 ± 5.0

Data are mean ± SE, $n = 6$. *$p < 0.05$ vs control.

CD36 Transfection Also Confers Phagocytic Capacity upon COS-7 Cells. To establish that the ability of transfection with CD36 to confer capacity for phagocytosis of apoptotic cells upon Bowes cells was not an idiosyncrasy of this cell type, we transiently transfected COS-7 cells with cdm8 vector incorporating CD36 cDNA (12, 14). Despite yielding ~10-fold lower levels of CD36 expression than stable transfection of Bowes cells, this conferred specifically increased capacity to phagocytize apoptotic neutrophils when compared with nontransfected COS-7 cells, or cells transfected with an irrelevant phagocytic receptor, FcR1 (Fig. 4).

Discussion

CD36 (also known as glycoprotein IV) is an 88-kD monomeric cell surface molecule expressed by a limited range of cell types, namely monocyte-macrophages, microvascular endothelium, mammary epithelium, platelets and megakaryocytes, and some erythroid cells (27). CD36 was originally characterized as an adhesion receptor for thrombospondin (11, 26) and collagen (28), and was later implicated in microvascular endothelial cell binding of erythrocytes parasitized by *Plasmodium falciparum* (29, 12). Our own antibody inhibi-

Table 2. *CD36+ Bowes Cell Phagocytosis of Apoptotic Neutrophils Exhibits Characteristics of an $\alpha_v\beta_3$ Vitronectin Receptor-dependent Rather Than a Phosphatidylserine Receptor-dependent Mechanism*

Inhibitor	Number of PMNs ingested per 100 Bowes cells
Nil (medium control)	56 ± 10.8
OX7 (irrelevant mAb)	50 ± 8.7
OKM5 (CD36 mAb)	17 ± 1.5*
C6.7 (TSP mAb)	15 ± 5.6*
23C6 ($\alpha_v\beta_3$ mAb)	5 ± 2.7*
15.4.2 (β_3 mAb)	17 ± 2.0*
Phospho-L-serine	60 ± 6.7

Data are mean ± SE, $n = 6$. *$p < 0.05$ vs medium control. Inhibitors were employed as described in Materials and Methods.

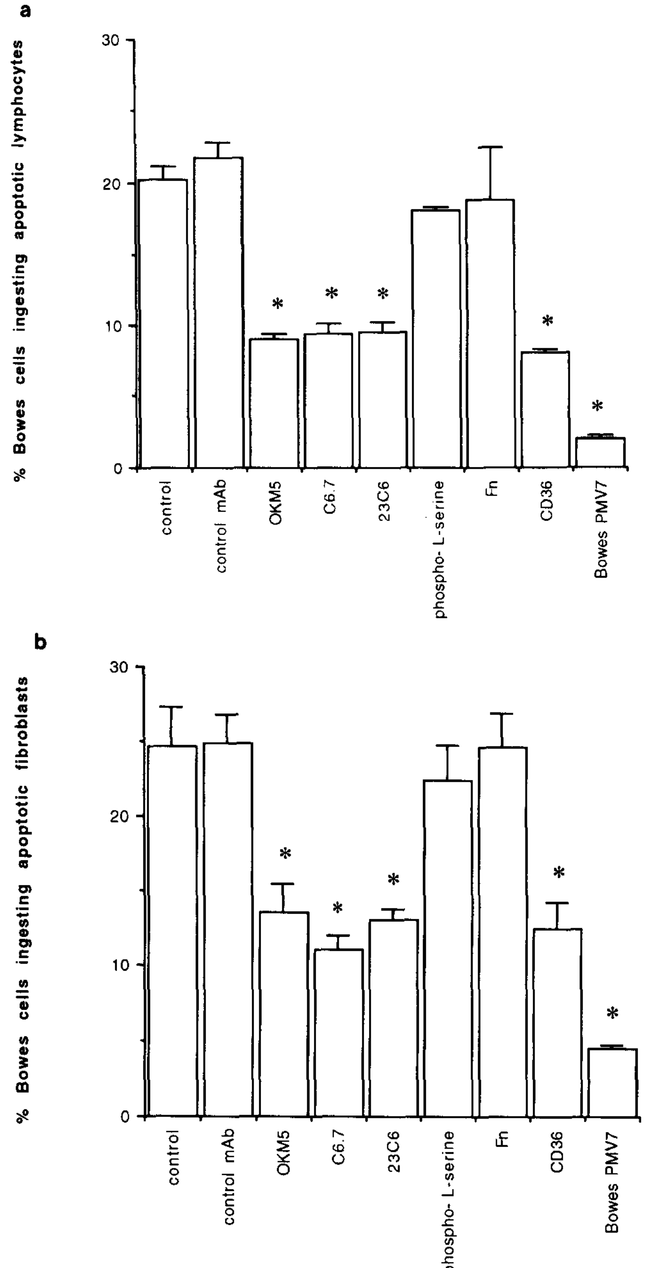

Figure 3. CD36+ Bowes melanoma cells specifically phagocytize apoptotic lymphocytes and fibroblasts. (*a*) Specific phagocytosis of apoptotic cells from B lymphocyte line. Note inhibition by soluble CD36 (at 10 μg/ml) and mAbs to CD36 (*OKM5*), $\alpha_v\beta_3$ (*23C6*), and TSP (*C6.7*), together with lack of effect of fibronectin (Fn, at 10 μg/ml) and irrelevant mAb (OX7) controls, and failure of phospho-L-serine (1 mM) to inhibit. Antibodies were used at concentrations given in Materials and Methods. Note also that nonapoptotic lymphocytes were not ingested by CD36+ Bowes cells, and that Bowes cells transfected with PMV7 vector alone exhibited minimal phagocytosis of apoptotic B cells. *$p < 0.05$. (*b*) Specific phagocytosis of apoptotic transformed murine fibroblasts. The same inhibitors were used. *$p < 0.05$.

