Characterization of MAX.3 antigen, a glycoprotein expressed on mature macrophages, dendritic cells and blood platelets: identity with CD84

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MAX.3 is a monoclonal antibody that preferentially reacts with mature macrophages (MAC), monocyte-derived dendritic cells, megakaryocytes and platelets. In this study, we describe the characterization, purification and identification of the MAX.3 antigen. Immunoprecipitation and SDS/PAGE revealed different molecular masses of MAX.3 antigen in MAC (60–90 kDa) and platelets (58–64 kDa), whereas a similar size (45 kDa) was observed in both cell types after digestion with N-glycosidase F. Lectin affinity and sequential treatment with different glycosidases suggests complex type glycosylation of MAX.3 antigen in MAC and hybrid type glycosylation in platelets. Amino acid sequencing led to the identification of a corresponding cDNA clone and showed its identity to the sequence of the CD84 antigen, a member of the CD2 family of cell surface molecules. MAX.3/CD84 was further studied by immunohistochemistry and a variable expression was found on tissue MAC, confirming this antigen to be mainly a marker for MAC *in situ*.

Key words: cell differentiation, glycosylation, monocyte.

INTRODUCTION

Mononuclear phagocytes participate in both specific and non-specific immune responses and play a critical role in extracellular matrix remodelling and wound healing [1]. They differentiate from bone marrow precursors in a multistep process being only partly understood [2]. Peripheral blood monocytes (MO) provide the common precursor of tissue macrophages (MAC), the latter being considered mature effector cells. In response to so far unknown signals, MO attach to the wall of blood vessels and start to infiltrate tissues and body cavities. There depending on the microenvironment and tissue-specific factors, they develop into different types of MAC with a wide spectrum of functional heterogeneity (e.g. alveolar MAC in the lung, Kupffer cells in the liver, osteoclasts and many others [2,3]). Under *in vitro* conditions, MO also adhere tightly to cell culture substrates and a similar process of maturation from human blood MO to MAC takes place, accompanied by specific changes of morphological, cytochemical, phenotypic and functional properties [4,5]. Several cell surface antigens can be used as phenotypic markers to distinguish mature MAC from blood MO. For example, the low-affinity Fc receptor (CD16), the α-chain of the vitronectin receptor (CD51), the transferrin receptor (CD71), endoglin (CD105) and carboxypeptidase M are preferentially detectable on mature MAC [6–8], whereas, e.g., the B148.4 antigen is mainly expressed on peripheral blood MO [9]. In the last few years it has been shown that, under appropriate conditions, dendritic cells (DC) as professional antigen presenting cells can also differentiate from MO [10,11], although it is not completely clear, if this pathway of differentiation is also operative *in vivo*.

MAX.3 monoclonal antibody had been produced by immunizing mice with human mature MAC and screening the resulting hybridomas for antibodies reacting with MAC but not with fresh blood MO as described previously [7]. MAX.3 antigen is not detected on MO or is expressed very weakly, but is upregulated during *in vitro* differentiation towards MAC. Furthermore, it is expressed on platelets and megakaryocytes, but not on other cells in the blood or bone marrow [7]. *In vivo* differentiated MAC in serous cavities (e.g. pleural and peritoneal cavity) express MAX.3 at a slightly lower level compared to *in vitro* derived MAC [12].

In the recent years, MAX.3 antibody has been used to analyse MAC differentiation under various conditions and it has emerged that MAX.3 antigen expression is closely linked to the morphological and functional features of mature MAC under a variety of experimental conditions [8,13–16]; the corresponding molecule has not yet been defined. Here we report the biochemical and molecular characterization of the antigen detected by MAX.3 antibody. Different molecular masses and glycosylation patterns were found and analysed on MAC and platelets, the two major MAX.3 antigen expressing cell types. Amino-acid sequencing of affinity-purified protein led to the identification of a corresponding cDNA. The recent cloning of an overlapping cDNA [17] shows identity of MAX.3 antigen with CD84.

MATERIALS AND METHODS

Antibodies, chemicals and cells

All chemical reagents used were purchased from Sigma (Deisenhofen, Germany) unless otherwise noted. MAX.3 antibody was purified from hybridoma supernatants by ammonium sulphate precipitation (55 %) followed by Protein G–Sepharose chromatography. The purity and immunologic reactivity of the purified antibody was documented by SDS/PAGE and used in flow cytometry, respectively.

Abbreviations used: ConA, concanavalin A; DC, dendritic cells; MAC, macrophages; MO, monocytes; WGL, wheat germ lectin.

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Sequence information about cDNA clone HDPFF19 is deposited at EMBL data bank (accession number AJ223324).

Peripheral blood mononuclear cells were separated by leukapheresis of healthy donors, followed by density gradient centrifugation over Ficoll/Hypaque. MO were isolated from mononuclear cells by countercurrent centrifugal elutriation in a J6M-E centrifuge (Beckman, Munich, Germany) as previously described [18]. MO were about 90% pure as determined by morphology and expression of CD14 antigen. Platelets were also retrieved from the counter-current elutriation by collecting the early elutriation fraction containing the smallest cells [19]. Isolated MO were cultured in RPMI 1640 medium (Biochrom KG, Berlin, Germany) supplemented with vitamins, antibiotics, pyruvate, non-essential amino acids (all from Gibco–BRL, Eggenstein, Germany), 5×10^{-8} M mercaptoethanol, and 2% human pooled AB-group serum either on teflon foils or bacterial grade plastic Petri dishes (Greiner) or in polypropylene bottles rotating slowly in a horizontal position for a period of up to 8 days as indicated in the text. In some experiments, dendritic cell differentiation was induced by culture of MO for 1 week in medium containing 10% fetal bovine serum, interleukin-4 and granulocyte-macrophage colony-stimulating factor as described previously [11,20]. Differentiation of megakaryocytes *in vitro* from human affinity-purified $CD34^+$ haematopoietic progenitor cells [20] was induced by a combination of interleukin-3, interleukin-6 and thrombopoietin in serum-free cultures (Easymega kit, Hemeris, Sassenage, France).

