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Endoglin: a 180-kD endothelial cell and macrophage restricted differentiation molecule

P. J. O'CONNELL*, A. McKENZIE, N. FISICARO, S. P. ROCKMAN†, M. J. PEARSE & A. J. F. D'APICE
*Department of Clinical Immunology, St Vincent's Hospital, Fitzroy, and Departments of *Nephrology and †Haematology, Royal Melbourne Hospital, Victoria, Australia*

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SUMMARY

A MoAb RMAC8 was generated by immunizing Balb/c mice with cultured human umbilical vein endothelial cells (HUVE). The molecule recognized had a mol. wt of 180 kD non-reduced, 95 kD after reduction and 66 kD in its reduced and N-deglycosylated form. Sequential immunoprecipitation studies with the MoAb 44G4, which recognizes the O- and N-glycosylated homodimer endoglin, showed that both MoAbs recognize the same molecule on HUVE and phorbol myristate acetate (PMA)-stimulated U937 cells. The distribution of the RMAC8-recognized molecule was the same as that described for endoglin, i.e. arterial and venous endothelium, myelomonocytic and pre-B leukaemia cells and cell lines; however, unlike 44G4, RMAC8 also reacted weakly with monocytes and strongly with *in vitro* differentiated macrophages as well as peritoneal and alveolar macrophages. This distribution of endoglin was confirmed by Northern blot analysis using a full length endoglin cDNA probe. These studies suggest that endoglin is a differentiation marker on macrophages.

Keywords endothelial cell macrophage differentiation marker endoglin monoclonal antibody

INTRODUCTION

The mononuclear phagocyte system consists of a wide variety of cell types and performs a vast array of functions. It plays a major role in the modulation of local inflammatory responses, the processing and presentation of antigen and in scavenging extracellular debris [1]. Tissue macrophages originate from early haematopoietic progenitor cells in the bone marrow. From there, these cells undergo continuous differentiation which finally results in the replenishment and replacement of tissue macrophages. In this context, circulating blood monocytes are an intermediary step in the differentiation process. Upon migration from the vasculature into tissues these cells undergo further differentiation to the mature macrophage. This differentiation results in morphological, phenotypic and functional changes and is influenced by local environmental constraints [2]. To some extent this differentiation process can be imitated *in vitro* by culturing peripheral blood monocytes in the presence of serum [3]. This results in the expression of maturation-associated molecules which are absent on circulating monocytes but are expressed on terminally differentiated macrophages. Such molecules include CD16, CD51, CD71 and the macrophage mannose receptor [4–7]. Not only is the expression of these molecules important in studying the differentiation of macrophages, they are also important in determining functions unique to macrophages which are not performed by monocytes [4].

This study describes a novel macrophage differentiation molecule which was weakly expressed or absent on monocytes and showed increasing expression as myeloid cells differentiated toward a macrophage morphology. This molecule was identified by the MoAb RMAC8 which was raised against human umbilical vein endothelial cells (HUVE) and upon further characterization was found to identify the cell surface molecule, endoglin. Endoglin was originally identified by the MoAb 44G4 and was previously thought to have restricted expression on endothelial cells, pre-B cells and myeloid leukaemic cells [8–12]. As demonstrated in this report, RMAC8 can identify this molecule on *in vitro* and *in vivo* differentiated macrophages and endoglin surface expression corresponded with transcription of endoglin mRNA in macrophages. A recent report by Lastres *et al.* [13] presents essentially similar data and conclusions.

Correspondence: Dr Anthony J. F. d'Apice, Department of Clinical Immunology, St Vincent's Hospital, Fitzroy, Victoria 3065, Australia.

MATERIALS AND METHODS

Endothelial cells

HUVE were isolated from human umbilical veins by the method of Gimbrone [14] and serially passaged. The cells were plated onto 75 mm^2 tissue culture flasks (Costar, Cambridge, MA) and cultured in RPMI 1640 (Flow Laboratories, Sydney, Australia) supplemented with 20% FCS (Flow), 20 μg/ml endothelial cell

growth supplement (Sigma, St Louis, MO), 100 U/ml bovine heparin, 4 mM glutamine, 100 U/ml penicillin and 1 μg/ml streptomycin. The flasks were incubated at 37°C in 5% CO_2 and the cells were used on their third to ninth subculture.