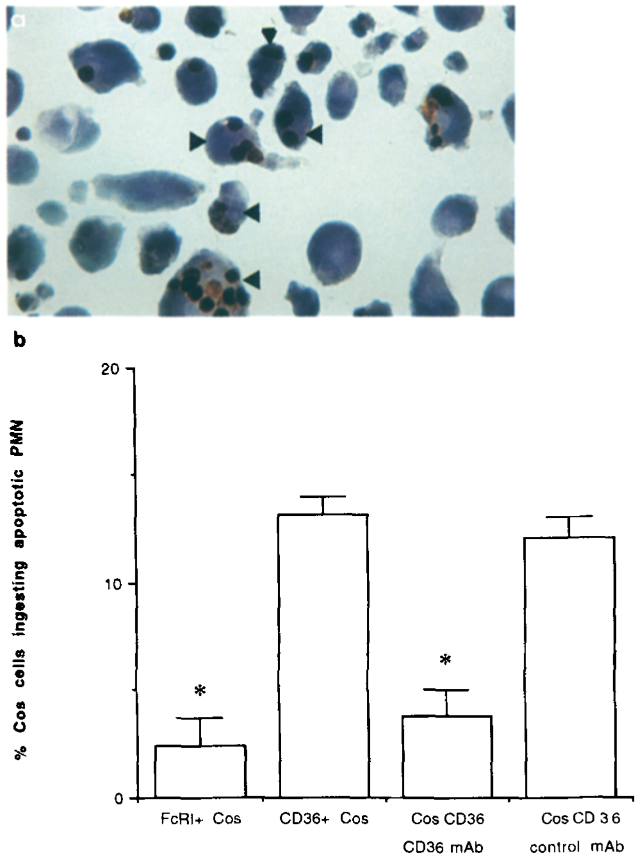

Figure 4. Phagocytosis of apoptotic neutrophils by monkey COS-7 cells transiently transfected with CD36. (*a*) Appearance of cytocentrifuge of trypsinized CD36-transfected COS-7 cells after 3-h interaction with apoptotic neutrophils. Note many COS-7 cells contain brown-staining apoptotic neutrophils (examples arrowed). ×300. (*b*) Specificity of phagocytosis. Note minimal ingestion of apoptotic neutrophils by COS-7 cells transfected with FcR1 (p135), an irrelevant phagocytic receptor. CD36 mAb, but not irrelevant mAb 28, inhibits uptake of apoptotic neutrophils by CD36-transfected cells. **p* <0.05.

tion experiments subsequently implied a previously unsuspected role for macrophage CD36 in mediating phagocytosis of apoptotic neutrophils (5) and lymphocytes (18). However, because cross-linking of macrophage CD36 was known to elicit signals such as superoxide generation (30), it remained possible that such effects of CD36 mAbs might mediate spurious inhibition of Mø phagocytosis of apoptotic neutrophils. The current data are therefore the first direct demonstration that the functional repertoire of CD36 includes the capacity to promote efficient phagocytosis of apoptotic cells.

This observation is also of potential importance because it is the first example of a gene transfer strategy by which clearance of apoptotic cells could be increased. As understanding of the mechanisms by which apoptotic cells are phagocytized grows, genes other than CD36 may be found to have similar capacity to promote clearance of cells being eliminated by this mechanism. If treatments are developed which induce apoptosis in undesirable cells (10), then it may be possible to bolster local phagocytic capacity against a massively increased load of dying cells by targetting phagocytic genes to such sites. Because of its limited tissue distribution, CD36 holds some promise as a tool for promoting clearance of apoptotic cells in vivo. However, it will be important to understand the molecular mechanisms of CD36-potentiated phagocytosis, which should include detailed investigation of functional cooperation with the $\alpha_v\beta_3$ vitronectin receptor integrin and thrombospondin as observed in human Mø and CD36+ Bowes cells.

Finally, the current findings emphasize that the biological role of CD36 is not merely limited to that of an adhesion molecule. Recently, CD36 has been implicated in endocytosis of oxidized low density lipoprotein (31, 32) and fatty acids (33), and in phagocytosis of retinal photoreceptor outer segments (34). Whether CD36 has a wider role in phagocytosis and endocytosis is worth further investigation.

Dr. C. Sarraf (Histopathology, Royal Postgraduate Medical School, London, UK) is thanked for electron microscopy, and Dr. B. Seed (Harvard Medical School, Cambridge, MA) generously gave the CD36 cDNA. Dr. M. Horton, Dr. N. Hogg, Dr. V. Dixit (University of Michigan Medical School, Ann Arbor, MI), and Dr. M. Ginsburg are thanked for gifts of antibodies, and Professor J. Hickman and Dr. D. Ucker generously gave cell lines.

This work was supported by the Wellcome Trust (031358 and 039737) and National Institutes of Health (Rol HLA 42540, PO1 HL 46403, P50 HL18828). J. Savill was a Wellcome Trust Senior Research Fellow in Clinical Science.

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Received for publication 1 December 1994 and in revised form 27 January 1995.

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