Surface biotinylation and protein purification

Cells were surface-labelled using biotin as described elsewhere [21]. Briefly, $5–10 \times 10^6$ MAC or 50 mg of thrombocytes were labelled in 1 ml of PBS containing 150 μg/ml 6-(+)-biotinylamidohexanoic acid *N*-hydroxysulphosuccinimide ester sodium salt (NHSS-LC-biotin, Serva, Heidelberg, Germany) for 15 min at room temperature. The reaction was stopped by adding NH_4Cl to a final concentration of 10 mM, and cells were washed twice with PBS.

For immunoprecipitation, biotin-labelled cells were resuspended in 500 μl TBS containing 20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 2 μg/ml aprotinin (Boehringer–Mannheim, Germany) and 0.5 μg/ml leupeptin (Boehringer–Mannheim, Germany), and solubilized by addition of 500 μl lysis buffer containing 1% Nonidet P-40 (Boehringer–Mannheim, Germany) and 1% BSA in TBS. Lysis was performed for 45 min on ice, then nuclei and cell debris were removed by centrifugation (12000 ***g***, 4 °C, 30 min). Supernatants from cell lysis were precleared with 30 μl Protein G–Sepharose (Pharmacia Biotech, Uppsala, Sweden) overnight at 4 °C on a rotating mixer. Immunoprecipitation was done by incubating the precleared supernatant with antibody (10 μg/ml) for 2 h at 4 °C and precipitation with 30 μl Protein G–Sepharose for 45 min on a rotating mixer. Sepharose pellets were washed five times with ice-cold TBS, suspended in SDS sample buffer [22] or processed for deglycosylation as described below.

For immunoaffinity column purification, platelets were suspended in TBS containing 20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 2 μg/ml aprotinin (Boehringer–Mannheim), and 0.5 μg/ml leupeptin (Boehringer–Mannheim), and solubilized by adding 500 μl of lysis buffer containing 1% Nonidet P-40 (Boehringer–Mannheim), and 1% BSA in TBS solution. Following lysis for 45 min on ice, nuclei and cell debris were removed by centrifugation (12000 ***g***, 4 °C, 30 min). Supernatants were applied to a 1.5 ml immunoaffinity column [4.7 mg/ml antibody covalently bound to 6-(+)-aminohexanoic acid *N*-hydroxysulphosuccinimide ester-Sepharose] equilibrated with binding buffer (1:1 mixture of PBS and TSS buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate) over a 5 ml preclearing column (5 ml Sepharose CL-4B, Pharmacia, Freiburg, Germany). After sample loading, the column was washed sequentially with 5 column volumes of each of the following buffers: TSS buffer, Tris buffer, pH 8.0 (50 mM Tris/HCl, pH 8.0, 0.5 M NaCl), and Tris buffer, pH 9.0 (50 mM Tris/HCl, pH 9.0, 0.5 M NaCl). Bound material was eluted with elution buffer (50 mM Glycin/HCl, pH 2.5, 0.1% Nonidet P-40, and 150 mM NaCl) in 1.6-ml fractions, and neutralized immediately with 0.2 volumes of 1.5 M Tris/HCl, pH 8.8. Eluates were analysed by SDS/PAGE to identify antigen-containing fractions.

For lectin affinity purification, a 200 μl lectin affinity column (wheatgerm lectin-Agarose or concanavalin A (ConA)-Sepharose, Pharmacia, Freiburg, Germany) was equilibrated with 10 ml PBS and incubated with up to 9 ml glycoprotein solution dialysed against PBS for 3 h at 4 °C on a rotating mixer. After washing with 5 column volumes of wash buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate) the column was saturated with approx. 0.6 column volumes of elution buffer (Tris-buffered saline containing 20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 0.3 M *N*-acetyl-D-glucosamine (WGL-Agarose, where WGL is wheat germ lectin) or 0.3 M α-D-mannose (ConA-Sepharose) containing phenol red and incubated for 90 min at room temperature. Elution was performed with approx. 3 column volumes of elution buffer without colorant until complete decolorization of the matrix was achieved.

For the detection of biotinylated protein, samples from immunoprecipitation were electrophoresed on 12% SDS-polyacrylamide gels under reducing conditions [22] along with biotinylated molecular mass marker proteins (Bio–Rad, Munich, Germany) and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) for 1 h at 0.8 mA/cm² [24]. Membranes were blocked overnight at 4 °C in blocking solution (20 mM Tris/HCl, pH 7.4, 150 mM NaCl) containing 5% BSA. After washing with 0.1% Tween-20 in blocking solution the membrane was incubated for 1 h at room temperature with a streptavidine-coupled horseradish peroxidase complex (Amersham, U.K., diluted 1:2500 in blocking solution containing 5% BSA). After extensive washing, biotinylated proteins on the nitrocellulose membrane were visualized by enhanced chemiluminescence detection system (ECL, Amersham, Freiburg, Germany).

Immunoaffinity-purified MAX.3 antigen from platelets was concentrated by lectin affinity chromatography and methanol/chloroform precipitation [25]. The resulting protein was subjected to SDS/PAGE (15% polyacrylamide gel, reducing conditions) and blotted to PVDF membrane (Millipore, Eschborn, Germany). After staining with Coomassie Blue, a specific band was excised and the N-terminal amino acid sequence was determined by automated Edman degradation utilizing an Applied Biosystems protein sequencer (model 477A) with an online PTH-analyser (model 120A).