Production of RMAC8

MoAb RMAC8, an IgG2a antibody, was produced by immunizing Balb/c mice intraperitoneally with HUVE on days 31 and 3 before fusion. Sensitized spleen cells were then fused with the murine myeloma cell line Sp2/0-Ag14 [15] and grown in selective medium. Hybridomas recognizing anti-HUVE cell determinants were detected by ELISA, where the primary antigen was paraformaldehyde (0·1%) fixed HUVE cells. Selected positive hybrids were cloned by limiting dilution and RMAC8 was obtained from mouse ascites by ammonium sulphate precipitation.

Monoclonal antibodies

OKMI was purchased from Ortho Diagnostics (Raritan, NJ) and recognizes the α chain of the C3bi receptor (CD11b) on myelomonocytic cells. RM5.112 was raised in our laboratory and recognizes a monomorphic determinant on MHC class II molecules. 44G4 recognizes endoglin [8–12]. RM2.184 recognizes a polymorphism of CD11b [16] and RMAC11 recognizes CDw49b [17] and were used as negative controls.

Cells

Granulocytes were separated from heparinized blood by sedimentation with dextran followed by Lymphoprep (Nygaard, Oslo) density gradient sedimentation to remove mononuclear cells. Mononuclear cells were separated from heparinized blood by Lymphoprep density gradient sedimentation. Monocytes were separated from lymphocytes by adherence to human AB serum-coated tissue culture flasks (Costar) and eluted with 0·2% EDTA. T and B lymphocytes were then separated by passage through a nylon wool column. Splenic B cells were obtained from normal human spleen which was removed at the time of cadaveric organ donation. Monocytes were removed by adherence to plastic culture flasks and B cells were obtained by adherence to nylon wool and were further depleted of T cells by removal of E-rosetting cells. In certain experiments monocytes were allowed to differentiate by culture on plastic tissue culture flasks in RPMI 1640 supplemented with 10% fetal calf serum (FCS) for 16 or 60 h and in others T cells were cultured with 10 μg/ml phytohaemagglutinin (PHA) or 5 μg/ml concanavalin A (Con A) for 48 h.

Alveolar macrophages were obtained from bronchial lavage fluid from patients undergoing bronchial lavage for a variety of diagnoses. Mucus was removed by filtration through a double layer of gauze and the cells separated by Lymphoprep sedimentation. Peritoneal macrophages were obtained from patients commencing peritoneal dialysis for end stage renal failure. The dialysis fluid was centrifuged and the pelleted cells were freed of contaminating cells by Lymphoprep sedimentation. Preparations of cells were stained with non-specific esterase to determine the number of macrophages and those containing less than 85% macrophages were discarded.

Cell lines

The T cell lines RPMI-8402, Peer, Jurkat, CEM, HSB-2 and Molt-4, the B cell lines Daudi, M4B, Mann, WT49 and LDB, the myelomonocytic lines HL-60 and U937, the pre-B leukaemia cell lines Nalm-1, REH, LK63 and Lila-1 and the undifferentiated lines K562 and U299 were grown in RPMI 1640 with 10% FCS.

Indirect immunofluorescence and flow cytometry

Cells ($2·5 \times 10^5$) in 50 μl of ice cold PBS plus 5% FCS and 0·1% NaN_3 were incubated at 4°C for 45 min with 50 μl of MoAb (0·1 mg/ml) which had been determined to be a saturating concentration of RMAC8 by reactivity in FACS assay with phorbol myristate acetate (PMA)-stimulated U937 cells. After washing three times the cells were stained with 50 μl of FITC-conjugated sheep anti-mouse immunoglobulin (Silenus, Melbourne, Australia) at saturating concentrations. After 30 min incubation at 4°C the cells were washed three times and fluorescence was measured by an EPICS (Coulter, Hialeah, FL) fluorescence analyser. For specific analyses HL-60, U937, M4B and RPMI-8402 were stimulated with 100 ng/ml of PMA (Sigma) for 3 days. A total of 10 000 viable cells, gated on the basis of light scatter signals, were scored in each sample. Data were acquired by the flow cytometer on a logarithmic scale. The data were subsequently converted to a linear scale before calculation of mean fluorescence. Data are presented in tabular form as percentage positive cells and/or mean linear fluorescence intensity after subtraction of background fluorescence with a non-reactive MoAb (RM2.184) and in graphic form on a logarithmic scale as channel numbers.

