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Identification and Immunocytochemical Analysis of DCNP1, a Dendritic Cell-Associated Nuclear Protein

Masako Masuda,*,† Satoru Senju,* Shin-ichiro Fujii,‡ Yasuhiro Terasaki,§ Motohiro Takeya,§ Shin-ichi Hashimoto,¶ Kouji Matsushima,¶ Eiji Yumoto,† and Yasuharu Nishimura*,[1]

**Division of Immunogenetics, Department of Neuroscience and Immunology, Kumamoto University Graduate School of Medical Sciences, Kumamoto 860-0811, Japan; †Department of Otolaryngology—Head and Neck Surgery, Kumamoto University School of Medicine, Kumamoto 860-8556, Japan; ‡Institute for Clinical Research, Kumamoto National Hospital, Kumamoto 860-0008, Japan; §Second Department of Pathology, Kumamoto University School of Medicine, Kumamoto 860-8556, Japan; and ¶Department of Molecular Preventive Medicine, School of Medicine, University of Tokyo, Tokyo 113-0033, Japan*

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Dendritic cells (DCs) are potent antigen-presenting cells (APCs). Among so-called professional APCs, only DCs can activate naive T cells to initiate immune response. To better understand molecular mechanisms underlying unique functions of DCs, we searched for genes specifically expressed in human DCs, using PCR-based cDNA subtraction in conjunction with differential screening. cDNAs generated from $CD34^+$ stem cell-derived $CD1a^+$ DC were subtracted with cDNA from monocytes and used for generation of a cDNA library. The cDNA library was differentially screened to select genes expressed in DCs more abundantly than in monocytes. We identified a gene encoding a protein composed of 244 amino acids, which we designated as DCNP1 (dendritic cell nuclear protein 1). In Northern blot analysis, DCNP1 mRNA was highly expressed in mature DCs and at a lower level in immature DCs. In contrast, monocytes and B cells do not express the gene. In multiple human tissue Northern blot analysis, expression of DCNP1 was detected in brain and skeletal muscle. To examine subcellular localization of DCNP1, we performed immunofluorescence analysis using an anti-DCNP1 polyclonal antibody and found the molecule to be localized mainly in the perinucleus. In an immunohistochemical analysis, we compared the expression of DCNP1 with CD68, a marker for DCs and macrophages, in spleen, lymph node, liver, and brain. While DCNP1-positive cells showed a similar tissue distribution to CD68-positive cells, the number of DCNP1-positive cells was much smaller than that of CD68-positive cells. Our findings are consistent with the proposal that DCNP1 is specifically expressed in DCs.

Key Words: **dendritic cell; DCNP1 (dendritic cell nuclear protein 1); PCR-based cDNA subtraction; nuclear protein.**

Dendritic cells (DCs) are widely distributed throughout the body, and these are the most potent antigen-presenting cells (APC) which are particularly involved in initiation of antigen-specific immune responses (1). Immature DCs have a specialized antigen uptake and processing machinery, whereas mature DCs efficiently present antigens to T cells. The unique properties of DCs to induce and sustain primary immune responses make them optimal means of anti-cancer immunotherapy (2). DCs loaded with appropriate tumor-associated antigens can induce protective and therapeutic-immune responses in animal models (3–5) and promising data on humans have been reported (6–8). Recent studies suggest that DCs also play critical roles in the maintenance of immunological self tolerance (9, 10). Prevention of allograft rejections and treatment of autoimmune diseases with DCs has been reported. Allogenic DCs genetically engineered to express Fas ligand prolonged the survival of allografts (11). Adoptive transfer of DCs stimulated *in vitro* with interferon-gamma inhibits the development of diabetes in NOD mice (12) and treatment using DCs with forced expression of IL-4 was effective for collagen-induced arthritis (13). These therapies were not accompanied by any nonspecific immuno-suppression.

Abbreviations used: DC, dendritic cell; DCNP1, dendritic cell nuclear protein 1; APC, antigen-presenting cell; PCR, polymerase chain reaction; GM-CSF, granulocyte–macrophage colony-stimulating factor; TNF-α, tumor necrosis factor-α; IL-4, interleukin-4; PBMC, peripheral blood mononuclear cell; EST, expressed sequence tag; mAb, monoclonal antibody.

[1] To whom correspondence and reprint requests should be addressed at Division of Immunogenetics, Department of Neuroscience and Immunology, Kumamoto University Graduate School of Medical Sciences, Honjo 2-2-1, Kumamoto 860-0811, Japan. Fax: +81-96-373-5314. E-mail: mxnishim@gpo.kumamoto-u.ac.jp.

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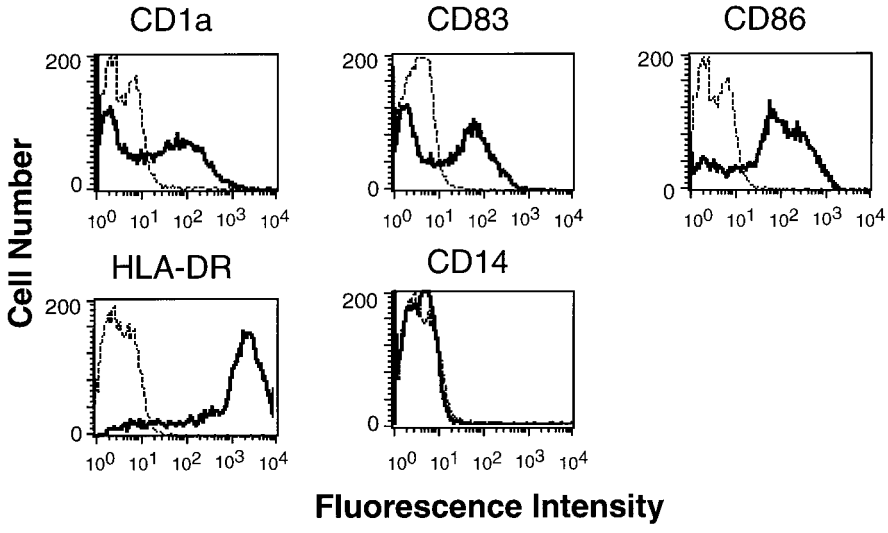