The protein concentrations were determined using bicinchoninic acid according to the described procedure [26] with albumin as the standard.

Glycosidase treatment

The glycosidases used were neuraminidase, β-galactosidase, N-acetyl-β-D-glucosaminidase, endoglycosidase H (obtained from Boehringer–Mannheim) and O-glycosidase (from *Diplococcus pneumoniae*, Roche Molecular Biochemicals, Mannheim,

Germany). Following immunoprecipitation, sequential deglycosylation of MAX.3 glycoprotein still coupled to Protein G–Sepharose involved washing the pellet with the corresponding glycosidase buffer (neuraminidase: 50 mM sodium acetate, 4 mM calcium chloride, 0.01 % BSA, pH 5.5; galactosidase and endoglycosidase H: 50 mM sodium acetate, pH 6.0; glucosaminidase: 50 mM sodium citrate, pH 4.8) prior to incubation with the respective glycosidase for 2 h at 37 °C. Complete removal of *N*-linked glycans was performed using *N*-glycosidase F (Boehringer–Mannheim) as described previously [23].

Transient transfection of COS7 cells

COS7 cells were split and seeded into six well plates in order to obtain semiconfluent growth on the day before transfection. Transfection was performed using Lipofectamine (Life Technologies, Eggenstein, Germany) as recommended by the manufacturer with either ELAM I cDNA in vector pCDMA [27] or clone HDPFF19 in vector pCMV Sport 3.0. On day 2 after transfection, COS7 cells were harvested from the dishes by vigorous pipetting after incubation in 0.5 mM EDTA in PBS, and analysed on adhesive slides by immunoperoxidase as described [28] using MAX.3 and anti ELAM I control antibody (4D10, Dianova, Hamburg, Germany).

Immunoaffinity staining and flow cytometry

Freshly isolated MO or cultured cells were washed twice with cold PBS containing 0.06 % immunoglobulin (Sandoz Pharma AG, Basel, Switzerland) and 5×10^5 cells were incubated with saturating amounts MAX.3 antibody or IgG control (Jackson Immuno Research, West Grove, PA, U.S.A) for 30 min at 4 °C. After two further washes, cells were incubated with saturating concentrations of FITC-conjugated goat anti mouse IgG (Jackson Immuno Research) for 30 min at 4 °C. After two more washes, cells were fixed with 1 % paraformaldehyde in PBS. Analysis was performed using a FACScan (Beckton–Dickinson, San Jose, CA, U.S.A.). MO and lymphocyte populations were gated according to their forward and side scattering and analysed separately.

For immunohistology, frozen sections of different tissues were stained by a sensitive immuno-alkaline phosphatase technique as described [16].

Northern blots and DNA sequence analysis

Total RNA was isolated from different cell types by the guanidine thiocyanate/acid phenol method [29]. The RNA (10 μg/lane) was separated by electrophoresis on 1 % agarose/formaldehyde gels, and transferred to nylon membranes (Magna NT, MSI, Westbrough, MA, U.S.A.). Oligonucleotides corresponding to the MAX.3 cDNA nucleotide sequence were synthesized for PCR amplification of a 600 bp DNA stretch to be used as a probe for Northern hybridization. The sense primer corresponded to nucleotides 27–45, the antisense primer corresponded to nucleotides 607–626 of the HDPFF19 sequence. The amplified PCR product was inserted into the plasmid vector pCRII (TA Cloning Kit, Invitrogen, San Diego, CA, U.S.A.). Hybridization was performed using ^{32}P-labelled cDNA insert (Random Primed DNA Labeling Kit, Boehringer–Mannheim) [30]. To provide an internal control, membranes were reprobed with an oligonucleotide against 18 S rRNA labelled by T4-Kinase (5′-end labelling kit, Amersham, U.K.). Autoradiography was performed at −70 °C. Alternatively, Northern blot analysis of whole tissues was performed by using Clontech (Palo Alto, CA, U.S.A.) multiple tissue mRNA Northern blots (Clontech 7770-1, 7750-1, 7751-1, 7754-1, 7755-1, 7756-1, 7757-1, 7759-1, 7760-1, 7766-1, 7768-1), consisting of 2 μg of poly(A)$^+$ RNA per lane. In this case, hybridization was overnight in Hybrisol solution (Oncor, Gaithersburg, MD, U.S.A.) at 42 °C, followed by two subsequent washes in 2 × SSC/0.1 % SDS and 0.2 × SSC/0.1 % SDS at the same temperature.

Computer based DNA analysis was performed with the HUSAR software package at the GENIUSnet in Heidelberg, Germany.

RESULTS

Biochemical characterization of MAX.3 antigen

For a biochemical characterization of antigens detected by antibody MAX.3, we surface-labelled *in vitro* differentiated MAC or blood platelets with NHSS-LC-biotin and immunoprecipitated antigens from the cell lysates. Apparent molecular mass was 60–90 kDa in MAC and 58–64 kDa in platelets (Figure 1). Subsequent digestion of the immunoprecipitated material with *N*-glycosidase F resulted in a shift to seemingly identical bands with an apparent size of 45 kDa (Figure 1) in both platelets and MAC, indicating that MAX.3 detects the same, but differentially glycosylated protein in both cell types. In control reactions, a dominant band at 110 kDa was detected on platelets with a CD61 specific antibody and no antigen could be precipitated from platelets using a CD11c specific antibody.