Cell surface iodination

Cells ($1–2 \times 10^6$ per sample) were resuspended in 150 μl of PBS and were iodinated with Na ^{125}I (Amersham, UK) by the lactoperoxidase method [18]. Cell membrane molecules were solubilized by resuspending the cells in lysis buffer (0·5% Triton X-100 and 2 mM PMSF in PBS) for 90 min at 4°C. The suspension was then centrifuged at 2500 ***g*** for 15 min at 4°C to remove nuclear debris.

Immunoprecipitation and SDS–PAGE analysis

The cell lysates were precleared by the addition of 2 μl of a non-reactive MoAb (RM2.184 at 10·2 mg/ml or RMAC11 at 8·3 mg/ml) for 1 h at 4°C followed by 200 μl of 10% suspension of *Staphylococcus aureus* Cowan strain I cells (SAC) (Sigma) in PBS with 0·5% Triton X-100, 4·8 mM potassium iodide and 0·02% NaN_3. After incubating overnight at 4°C the precleared cell membrane extract was incubated with MoAb (2 μl of RMAC8 at 9·6 mg/ml or the non-reactive MoAb (concentration as above) or 20 μl of X5 concentrated 44G4 supernatant) for 1 h at 4°C and then for 30 min with 200 μl of 10% SAC. The SAC was washed twice with 0·1 M Tris, 0·4 M NaCl buffer pH 8·3 plus 0·02% NaN_3 and resuspended and boiled for 3 min in 0·1 M Tris pH 6·8 containing 10% glycerol and 2% SDS. The samples were applied to 7·5% SDS–PAGE in the presence or absence of 50 mM dithiothrietol (Biorad, Richmond, CA). After electrophoresis the gels were fixed, dried and autoradiographed using Kodak XRP-5 x-ray film and Dupont Cronex Hi-plus intensifying screens.

Sequential immunoprecipitations were performed as previously described [16]. Briefly, single aliquots of precleared ^{125}I-labelled lysates of 10^7 cells were repeatedly immunoprecipitated with RMAC8 (or 44G4) and then reprecipitated with 44G4 (or RMAC8). At each sequential immunoprecipitation step, either

50 μl of 44G4 as a X5 concentrated supernatant or 4 μl of RMAC8 at 9·6 mg/ml was used. These amounts of antibody were determined empirically as sufficient to remove completely all endoglin from lysates of 10^7 HUVE or PMA-stimulated U937 cells in a single immunoprecipitation.

RNA hybridization analysis

Total cytoplasmic RNA was prepared from the following cell populations using the NP-40 lysis method as previously described [19]: Sp2/0.Ag14 (murine hybridoma line), Raji (human Burkitt lymphoma cell line), cultured HUVE, peritoneal macrophages, the human myelomonocytic cell lines U937 and HL-60 with and without prior stimulation with PMA as above and freshly isolated and 16 h cultured human peripheral blood monocytes. Equal amounts of RNA, as determined spectrophotometrically (OD 260 nm), were fractionated on a 1% agarose/formaldehyde minisub gel. The gel was then washed exhaustively in deionized distilled water to remove the formaldehyde and stained with ethidium bromide to verify that equal amounts of RNA had been loaded in each lane. The RNA was then transferred to a Hybond-N nylon hybridization filter (Amersham). Filters were hybridized with a ^{32}P-oligo-labelled cDNA probe for endoglin (clone 18A, 2·6 kb *Eco*RI fragment), provided by A. Gougos and M. Letarte [12]. Labelled filters were rinsed in 2 × SSC/0·1% SDS and then washed successively in 2 × SSC/0·1% SDS for 15 min at 42°C, 1 × SSC/0·1% SDS for 30 min at 65°C and finally in 0·1 × SDS/0·1% SDS for 15 min at room temperature. Autoradiography was performed with Amersham Hyperfilm, using two Cronex Lightning Plus intensifying screens at −70°C for 7 days.

