Polymerase Chain Reaction Selects a Novel Disintegrin Proteinase from CD40-Activated Germinal Center Dendritic Cells

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Summary

To identify genes expressed by a specific subset of dendritic cells found in vivo a polymerase chain reaction–based cDNA subtraction technique was applied to the recently described germinal center dendritic cells. A novel member of the disintegrin metalloproteinase family was cloned which comprises a not typical zinc-chelating catalytic site most similar to a bacterial metalloproteinase. Dendritic cell precursors or immature dendritic cells express no or low levels of the message. It is induced to high levels upon spontaneous or CD40-dependent maturation and in a mixed lymphocyte reaction. In situ hybridization showed distinct expression of this gene in the germinal center. This, together with the findings that certain disintegrin metalloproteinases regulate the activity of tumor necrosis factor α and that metalloproteinases have also been implicated in FasL processing, suggest that this novel molecule may play an important role in dendritic cell function and their interactions with germinal center T cells.

Dendritic cells (DCs) represent a heterogeneous population of hematopoietic-derived cells that display potent capacity to prime naive T cells and to stimulate memory T cells. DC can be found in an immature form, characterized by a high capacity for antigen capture and processing and a low ability for T cell stimulation (1).

Two populations of immature DC have been described: (a) Langerhans cells within the epidermis of skin and mucosa, (b) marginal DCs within the spleen, and (c) CD4+CD11c+ DCs in blood (human). Upon antigen capture, both Langerhans cells and marginal zone DCs migrate into the T cell areas of regional lymph nodes (through lymph) or of spleen (crossing marginal zone sinuses) where they become interdigitating cells expressing high levels of MHC class II and costimulatory molecules and strongly stimulate antigen-specific naive T cells (2, 3). Blood CD4+CD11c+ DCs may play an important role in capture and transport of blood antigens into the secondary lymphoid tissues. Recently, CD4+CD11c+ DCs (GCDCs) have been identified in tonsillar germinal centers (4), suggesting that blood DCs may penetrate into B cell follicles after crossing the high endothelial venules. Purified GCDCs express low levels of CD40, MHC II, and CTLA-4 ligands, but upregulate these molecules after spontaneous maturation. They induce strong stimulation of CD4+ T cells in vitro and are likely to play a pivotal role in germinal center reactions.

The molecular mechanisms that regulate generation, migration, maturation, or function of different DC subsets in vivo are poorly understood. DCs are hard to isolate and techniques such as producing DC-specific monoclonal antibodies and cloning DC-specific genes by cDNA library subtraction have only been applied to large numbers of DCs generated in vitro from hematopoietic progenitor cells or blood monocytes. Here we describe the application of a PCR-based subtraction technique to three million CD40-activated CD4+CD11c+ GCDCs isolated from human tonsils with the cloning of a novel member of the disintegrin metalloproteinase family. The gene is strongly expressed in mature DC and in situ can be localized to germinal centers. This novel proteinase may be involved in germinal center reactions, for example, by regulating activities of TNF family members.

Materials and Methods

Cell Preparations. GCDCs were purified from human tonsils according to Grouard et al. (4). After collagenase IV and DNAse digestion of tonsils, cells were centrifuged through a 50% Percoll gradient for 20 min at 400 g. CD3+ T cells, CD19+ B cells, CD14+ monocytes (anti-CD3eOKT3), CD19 [4G7], and CD14 [MOP9] mAbs were purified from ascites (our laboratory), and CD16+CD56+ NK cells (ION16; Immunotech, Marseille, France;
and NK1; Ortho Diagnostic System, Raritan, NJ) were removed from the collected low density cell population by magnetic beads (anti-mouse Ig-coated Dynabeads M 450; Dylina, Oslo, Norway). The remaining cells were stained with mouse anti-CD4-PE-Cy5 (Immunotech), anti-CD11c-PE (Becton Dickinson, Mountain View, CA), anti-CD3-FITC and CD34-FITC (Immunotech), anti-CD20-PE-FITC and anti-CD16-FITC (Becton Dickinson) anti-CD1a-FITC (Ortho Diagnostic System). CD4+CD11c+CD3-CD20-CD1a- GCDCs were isolated by cell sorting using FACStar Plus® (Becton Dickinson). In average, a tonsil pair yielded 3 × 10^6 cells of 98% homogeneity, a total of 12 tonsil pairs was needed to collect 3 × 10^6 cells.