Lectin binding experiments with MAX.3 antigen from platelets and MAC showed that glycoprotein from platelets bound

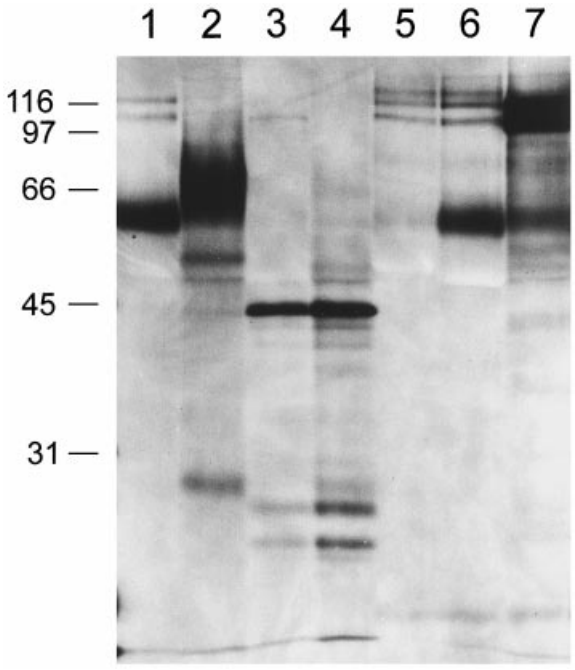

Figure 1 Molecular mass of MAX.3 antigen on MO-derived MAC and platelets

Biotinylated surface antigens were purified from MAC (lanes 2, 4) or platelets (lanes 1, 3) and analysed by SDS/PAGE and streptavidine–peroxidase detection as described in the Materials and methods section. Precipitated proteins were either subjected to N-glycosidase F treatment before SDS/PAGE (lanes 3, 4) or analysed undigested (lanes 1, 2). Control reactions were done with platelet antigens of a different donor and third party antibodies: CD11c (lane 5, no specific band), MAX.3 (lane 6), CD61 (lane 7, specific band at 110 kDa).

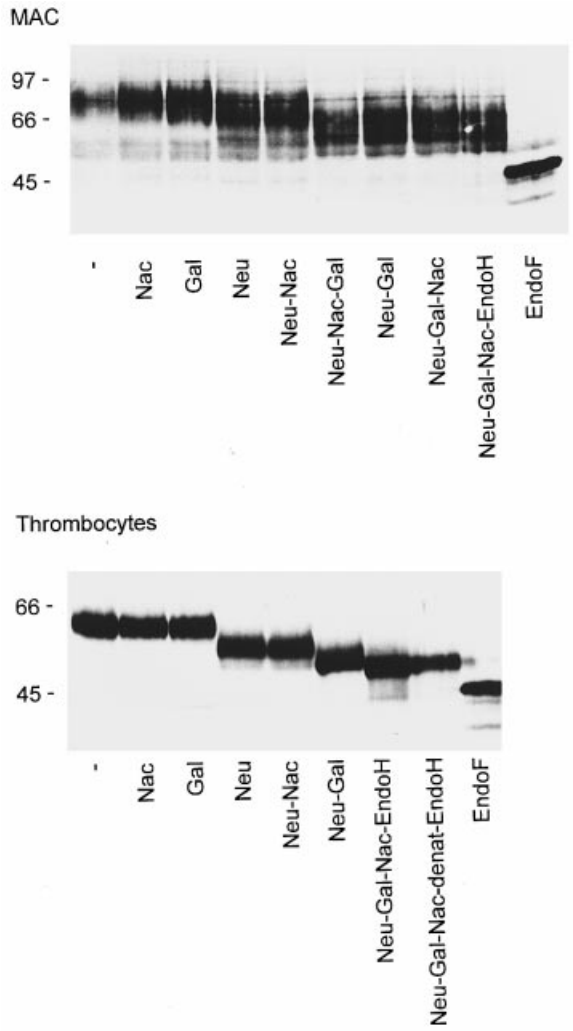

Figure 2 Analysis of differential glycosylation of MAX.3 antigen on MAC or platelets

Antigen precipitated from MAC or platelets was subjected to sequential deglycosylation and subsequently analysed by SDS/PAGE. The enzymes are listed in the order they were sequentially applied to the antigen sample (—, undigested; Nac, N-acetyl-D-glucosaminidase; Gal, β-galactosidase; Neu, neuraminidase; EndoH, endoglycosidase H; EndoF, endoglycosidase F; denat, a boiling step was switched between two enzyme digestions).

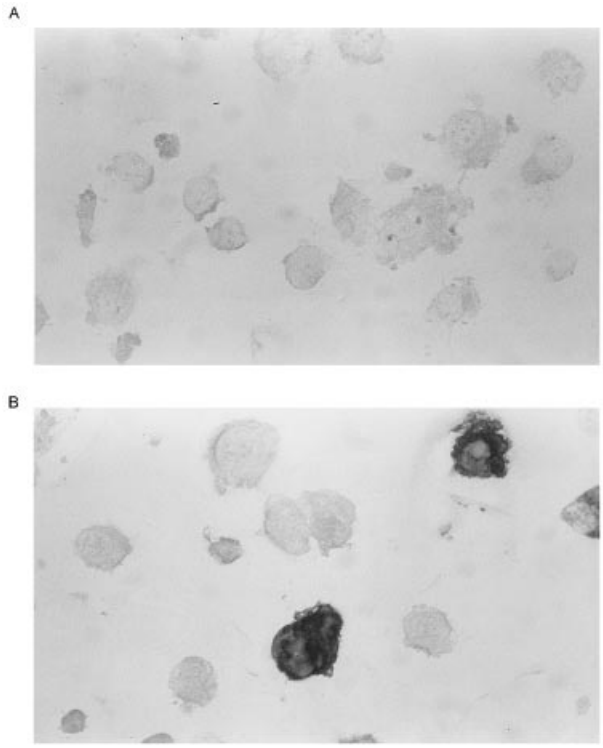

Figure 3 MAX.3 immunoreactivity on COS7 after transfection with HDPFF 19 coding for CD84