RESULTS

Reactivity of RMAC8 with leucocytes and cell lines

Leucocytes were examined by indirect immunofluorescence for the presence of the RMAC8 recognized molecule. T cell, splenic B cell, and granulocyte enriched populations did not react with RMAC8 and monocytes which were isolated on plastic tissue-culture plates demonstrated only low levels of expression (Table 1). RMAC8 reactivity was also examined on six T cell lines (RPMI-8402, Peer, CEM, Jurkat, HSB-2 and Molt 4), five B cell lines (Daudi, M4B, LDB, Mann and WT49), the myelomonocytic leukaemia cell lines U937 and HL-60, the pre-B leukaemia cell lines Nalm-1, REH, LK63 and Lila-1, and the undifferentiated cell lines U299 and K562. The RMAC8 molecule was not present on the T or B cell lines, K562 or U299 (Table 1). The pre-B cell lines REH, LK63 and Lila but not Nalm-1 had low to moderate levels of expression of the RMAC8 molecule, as did HL-60 and U937 (Table 1).

RMAC8 recognizes the homodimer endoglin

Lysates of ^{125}I-labelled HUVE were immunoprecipitated with RMAC8 and analysed by SDS–PAGE (Fig. 1). Under non-reducing conditions the molecule recognized had a mol. wt of approximately 180 kD. After reduction the molecule appeared as a single band at approximately 95 kD, indicating that it is a disulphide linked dimer of similar or possibly identical polypeptide subunits.

The reported molecular characteristics [11] and the distribution [8–10] of endoglin were similar to that of the RMAC8-recognized molecule. Sequential immunoprecipitations with RMAC8 and 44G4 were then performed to determine if the RMAC8 recognized endoglin on these cells. Sequential immunoprecipitations of endothelial lysates (Fig. 2) and PMA-stimulated U937 (data not shown) showed that both MoAbs were able to deplete cell lysates of the molecules recognized by the other and the molecules immunoprecipitated were of the same apparent mol. wt (Fig. 2). This result was supported by further molecular characterization of the RMAC8 recognized molecule which migrated as a single band of 66 kD under reducing conditions when it was immunoprecipated from HL-60 cells incubated with tunicamycin (data not shown). These data are consistent with the mol. wt of the non-glycosylated form of the 44G4-recognized molecule identified by Gougos *et al.* [11].

Endoglin is a differentiation marker on macrophages

Analysis of monocyte reactivity with RMAC8 by indirect immunofluorescence indicated that monocytes have no or only weak surface expression of endoglin. 44G4 also showed no reactivity with peripheral blood monocytes. However, when

Table 1. Expression of the RMAC8 molecule on leucocytes and cell lines

Cell	RMAC8 (%)	OKM1 (%)	5.112 (%)
Myelomonocytic cells/lines			
Granulocytes	0* (3)†	23·9 (94)	ND
Monocytes	1·4 (28)	36·0 (90)	25·1 (86)
Macrophages 16 h‡	5·6 (36)	55·0 (94)	73·0 (93)
Macrophages 60 h	19·5 (94)	22·7 (93)	21·9 (82)
HL-60	1·1 (4)	0 (0·1)	ND
HL-60 + PMA	4·3 (36)	9·1 (53)	ND
U937	1·9 (52)	0·4 (2)	ND
U937 + PMA	8·8 (93)	2·5 (20)	ND
Pre-leukaemic cell lines			
Nalm-1	0 (0)	ND	19·6 (70)
REH	2·5 (10)	ND	57·0 (72)
LK63	2·1 (80)	ND	ND
Lila-1	2·2 (93)	ND	ND
B lymphocytes/cell lines			
B cells	0 (0)	0·7 (7)	35·1 (80)
B cell lines (5)§	0 (0)	ND	Range 79·3–155·8
M4B + PMA	0 (0)	ND	ND
T lymphocytes/cell lines			
T cells	0 (0)	ND	1·5 (6)
T cells + PHA	0 (0)	ND	5·5 (24)
T cells + Con A	0 (0)	ND	3·1 (14)
T cell lines (6)¶	0 (0)	ND	ND
RPMI-8402 + PMA	0 (0)	ND	ND

* Expression of RMAC8 reactivity as linear mean fluorescence after subtraction of background fluorescence.