Blood CD4+CD11c+ DCs were prepared from PBMCs essentially following the procedure for GCDCs. After cell sorting, the purity was >95%. GCDCs and blood DCs were prepared for 24 h in complete medium in the presence of 10 μg/ml anti-CD40 antibody G2B4 (provided by Dr. E. Clark). Complete medium is RPMI 1640 (GIBCO BR L, Gaithersburg, MD) supplemented with 10% (vol/vol) FCS (Flow Laboratories, Irvine, UK), 10 mM Heps, 2 mM l-glutamine, 5 × 10^-2 M 2-mercaptoethanol, and 0.08 μg/ml gentamycine (Scheringer-Plough, Levalois Perret, France).

Stem cell-derived DCs were obtained after 6 and 12 d of culture of CD34 progenitor cells in the presence of TNF-α and GM-CSF (5, 6). Monocyte-derived DCs were produced by incubating purified human monocytes for 5 d in the presence of GM-CSF and IL-4 (7). For CD40 activation, the in vitro–generated DCs were cultured under irradiated murine CD40L–transfected L cells established in this laboratory (8).

Blood mononuclear cells were obtained from human peripheral blood by Ficoll-Hypaque centrifugation. Cells were collected at the interphase and washed twice in complete medium.

T lymphocytes were purified from PBMCs by immunomagnetic depletion using a cocktail of mAbs (IOM2 [CD14], ION16 [CD16], IOT17 [CD35], and ION2 [HLA-DR]) from ImmunoTech (Biocytomation, Uppsala, Sweden), and 0.5 units AmpliTaq® DNA polymerase (Boehringer Mannheim GmbH, Mannheim, Germany) in the presence of R N Ase inhibitor (R N Ase; Promega, Madison, WI) in standard reaction conditions. After phenol/chloroform extraction, the RNA was reverse transcribed using SuperScript™ II (GIBCO BRL, Gaithersburg, MD) and a dT<sub>12,18</sub> oligonucleotide, according to instructions (GIBCO BRL). The reaction was stopped, and nucleic acids were ethanol precipitated and resuspended in water. About 20 ng cDNA was used in one PCR reaction. For all other cells, total RNA was prepared from ~100,000 cells, reverse transcribed using Superscript™ II (GIBCO BRL) and a random hexaoligonucleotide (pd[N]<sub>6</sub> Phosphoramidite; Pharmacia) in a 20 μl reaction. 1 μl was used in a PCR reaction. The 50 μl PCR reaction contained 100 ng primers, 200 μM dNTP (Pharmacia, Uppsala, Sweden), and 0.5 units AmpliTaq® DNA polymerase (Roche Molecular Systems Inc., Branchburg, N J ) in standard PCR buffer (Roche Molecular Systems) and was subjected to 28 or 35 cycles of denaturing (30 s, 94°C), annealing (30 s, 60°C), and extension (2 min, 72°C) on the Perkin-Elmer thermal cycler 480. Decysprimers U 137 and L 677 are shown in Fig. 3A. β actin primers were purchased (Stratagene, La Jolla, CA). The Marathon™ kit (Clontech) was used for RACE PCR and performed as recommended by the supplier. The outward primers were 5'-CCATCCAGACAGATTTTCCATACCTACC (upstream) and 5'-CCATCTTCGTTGCTGTATGGCGT (downstream), and were used with the Marathon™ recommended cycling program 1. The two distinct PCR products were cloned into the T/A-vector and sequenced.

Northern and In Situ H ybridization. Poly A+ RNA blots of human tissues were purchased (Clontech). The original cloned 744-bp fragment was labeled by random priming with [32P]dCTP (3,000 Ci/mmol; Amersham Intl., Buckinghamshire, U K ; and HPrime; Boehringer Mannheim GmbH) and uncropped nucleotides were removed by spin column chromatography (Chromaspin-100; Clontech). Membranes were blocked with G block (Renaissance; Dupont) and hybridized at 65°C in Church solution (0.5 M NaHPO<sub>4</sub> pH 7.2, 7% SDS, 0.5 mM EDTA), heat-denatured probe was added, and incubated overnight at 65°C. The membranes were washed under high stringency conditions (0.1 × SSC/0.1% SDS at 65°C) and exposed for 2 wk.
In situ hybridization was done as described (12). Sense and antisense $^{35}$S probes were made by runoff transcription of the 744-bp fragment. 6-μm sections of tonsils were fixed in acetone and 4% paraformaldehyde followed by 0.1 M triethanol amine/0.25% acetic acid. The sections were hybridized overnight, RNase A treated, and exposed for 3 wk. After development, the cells were stained with hematoxyline.