COS7 cells were transfected with a control cDNA (ELAM I) (**A**) or with HDPFF19 cDNA (**B**) and stained with antibody MAX.3 using immunoperoxidase.

strongly to both WGL and ConA, while MAC-derived antigen did not bind to ConA and only very weakly to WGL (not shown in detail). For further analysis of the different glycosylation patterns found on platelets and MAC, biotinylated antigen from both cell types was immunoprecipitated and sequentially digested with three different exoglycosidases (neuraminidase, β-galactosidase, *N*-acetyl-D-glucosaminidase) and endoglycosidase H while still bound to the MAX.3-Protein G–Sepharose conjugate. The loss in apparent mass due to each digestion step was analysed by SDS/PAGE (Figure 2). Sequential application of neuraminidase and β-galactosidase resulted in cleavage of terminal sialic acid and adjoining galactose residues from MAC – as well as platelet-derived MAX.3 antigen. Successive treatment with N-acetyl-D-glucosaminidase only showed an effect with platelet-derived antigen (Figure 2) as did subsequent digestion with endoglycosidase H (not shown). Without neuraminidase pretreatment, neither galactosidase nor N-acetyl-D-glucosaminidase led to mass reduction in SDS/PAGE (Figure 2). Without pretreatment by neuraminidase, endoglycosidase H did not lead to any visible weight reduction in either platelets or MAC (not shown). Furthermore, O-glycosidase, either applied directly or after pretreatment with neuraminidase, did not lead to any detectable antigen deglycosylation from platelets or MAC (results not shown).

Sequence identity of MAX.3 antigen with CD84

Since the eluate from immunoaffinity columns was not sufficiently pure for amino acid sequencing (not shown), an additional lectin affinity was performed. The lysate of 7.8 g platelets was subjected to immunoaffinity column chromatography over MAX.3 covalently linked to Sepharose in two sequential batches. After washing, bound material was eluted in small fractions which were analysed for protein content using SDS/PAGE. Fractions containing the antigen were pooled and further purified over a lectin affinity column containing WGL-Agarose. The protein content of the eluate was chloroform/methanol precipitated and subjected to SDS/PAGE. The prominent band at about 58–64 kDa was cut out and used for NH_2-terminal amino acid sequencing yielding the following sequence: (Lys)-Asp-Ser-Glu-Ile-Phe-Thr-Val-Asn-Gly-Ile-Leu-Gly-Glu.

A search of the public databases initially revealed no identity of the peptide with any known sequence. Therefore we performed a computer search in the private database of Human Genome Sciences. We identified three clones in a cDNA library originating from interleukin-4/granulocyte-macrophage colony-stimulating

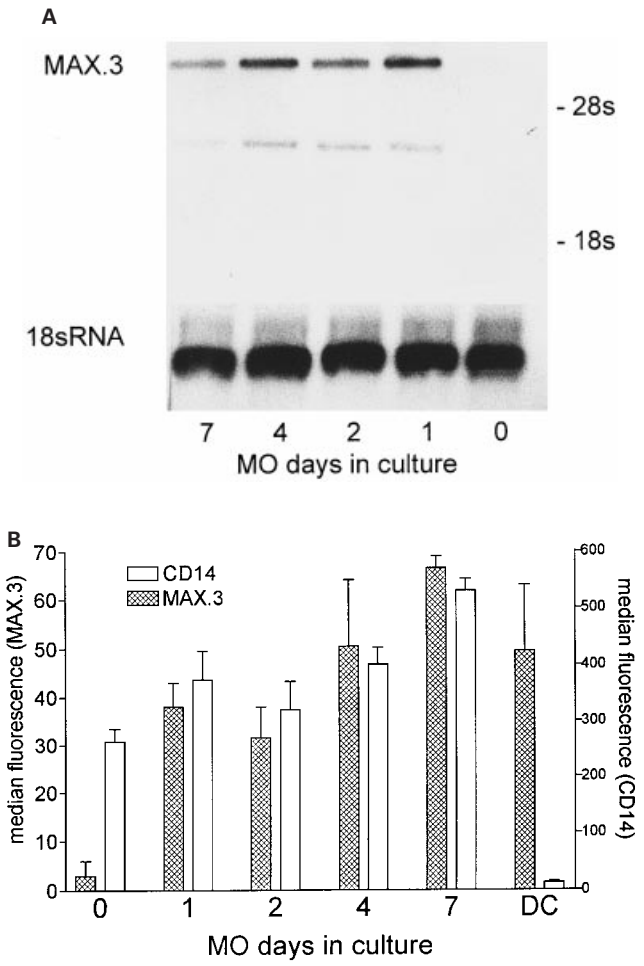

Figure 4 Expression of MAX.3/CD84

MO were allowed to differentiate into MAC *in vitro* and tested at different time points as indicated. Alternatively, differentiation into DC was induced as described in the Materials and methods section. (**A**) Northern blots detecting MAX.3/CD84 RNA, with 18 S RNA as an internal control. (**B**) Levels of MAX.3 immunoreactivity of MO/MAC or DC harvested from teflon bags as determined by flow cytometry.