† Expression of RMAC8 reactivity by indirect immunofluorescence as percentage positive cells after subtraction of background fluoresence.

‡ Macrophages were peripheral blood monocytes cultured on plastic culture flasks for 16 or 60 h.

§ M4B, LDB, Daudi, Mann and WT49.

¶ PEER, CEM, HSB-2, Jurkat, Molt-4 and RPMI-8402.

PMA, Phorbol myristate acetate; PHA, phytohaemagglutinin; Con A, concanavalin A; ND, not tested.

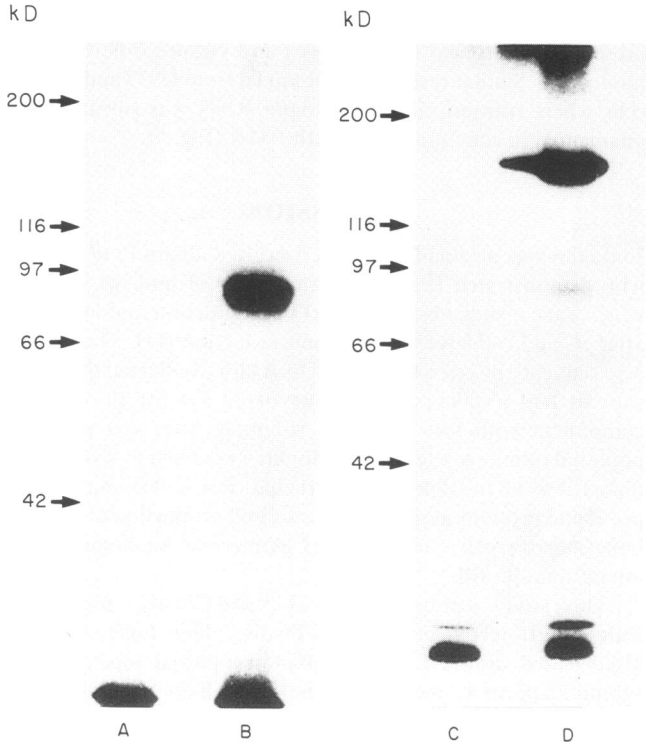

Fig. 1. SDS–PAGE of RMAC8 immunoprecipitates from ^{125}I-labelled human umbilical vein endothelial cells (HUVE). Lanes A and C, negative control (RM2.184). Lanes B and D, RMAC8, under reducing and non-reducing conditions, respectively.

monocytes were cultured for 16 or 60 h there was increased expression of the RMAC8 molecule (Fig. 3). Sixteen hours of culture caused a four-fold (1·4–5·6) increase in linear mean fluorescence and 60 h of culture resulted in a 14-fold (1·4 to 19·5) increase in linear mean fluorescence (Table 1). PHA and Con A stimulation of T cells did not induce expression of the RMAC8 molecule despite induction of other activation markers such as MHC class II antigen (Table 1).

PMA stimulation of HL-60 and U937 for 3 days, which induces myelomonocytic cells to differentiate towards a macrophage phenotype [20], caused an up-regulation of the RMAC8-recognized molecule by four-fold (1·1–4·3) and 4·6-fold (1·9–8·8) respectively (Table 1). These results were confirmed by immunoprecipitation of 24 h cultured monocytes (Fig. 4a, b) and peritoneal macrophages (Fig. 4c) with RMAC8 and 44G4. RMAC8 but not 44G4 immunoprecipitated the endoglin molecule from these cells even though 44G4 had been able to identify the molecule on endothelial cells (Fig. 2) and U937 cells.