**Results**

Construction of a Subtracted cDNA Library from Three Million CD40-activated GCDCs. Germinal center DCs were purified from human tonsils (4) and activated by anti-CD40 antibody G28-5 in complete medium for 24 h. From 12 tonsil pairs, a total of three million GCDCs could be obtained, which were estimated to be 98% pure but represented too little material for a conventional subtracted cDNA library without PCR amplification. By modification of the subtractive hybridization technique, termed PCR-select (11), the amount of tester cDNA necessary could be lowered to the 140 ng GCDC mRNA obtained. GCDC mRNA (tester) was cut with RsaI, adapters ligated, and after hybridization in the presence of competitor (driver) cDNA from human monocytic cell line U937, amplified. Thus, the resulting PCR products (Fig. 1A, lane 8) are restriction fragments of GCDC cDNA absent, or at least rare, in U937. Indeed, individual bands can be seen in lane 8 that are more clearly resolved in B. This is obviously different from amplified GCDC cDNA in absence of competitor (lane 6). As a positive control, subtracting skeletal muscle cDNA containing a trace amount of molecular weight marker against skeletal muscle cDNA only, was able to distinctly expose the added marker DNA (compare lanes 2 and 4). Given these results, GCDC cDNA fragments from panel B in the 0.7–1.4-kb size range were cloned, and 250 clones were sequenced. 30% of the clones contained human homologue of DEC205 1 45

| Gene | No. of clones of 250 total | Reference |
|------|---------------------------|-----------|
| MHC II | 66                        | 44        |
| CD83 | 10                        | 44        |
| DC tactin | 14                        | 45        |
| human homologue of DEC205 | 1 | 45 |
| Rel B | 6                          | 46        |
| IAP-c | 10                         | 47        |

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**Figure 2.** Restricted expression profile of decysin by RT-PCR. (A) Decysin is not detected in PMA/ionomycin-activated human cell lines TF1 (myeloid precursor cell), JURKAT (T cell), CHA, MRC5 (kidney epithelial and lung fibroblastic cells), and JY (B cell), but is expressed in PMA/ionomycin-activated stem cell-derived DCs. PCR with specific primers to decysin (Fig. 3A) and β actin was performed on reverse transcribed RNA from the cell lines, stem cell-derived DCs harvested at days 6 and 12 of cell culture (6) as well as GCDC library subtracted (Fig. 1, lane 7) and nonsubtracted (Fig. 1, lane 5). In the subtracted library, β actin could not be amplified. (B) A low level of cDNA is seen in PBMCs, monocytes and B cells. GCDCs were activated for 24 h by αCD40 antibody G28-5 in complete medium. Peripheral blood mononuclear cells, monocytes, and T lymphocytes were obtained from human peripheral blood, and B lymphocytes from human tonsils.
Among the unknown genes, we retained a clone containing a 744-bp insert because it was not expressed in U937, nor in the following tested human cell lines: TF1 (myeloid precursor cell), JURKAT (T cell), CHA, MRC5 (kidney epithelial and lung fibroblastic cells), and JY (B cell) (Fig. 2A). Yet, it is expressed in CD34 progenitor cell-generated DCs. As control, the cDNA is present in the nonsubtracted GCDC library, and enriched after subtraction. Among freshly isolated cells (B), the cDNA could be detected in PBMCs, monocytes, and B cells, but at a lower level compared to CD40-activated GCDCs where 28 PCR cycles were sufficient to generate a clearly visible product. Expression was never observed in T cells. As enough mRNA could not be obtained from GCDC, the expression of this gene in CD34 progenitor cell-generated DCs allowed us to clone the full-length cDNA.