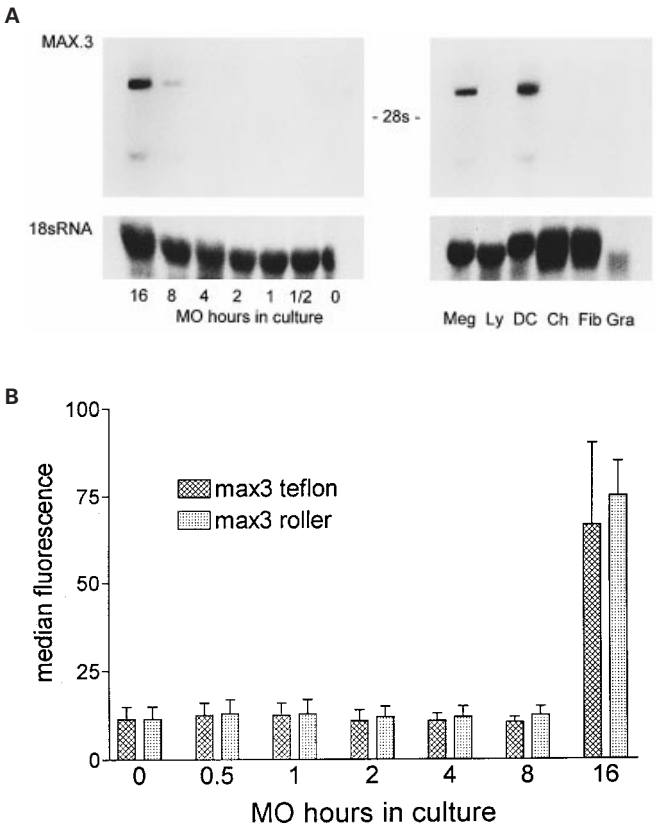

Figure 5 Expression of MAX.3/CD84

(**A**) Northern blots of MAX.3 RNA in different defined cell types (Gra, granulocytes; Fib, primary cultured fibroblasts; Ch, primary cultured chondrocytes; MO-derived DC; Ly, peripheral blood lymphocytes; Meg, *in vitro* derived megakaryocytes) and during short-time cultivation of MO. (**B**) Levels of MAX.3 immunoreactivity of MO harvested from teflon bags or roller bottles as determined by flow cytometry.

factor treated MO-derived dendritic cells (DC) [11], the longest clone (HDPFF 19) spanning a cDNA strretch of 3299 bp. Full length sequence information is deposited at EMBL data bank (accession number AJ223324). This clone contains an open reading frame of 984 bp, coding for 328 amino acids. Later during this study, a 1040 bp cDNA clone covering the identical open reading frame of MAX.3 became accessible in the GenBank database and assigned this DNA sequence as coding for the leucocyte antigen CD84 [17].

In order to prove that the HDPFF19/CD84 cDNA sequence was in fact coding for the MAX.3 antigen, the cDNA clone was transiently transfected into COS7 cells. Control cells were transfected with a cDNA coding for the ELAM I surface antigen [27]. After 2 days, immunoreactivity of transfected cells was tested by indirect immunoperoxidase staining. Anti ELAM I control antibody 4D10 reacted with 30% of COS7 transfected with ELAM I cDNA. Similarly, reactivity with antibody MAX.3 was detected on about 30% of HDPFF19 transfected cells, but not on ELAM-transfected cells (Figure 3), indicating that the HDPFF19/CD84 cDNA sequence is in fact coding for the antigen detected by MAX.3 and therefore, MAX.3 is an CD84-specific antibody.

Expression pattern of MAX.3 antigen and RNA

In the next series of experiments, RNA expression of CD84 was analysed in different cells of haematopoietic origin. As expected, RNA expression is upregulated during transition of MO into MAC. Two bands are visible on these blots: one with an RNA length of about 4.0 kb, the second band indicating a transcript length of about 7 kb (Figure 4A). A strong CD84 signal is seen in MO already after 1 day in culture. This result is in good accordance with the level of MAX.3 immunoreactivity as measured by flow cytometry (Figure 4B). Interestingly, a considerable degree of CD84/MAX.3 antigen upregulation is also observed during differentiation of MO into MO-derived DC that are functionally different from MAC in many aspects of function and phenotype [10]. In comparison, CD14, a typical marker of MO and MAC, is expressed already on circulating MO and downregulated on DC. Since a strong upregulation of CD84/MAX.3 antigen was already observed after 1 day in culture, shorter culture periods were examined and an increased RNA expression could be detected in adherent MO after 8 h (Figure 5A). Surface antigen expression was examined in parallel experiments by flow cytometry and was increased after a 16 h period

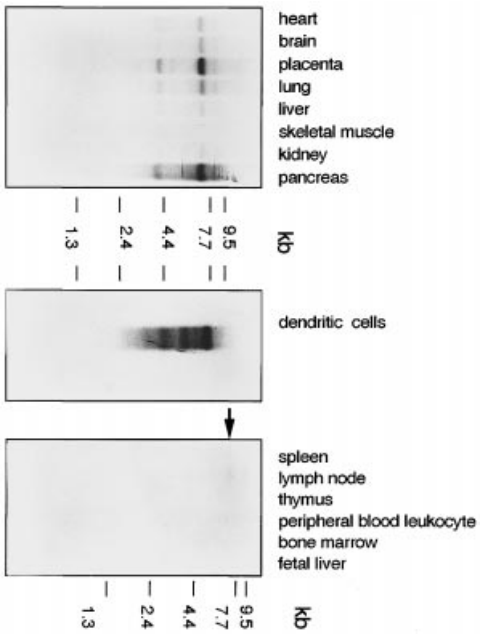

Figure 6 Expression of MAX.3/CD84 RNA in different whole tissue blots

Northern blots with poly(A)$^+$ RNA from a multitude of different tissues were tested for MAX.3/CD84 expression. Poly(A)$^+$ RNA from MO-derived DC was used as a control. Blots were exposed for 10 days (upper panel) or 1 day (middle and lower panels).