Northern blots

The ability of RMAC8 to bind macrophages and cultured monocytes but not freshly isolated monocytes suggests that endoglin expression distinguishes monocytes from cells of macrophage morphology. In order to determine if transcription of endoglin RNA corresponded with its observed surface expression, monocyte and macrophage RNA was electrophoresed and transferred to nylon paper by Northern blotting. The detection by Northern blotting of endoglin mRNA (Fig. 5) correlated exactly with the detection of endoglin on the cell surface by RMAC8 (Table 1 and Fig. 3). Significantly, endoglin

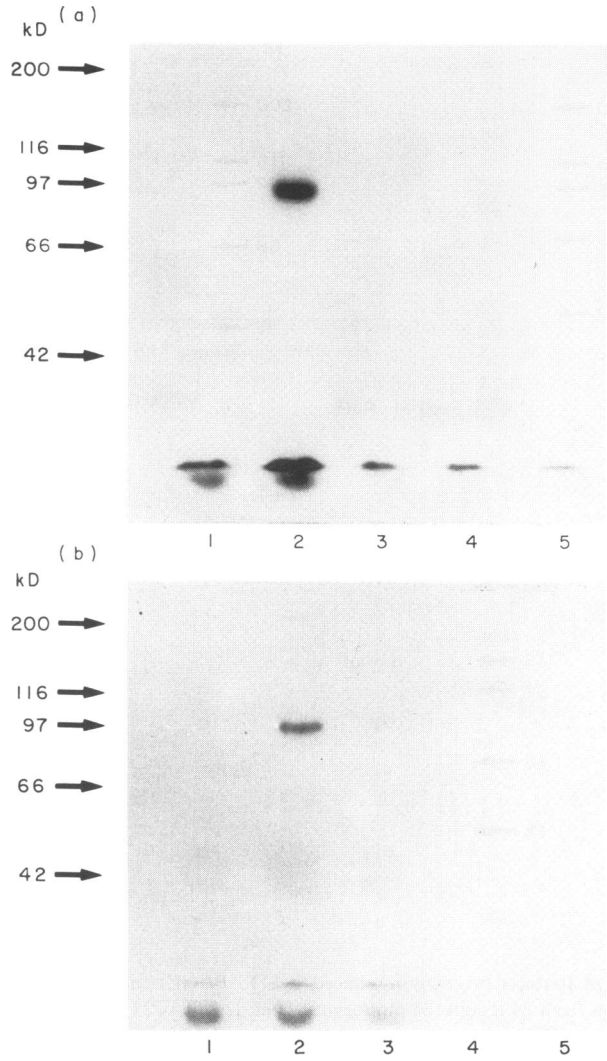

Fig. 2. SDS–PAGE (reducing conditions) of sequential immunoprecipitates from ^{125}I-labelled human umbilical vein endothelial cells (HUVE). (a) The preclearing of the HUVE lysate by RMAC8 before immunoprecipitation with 44G4. Lane 1, negative control (RMAC11). Lanes 2–4, sequential precipitates with RMAC8 followed by 44G4 (lane 5). (b) The preclearing of the HUVE lyaste by 44G4 before immunoprecipitation with RMAC8. Lane 1, negative control (RMAC11). Lanes 2–4, sequential precipitates with 44G4 followed by RMAC8 (lane 5).

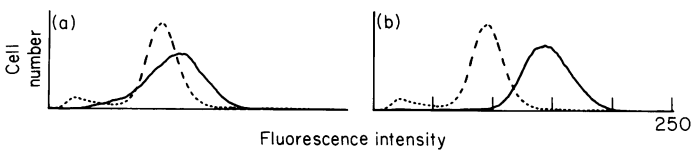

Fig. 3. Flow cytometric profiles of the reactivity of RMAC8 with monocytes which were freshly isolated (– – –) or cultured for 16 h (a) or 60 h (b) (——). The abscissa shows channel numbers and the scale is logarithmic.