**A Novel Member of the Disintegrin Metalloproteinases.** Several RACE PCR products were sequenced to determine the full-length 2,187-bp nucleotide sequence of the gene which we have named decysin (Fig. 3A). It encodes an open reading frame of 470 amino acids with strong homology to disintegrin metalloproteinases. It has two putative start codons, although the latter is likely to be used more regularly since it more closely resembles a Kozak consensus (13). It is followed by a hydrophobic signal sequence. As a member of the metalloproteinase family, decysin contains a prodomain with a cysteine-switch activation domain at cysteine 187 (14) and a furin cleavage site between residues 200 and 203 (15). Two zinc-chelating histidine (H) residues of the zinc binding pocket (B) are found at positions 352 and 356 as well as the methionine turn at residue 176 (16, 17). The glutamic acid after the first histidine is strictly conserved in active enzymes. However, distinct from all known mammalian disintegrin or matrix metalloproteinases (18, 19), the third zinc-chelating histidine is replaced by an aspartic acid (residue 362). In the bacterial metalloproteinase ScnP (20), an aspartic acid found at exactly this position was recently identified as the third zinc-chelating amino acid (residue 362). In the bacterial metalloproteinase ScnP (20), an aspartic acid found at exactly this position was recently identified as the third zinc-chelating amino acid (21; B). All mammalian disinte...
Grin metalloproteinases share an ~90 amino acid stretch with snake venom disintegrins (22; C). Decysin comprises many of these conserved residues, but its open reading frame terminates half way along the consensus. It lacks a transmembrane region that together with the signal peptide, suggests that it is secreted. In summary, by a number of common criteria, the gene codes for a novel member of the disintegrin metalloproteinases with unique features so far unobserved in any other mammalian metalloproteinases.

Decysin is Induced or Upregulated During DC Maturation.

Since decysin was identified in CD40-stimulated GCDCs, we wondered whether the metalloproteinase might be expressed at high levels in CD40-activated DCs. Indeed, 28 cycle PCR coupled to reverse transcribed RNA (RT-PCR) on freshly isolated GCDCs failed to detect decysin (Fig. 4A). Its expression is induced by spontaneous maturation in culture (4) and increases in response to CD40 activation. Similarly, in another ex vivo isolate, blood CD11c+ DCs do not contain detectable decysin mRNA, but maturation in culture (23) and more importantly CD40 activation result in decysin induction. In vitro generated DCs from CD34 progenitor cells or monocytes rapidly synthesize the message in response to CD40 ligation and a mixed lymphocyte reaction (B) results in decysin expression together with CD83. Thus, the novel metalloproteinase represents a DC maturation marker synthesised in response to T cell signals.

Decysin is Strongly Expressed in Tissues of Chronic Antigen Stimulation.

By Northern blot analysis on different human tissues (Fig. 5), decysin is expressed as a single 2.4-kb message and is highly abundant in the small intestine and appendix. Database searches produced a single partial expressed sequencing tag from pig small intestine (not shown). Expression is also seen in lymph node, mucosal lining of the colon, thymus, spleen, and very weakly in bone marrow. Peripheral blood, ovary, testis, prostate, and fetal liver are negative, as well as other blots containing tissues such as heart, lung, or liver (data not shown).

In Situ Hybridization Detects Decysin Message in Germinal Centers.

To localize decysin mRNA in human lymph nodes in situ hybridization was performed (Fig. 6). A tonsil section probed with the antisense RNA strand shows distinct hybridization signals primarily within germinal centers (A and C) in contrast to the same follicles probed with the sense strand (B). The follicle marked by an arrow is shown in higher magnification (C). Silver grains are in focalized clusters evenly distributed with the germinal center. This profile is identical to that obtained by anti-CD11c immunostaining of GCDCs (compare C and D), and confirms that DCs of the germinal center express high levels of decysin.

Discussion

The study of DCs is hampered by their scarcity in vivo. In this paper, a PCR-based method was used to clone a cell type-specific cDNA from three million CD40-activated germinal center dendritic cells isolated from human tonsils.
Several lines of evidence indicate the power of this technique. (a) Among the 250 sequenced clones, <5% contained common housekeeping genes (β-actin could not be amplified from subtracted GCDC cDNA after 35 cycles of PCR [Fig. 2 A]). (b) A third were unknown genes. (c) 107 clones represented genes whose expression is either specific to or highly expressed by mature DCs (CD83, DC-tactin, DEC205, MHC class II, Rel-B, IAP-c). On average, we determined that 1 out of 10 clones corresponds to a gene expressed in tester GCDCs and not in driver U937. All these data suggest that the PCR-based subtraction method used here is well applicable to clone unique genes from low number of ex vivo cells, including different DC subsets.