Table 1 Detection of MAX.3 immunoreactive cells in different tissues by immunohistochemistry

Organ	MAX.3 reactivity	MAX.3 compared to CD68
Stomach	Some interstitial cells positive, more cells positive close to cancer	(+)*
Duodenum	Some interstitial cells positive in between crypts	(+)
Colon	Some interstitial cells positive in between crypts	(+)
Liver	Kupffer cells staining positive, additional cells positive close to cancer metastasis	+
Breast	Some interstitial cells positive, very faint unspecific staining in fat cells	(+)
Skeletal muscle	Negative	−
Nerve	Very faint diffuse staining, probably unspecific	−
Lymph node	Very strong staining of parafollicular area (unspecific inflammation)	++
Spleen	Positive cells in germinal centre, weak staining of numerous cells in the red pulp	+ (weak)
Thyroid gland	Few interstitial cells positive	(+)

* MAX.3 positivity was semiquantitatively scored compared to CD68 reactivity, a broadly MAC-specific antigen. Positive staining with MAX.3 on more than 50% of CD68$^+$ cells is indicated by '++', 20–50% of CD68$^+$ cells positive for MAX.3 is indicated by '+', 5–20% of CD68$^+$ cells positive for MAX.3 is indicated by '(+)', and '−' indicates less than 5% CD68$^+$ MAC are MAX.3 positive.

of MO either cultured in teflon bags or non-adherently in roller bottles (Figure 5B).

CD84/MAX.3 antigen expression could not be detected in either granulocytes or freshly isolated lymphocytes from peripheral blood. Strong immunoreactivity, however, is seen in platelets. Accordingly, a strong RNA message could be detected in megakaryocyte-like cells cultured *in vitro* from human haematopoietic progenitor cells in the presence of thrombopoietin (Figure 5A). Additional Northern blots with mRNA of a wide array of different tissues gave no clearly detectable signal at a standard exposure time of 1 day (Figure 6 and several additional blots, not shown) with the exception of a very weak signal in spleen and lymph node RNA, although poly(A)$^+$ RNA was used for these experiments. In contrast, very strong signals were seen in MO-derived DC (Figure 6). At long exposure times of 10 days, bands could be detected in most organs (Figure 6).

Subsequently, different tissues were analysed by immunohistochemistry in order to determine the source of the weak signals in tissue RNA described above. In most tissues, small numbers of cells were staining positive using MAX.3 antibody, most probably interstitial MAC. On the contrary, parenchymal cells of epithelial origin were all staining negative (see Table 1). In control sections of the same organ specimens, reactivity of the MAC marker CD68 was analysed. In most tissues, the number of MAC reacting with CD68 was considerably higher than the number of MAC expressing CD84/MAX.3 antigen. Strong CD84/MAX.3 signals were detected in two examples of a leucocytic infiltrate surrounding cancer metastasis and in a lymph node with sinus histiocytosis due to unspecific inflammation. No clear reactivity of B cells in lymph nodes was found.

DISCUSSION

The antibody MAX.3 was originally described as a marker for mature MAC. Here, we show that the cell surface antigen detected by MAX.3 is identical to CD84 antigen. The biochemical characterization of the MAX.3/CD84 antigen revealed different apparent molecular masses on MAC and platelets. This difference disappeared after deglycosylation, indicating a similar protein core in both cell types. Protein purification and N-terminal amino acid sequencing allowed the identification of the corresponding cDNA clone HDPFF19 and revealed its identity to the CD84 cDNA sequence [17]. The assumption that this sequence is in fact coding for MAX.3 antigen is supported by two facts. Firstly, the observed pattern of RNA expression correlates with MAX.3 antibody immunoreactivity. Secondly, MAX.3 immunoreactivity was specifically induced on COS7 cells transiently transfected with HDPFF19. The specific expression of CD84 mRNA in MO-derived MAC further supports identity of the protein core of MAC and platelets, because the amino acid sequence leading to the cDNA was independently deduced from platelets.

In previous studies, MAX.3 was described as a marker of terminal MAC differentiation (e.g. [7,8,13–16]). Earlier results obtained by immunoperoxidase staining could be confirmed by flow cytometry and Northern analysis in the work presented here. Staining for CD84 as detected by MAX.3 is virtually absent from circulating blood cells except platelets. In some experiments, very weak expression on freshly isolated MO was observed and remained unchanged during the first few hours *ex vivo*. Upon adhesion on teflon foils and initiation of terminal MAC maturation, the antigen is upregulated with a strong expression after 1 day in culture. Interestingly, upon separation from the blood, CD84/MAX.3 antigen is upregulated in adherent cells as well as in roller bottles, although no overt adhesion stimulus is present in the latter situation. It can be speculated that gradient centrifugation over ficoll, transient cell adhesion during cell pelleting by centrifugation, or short cell interactions in culture may induce this process. Physical separation of MO from each other by surrounding red blood cells may be responsible for the suppression of MAX.3 expression on circulating MO as it was

described previously for the surface molecule intercellular cell-adhesion molecule [31].

MAX.3/CD84 expression differs from 'late' markers of MAC such as carboxypeptidase M or the secreted protein HCgp39 that are only expressed in parallel with the complete morphologic changes occurring during terminal MAC differentiation [18,23]. Furthermore, upregulation of CD84 transcript and protein on MO-derived cells is not confined to classical MAC, but is also observed on MO-derived DC. Accordingly, the CD84 cDNA clone HDPFF19 was found in a DC cDNA library. This indicates that CD84/MAX.3 antigen may be important for cellular interactions, as soon as MO leave in vasculature and develop into different types of mature effector cells. On cells of the megakaryocyte lineage, CD84/MAX.3 is expressed already in the bone marrow [7] and, accordingly, it is expressed on *in vitro* differentiating megakaryocyte-like cells.