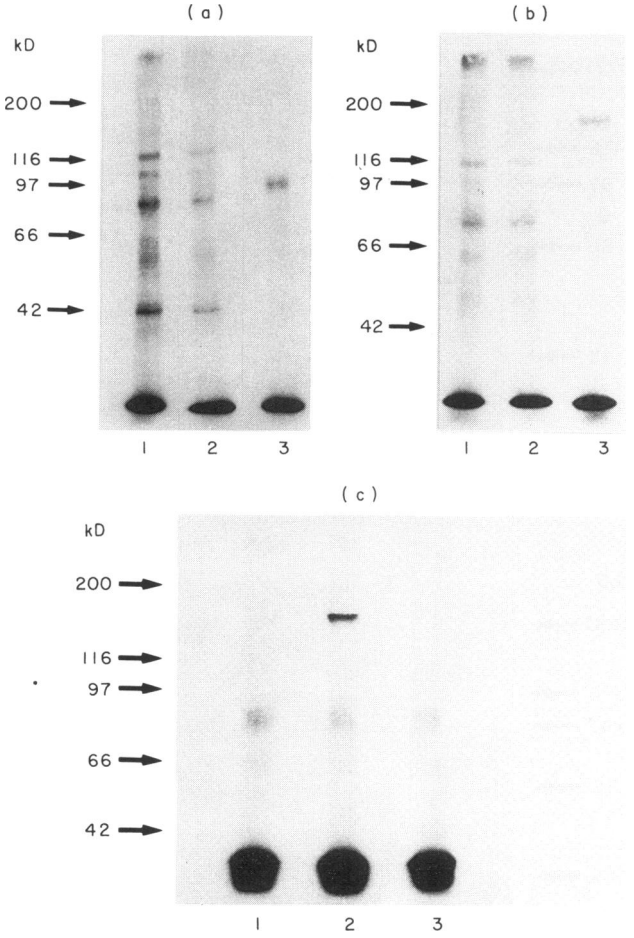

Fig. 4. Reduced (a) and non-reduced (b) SDS–PAGE of immunoprecipitates from 24 h cultured monocytes. Lane 1, RMAC11; lane 2, 44G4; lane 3, RMAC8. (c) Non-reduced SDS–PAGE of RMAC8 and 44G4 immunoprecipitates from ^{125}I-labelled peritoneal macrophages. Lane 1, negative control (RMAC11); lane 2, RMAC8; lane 3, 44G4.

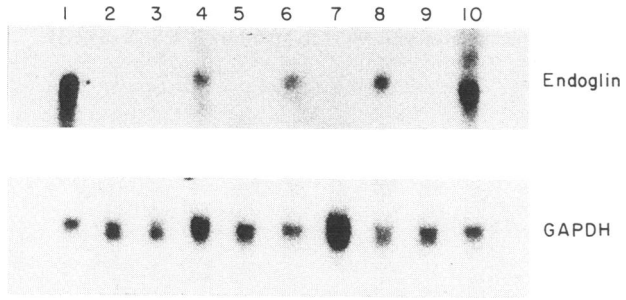

Fig. 5. Northern blots of mRNA from cells and cell lines hybridized with a full length endoglin cDNA probe. Equal amounts of RNA, as determined spectrophotometrically (OD 260 nm), were loaded in each lane. Lane 1, human umbilical vein endothelial cells (HUVE); lane 2, Raji cells; lane 3, Sp2/0.Ag14; lane 4, human peritoneal macrophages; lane 5, unstimulated U937 cells; lane 6, phorbol myristate acetate (PMA)-stimulated U937 cells; lane 7, unstimulated HL-60 cells; lane 8, PMA-stimulated HL-60 cells; lane 9, freshly isolated monocytes; lane 10, monocytes cultured for 16 h.

mRNA was not detected in freshly isolated monocytes but was present in peritoneal macrophages and culture differentiated monocytes. Similar results were obtained from U937 and HL-60 cells where transcription of endoglin RNA was substantially augmented in cells stimulated with PMA (Fig. 5).