In this subtracted library we identified a novel member of the disintegrin metalloproteinases. It is a large family of mostly membrane-anchored proteases with conserved disintegrin and cysteine-rich domains and a zinc-chelating pocket, although not all members are active enzymes (19). They encode an adhesive function to cell-surface proteins through the COOH-terminal disintegrin domain and a potential antiadhesive/cleavage function through the zinc-dependent metalloprotease domain. Members of this group encode diverse functions. Fertilin α and β have been implicated in sperm-egg binding and fusion (24), meltrin in muscle cell fusion (25), and Kuzbanian in neurogenesis (26). Snake venom disintegrins bind the β3α1 integrin and prevent its interaction with fibrinogen (27). By the use of specific inhibitors, metalloproteases have been implicated in shedding of a number of molecules which play critical roles in the immune system: TGF-α, TNF receptors p60 and p80, FasL, CD30, IL-6 receptor, and L-selectin (28-33). Recently, the enzymes that process TNF-α have been identified as disintegrin metalloproteinases TACE (34, 35) and ADAM-10 (36).

Decysin is a member of the disintegrin metalloproteinase family by the following criteria: it has a hydrophobic leader followed by a prodomain with a cysteine-switch consensus, a mechanism by which a prodomain cysteine ligates the active site zinc, and retains the zymogen in an inactive state. Decysin comprises a zinc-chelating catalytic site with a methionine turn and most of the disintegrin domain. Yet, it is unique in three points. First, the third zinc-chelating residue, a histidine in all other disintegrin and matrix metalloproteinases, is replaced by aspartic acid in analogy to a bac-

Figure 6. Localization of decysin mRNA to germinal centers by in situ hybridization. Human tonsil sections are hybridized with antisense (A, C) and sense (B) decysin 35S-labeled RNA probes. (A) and (B) are from serial sections. Original magnification: 40. The germinal center marked by an arrow original magnification: 100 (C). The sense probe generates diffuse background hybridization, whereas with the antisense probe, dense clusters of silver grains are seen mainly localized to the follicles. D is from reference 4, and shows immunohistological staining of CD4+CD11c+ GCDCs with anti-CD11c (red). In blue are proliferating cells stained by anti-Ki67. Original magnification: 100.
terial proteinase. Second, the disintegrin domain is truncated. Third, decysin lacks a transmembrane domain, and is therefore potentially secreted. It will be important to examine whether this gene might represent the first member of a new subclass of mammalian disintegrin metalloproteinases.

RT-PCR distribution analysis showed that decysin is moderately expressed by normal B cells and monocytes, but not by T cells and a wide range of human cell lines, even though they had been treated with PMA/ionomycin which is known to promote processing of \( \text{TGF-}\alpha, \text{TNF-}\alpha, \text{TNFR}, \text{IL-6R}, \) and L-selectin by metalloproteinases (28–30). Instead, decysin is induced or upregulated after spontaneous or CD40 ligand–promoted maturation of different types of immature DCs in vitro. These include blood CD4\(^+\)CD11c\(^+\) DCs, tonsillar CD4\(^+\)CD11c\(^+\) GCDCs, monocyte-derived DCs with GM-CSF, IL-4, and CD34\(^+\) progenitor-derived DCs with GM-CSF and TNF. In addition, a reaction with allogeneic T cells induces decysin synthesis in stem cell–derived DCs. CD40 ligand failed to upregulate decysin expression of human B cells (data not shown). In situ hybridization confirmed the expression of decysin in germinal centers in a pattern identical to CD11c staining of GCDCs. These data show that decysin is selectively expressed by DC and upregulated by signals of activated T cells. The cloning of decysin together with genes of mature DC markers CD83, DEC205, and DC tactin from CD40-activated GCDCs further support the idea of decysin as a novel DC maturation maker.

The impressive expression of decysin in the small intestine and appendix is likely due to the high abundance of DCs in gut epithelium, the lamina propria, and the Peyer’s patches which comprise a large population of lymphoid cells (37). The continuous and high antigenic load in these sites may induce chronic DC–T cell interactions. Since the members of disintegrin metalloproteinase such as TACE and ADAM-10 have been shown to process membrane-bond TNF-\( \alpha \)-precursors, and metalloproteinases have been implicated in processing of other members of the TNF family (Fas-L, CD30, and TNFR), decysin may represent a key molecule in regulating DC–T cell interaction.

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Note added in proof. DC tactin is identical to macrophage-derived chemokine recently published (Godiska et al., J. Exp. Med. 185:1595–1604).

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