In the 5th leucocyte antigen workshop, CD84 was described to be present on B-cells, MO, platelets and some cell lines of monocytic or B-cell origin. For many of the positive reactions, only a very weak signal could be demonstrated. A strong staining of MAC was mentioned, but not described in detail [32,33]. With antibody MAX.3 we only saw a very weak staining of circulating MO (completely negative in some experiments) and could not reproduce the weak staining of B lymphocytes. It is possible that other CD84 antibodies react with different epitopes and the epitope detected by MAX.3 may not be present or accessible on B cells. Alternatively, a crossreactive antigen with a slightly different protein core is expressed on B cells and is detected by other CD84 antibodies but not by MAX.3. Other CD84 specific antibodies could not be analysed side by side with MAX.3 in our laboratory, because none of them is commercially available.

Outside the haematopoietic lineages, MAX.3 expression is virtually absent [12]. Similarly to our experiments, in the work of de la Fuente et al. [17], only low levels of CD84 RNA were present in crude preparations of different organs. In our analysis of tissue samples by immunohistochemistry using a sensitive alkaline phosphatase detection method, we found some tissue MAC scattered between the parenchyma of most organs. However, MAX.3/CD84 antigen is selectively expressed only on a part of tissue MAC, since staining for CD68 detected more cells than staining with MAX.3. A strong MAX.3 reactivity was found in an unspecifically inflamed lymph node with sinus histiocytosis and in the leucocytic infiltrate surrounding tumour sites in pathological samples. Accordingly, MAX.3 was described earlier to be present in transplanted kidneys infiltrated due to a graft rejection [12], leading to the impression that only MAC when 'activated' to some degree will express this antigen. A systematical analysis of MAX.3/CD84 reactivity on tissue macrophages in a larger sample of normal and pathological histological specimen and on early haematopoietic cells is under way.

MAX.3/CD84 antigen is a heavily glycosylated protein, with a different glycosylation profile on platelets compared to MAC. The calculated molecular mass of the deglycosylated protein is lower than the apparent mass determined by SDS/PAGE. It is quite unlikely that this discrepancy is due to a sequencing error, introducing a 'false' stop codon, since additional stop codons are present in the following stretch of cDNA sequence in all three reading frames. Furthermore, an additional band of about 36 kDa can be seen in some experiments after deglycosylation with N-glycosidase F. Similar results were described by de la Fuente et al. [17]. We assume that secondary structure of the protein may hamper complete deglycosylation *in vitro* or some secondary structure distorting molecular mass estimation by SDS/PAGE is preserved despite denaturation.

The difference in apparent mass of the MAX.3 antigen on *in vitro* differentiated MAC and platelets suggests lineage restricted differences in glycosylation of the protein. A sequential deglycosylation method was employed for further analysis of glycosylation and revealed clear differences between MAX.3/CD84 antigen on platelets and MAC. *N*-Glycans can be classified into three types: firstly the high mannose type which contains only mannose and *N*-acetylglucosamine residues, the second is a complex type comprising of a common pentasaccharide structure, Manα1 $\rightarrow$ 6 (Manα1 $\rightarrow$ 3) Manβ1 $\rightarrow$ 4 GlcNAcβ1 $\rightarrow$ 4 GlcNAc, called the trimannosyl core with outer chains of high variability attached to the outer mannose residues of the core, usually containing sialic acid and α-L-fucosyl termini connected to β-D-galactosyl and joining *N*-acetylglucosamine residues. The third type, sharing features of both high mannose and complex type, is called hybrid type [34].

Sequential deglycosylation of antigen from both sources revealed terminal sialic acid as well as adjoining galactosyl residues, ruling out high mannose-type glycosylation [34]. This interpretation was supported by lectin binding studies, where platelet-derived MAX.3 antigen exhibited very high affinity for WGL. The low affinity of MAC-derived antigen for WGL despite its terminal sialic acid content might be due to fucosylation of the innermost *N*-acetyl-glucosyl residue which would efficiently block binding to WGL [35]. The MAC-derived antigen also did not bind to ConA indicating the lack of terminal α-mannosyl residues (hybrid type glycosylation) as well as absence of biantennary complex glycosylation [36]. Taken together these findings point to multiantennary complex glycosylation of the MAX.3 antigen on MAC and hybrid glycosylation on platelets.

Differential glycosylation patterns of a single gene product have been reported earlier, but only in a few instances could this differential glycosylation be linked to biological functions. An interesting example is the carbohydrate part of the CD2 surface molecule on a T cell line leading to MO activation, whereas the same effect is not induced by differentially glycosylated CD2 on normal T lymphocytes [37]. The differences in glycosylation of the CD84/MAX.3 antigen on platelets versus MAC may therefore be associated with distinct cell interactions, for example due to differential binding to selectins or other cell surface molecules.

MAX.3/CD84 is a member of the immunoglobulin superfamily of surface molecules and the strongest homology exists to Ly-9 and other members of the CD2 subfamily [17]. The function of Ly-9 is currently unknown, but other members of this family are involved in cell–cell interaction and cell activation. CD2 binds to CD58 (LFA-3) in humans and CD48 in mice, both of these molecules also belonging to the CD2 family. It was also suggested that ligand binding is involved in T-lymphocyte antigen recognition [38]. Assuming a role in cell–cell interactions, the identification of a natural ligand (or ligands) for MAX.3/CD84 would allow the investigation of a conceivable involvement in intracellular signalling events in myelomonocytic cells and platelets.

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