DISCUSSION

Endoglin was originally characterized by Gougos *et al.* [10,11] who demonstrated it to be a homodimer of mol. wt 170 kD which gave a single band at 95 kD on reduction and at 62 kD after N- and O-deglycosylation and reduction [11]. The amino acid sequence inferred from the cDNA clone indicated that each subunit had a calculated mol. wt of 68 kD for the protein component with four potential N-linkage sites and multiple potential O-linkage sites [12]. Endoglin, as defined by 44G4, was reported to be restricted to endothelial cells, leukaemia cells of pre-B and myelomonocytic origin, a small proportion of normal bone marrow cells and the renal glomerular mesangium and interstitium [8–10].

This study demonstrates that RMAC8 also recognizes endoglin. It recognizes a 180 kD disulphide linked heavily glycosylated dimer. Like the 44G4 recognized molecule the subunits appear to contain internal disulphide bridges as they migrate more slowly than would be expected from the apparent molecular weight of the unreduced molecule. The apparent molecular weight of the N-deglycosylated subunits is 66 kD. The RMAC8-recognized molecule, like endoglin, is expressed on endothelial and pre-B cells. Finally, the sequential immunoprecipitation experiments formally demonstrate that on endothelial cells and PMA-stimulated U937 cells, the 44G4 and RMAC8-recognized molecule is identical.

RMAC8 also reacts with cultured differentiated monocytes and peritoneal macrophages. Immunoprecipitates of differentiated monocytes and peritoneal macrophages by RMAC8 show that the molecule recognized on these cells appears to be identical to that recognized on endothelial cells. Furthermore, Northern blots established that transcription of endoglin mRNA corresponds exactly with endoglin expression on macrophages and PMA-stimulated myeloid leukaemic cell lines. However, 44G4 could not detect or immunoprecipitate endoglin from the culture differentiated macrophages, suggesting that the epitopes recognized by the two MoAbs differ in their presence or accessibility on different cell types. Recently, Lastres *et al.* [13] reported similar findings with the anti-endoglin MoAb 8E11. They demononstrated the presence of endoglin on culture differentiated macrophages and tissue macrophages of the red pulp of spleen but not on macrophages in lymph nodes, lung or tonsil. However, in contrast to the findings reported here, they found that 44G4 reacted with culture differentiated macrophages. An explanation for this conflict is not readily apparent as the batch of 44G4 used in this study was clearly active as it was reactive with endothelial cells and U937 cells.

The extensive screening of normal peripheral blood leucocytes and cell lines in this study, together with the findings of Lastres *et al.* [13], indicates that endoglin expression is restricted to endothelial cells, a small population of bone marrow cells and macrophages. Although strongly expressed on peritoneal and bronchial macrophages, it was not detected on peripheral blood granulocytes, B cells, resting or activated T cells, lymph node or splenic lymphocytes. Other molecules such as CD16, CD51 and

CD71 [4–6] are markers of macrophage differentiation, but they are widely expressed on other leucocyte subpopulations. Only the macrophage mannose receptor has a more macrophage-restricted expression than endoglin and its surface expression is difficult to detect [7]. This identifies endoglin as an important restriction marker of macrophages as endoglin cell surface expression can be easily detected using the RMAC8 MoAb and it has a cloned cDNA probe allowing for accurate detection of macrophage mRNA.

This report extends the knowledge of endoglin by confirming its strong expression on macrophages and weaker or absent expression on peripheral blood monocytes and indicates that transcription of the endoglin gene accompanies the differentiation process. These observations suggest that endoglin is a differentiation marker which is absent from or markedly down-regulated on circulating monocytes and then expressed on mature macrophages. The identification of such a restricted molecule provides a powerful tool for further studying the conditions and factors responsible for macrophage differentiation.

The function of this molecule is unknown. No doubt its expression on macrophages and endothelial cells is associated with a function common to these cells. Studies to date have shown that RMAC8 does not inhibit alloantigen-induced T cell proliferation nor T cell proliferation induced by macrophage-dependent mitogens such as OKT3 or Con A. Nor does it inhibit the adhesion of PMA-stimulated HL-60 cells to HUVE (data not shown). However, the demonstration of an RGD sequence in the molecule [12] suggests that it will be found to have an adhesion-related function.

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