FIG. 1. Surface phenotype of $CD34^+$ hematopoietic cell-derived DCs. $CD34^+$ cells were purified from human cord blood and cultured with GM-CSF and TNF-α. At days 11–14, cells were harvested and examined for expression of surface markers, using flow cytometry. Thick lines represent staining with specific antibodies, and the dotted lines represent staining with the isotype-matched controls. $CD1a^+$ DCs, purified from cultures using microbeads, were used for cDNA subtraction.

Although DC-based immunotherapies for cancers and autoimmune diseases have produced certain outcomes, much remained to be determined. Investigators attempted to modify the function of DCs by genetic engineering. From this point of view, identification of genes expressed in DCs may provide information useful not only for further understanding of biological function of DCs, but also for more effective DC-based immunotherapy. Efforts have been made to analyze the repertoire of genes expressed by human DCs (14, 15).

In the current study, we carried out a PCR-based cDNA subtraction combined with subsequent differential screening in order to isolate genes specifically expressed in human hematopoietic stem cell-derived DCs but not in monocytes. We identified a gene, DCNP1 (dendritic cell nuclear protein 1) which is expressed in mature and immature DCs but not in monocytes and B cells. We generated an anti-DCNP1 polyclonal antibody and used it to analyze subcellular and cellular localizations of the molecule.

MATERIALS AND METHODS

Cell preparations. Human umbilical cord blood and peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers. Human DCs used for cDNA subtraction were generated from umbilical cord blood by the method established by Caux *et al.* (16). In brief, $CD34^+$ hematopoietic progenitors were isolated from umbilical cord blood using $CD34^+$ Progenitor Cell Selection System (Dynal, Oslo, Norway). $CD34^+$ cells were cultured in the presence of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF, 100 ng/ml, from Kirin Brewery Co., Ltd., Japan) and tumor necrosis factor-α (TNF-α, 2.5 ng/ml, from Asahi Chemical Industry Co., Ltd., Japan) in RPMI 1640 medium containing 10% fetal calf serum (FCS). On days 11–14 of culture, $CD1a^+$ DCs were isolated from the culture using M-450 rat anti-mouse IgG1 (Dynal) Dynabeads and mouse anti-human CD1a monoclonal antibody (mAb) (Pharmingen). For Northern blots and immunocytochemical analysis, monocytes were purified from peripheral blood mononuclear cells (PBMCs), using M-450 CD14 Dynabeads or CD14 microbeads (Miltenyl Biotec). Immature DCs were prepared by culturing monocytes for 5 days in the presence of GM-CSF (100 ng/ml) and interleukin-4 (IL-4, 100 U/ml, from Ono Pharmaceutical Co., Ltd., Japan). For maturation, immature DCs were treated for 2 days with TNF-α (20 ng/ml). B cells were isolated from PBMCs, using Dynabeads M-450 CD19 (Dynal).

Flow cytometric analysis. Cells were washed with phosphate-buffered saline (PBS) containing 2% FCS and incubated for 30 min on ice with each test mAb diluted to the optimal concentration. Staining was done using the following mouse mAbs: phycoerythrin (PE)-CD1a and PE-CD83 were purchased from Immunotech, and fluoresceinisothiocyanate (FITC)-CD14, FITC-CD86, FITC-HLA-DR, FITC-mouse IgG1 control, PE-mouse IgG1 control, FITC-mouse IgG2a control, and PE-mouse IgG2b control were purchased from Pharmingen. Stained-cells were washed in PBS containing 2% FCS and analyzed using a flow cytometer (FACScan, Becton–Dickinson). Fluorescence signals from cells stained with specific antibodies were compared with those incubated with appropriate isotype-matched controls.

PCR-based cDNA subtraction and differential hybridization. Total cellular RNA was extracted from human DCs and monocytes using TRIzol reagent (Gibco BRL), and poly$(A)^+$ RNA were purified from total RNA using oligo(dT)–cellulose columns (mRNA purification kit, Amersham). cDNA synthesis and subtraction were done using PCR-Select cDNA Subtraction Kits (Clontech) following manufacturer's instruction but with some modification. mRNA of DCs was used as a tester, and mRNA of monocytes was used as a driver. Subtracted PCR products were cloned into a plasmid vector (pGEM-T Easy Vector, Promega) to obtain a subtraction library. DNA fragments were prepared from individual cDNA clones by PCR and spotted onto nylon membranes (Hybond N^+, Amersham). To prepare [^{32}P]dCTP-labeled hybridization probes, total cellular RNA from DCs and monocytes were used as templates to synthesize first-strand cDNA with oligo(dT) primers. Probes were purified by gel filtration chromatography on Sephadex G50 columns, and hy-

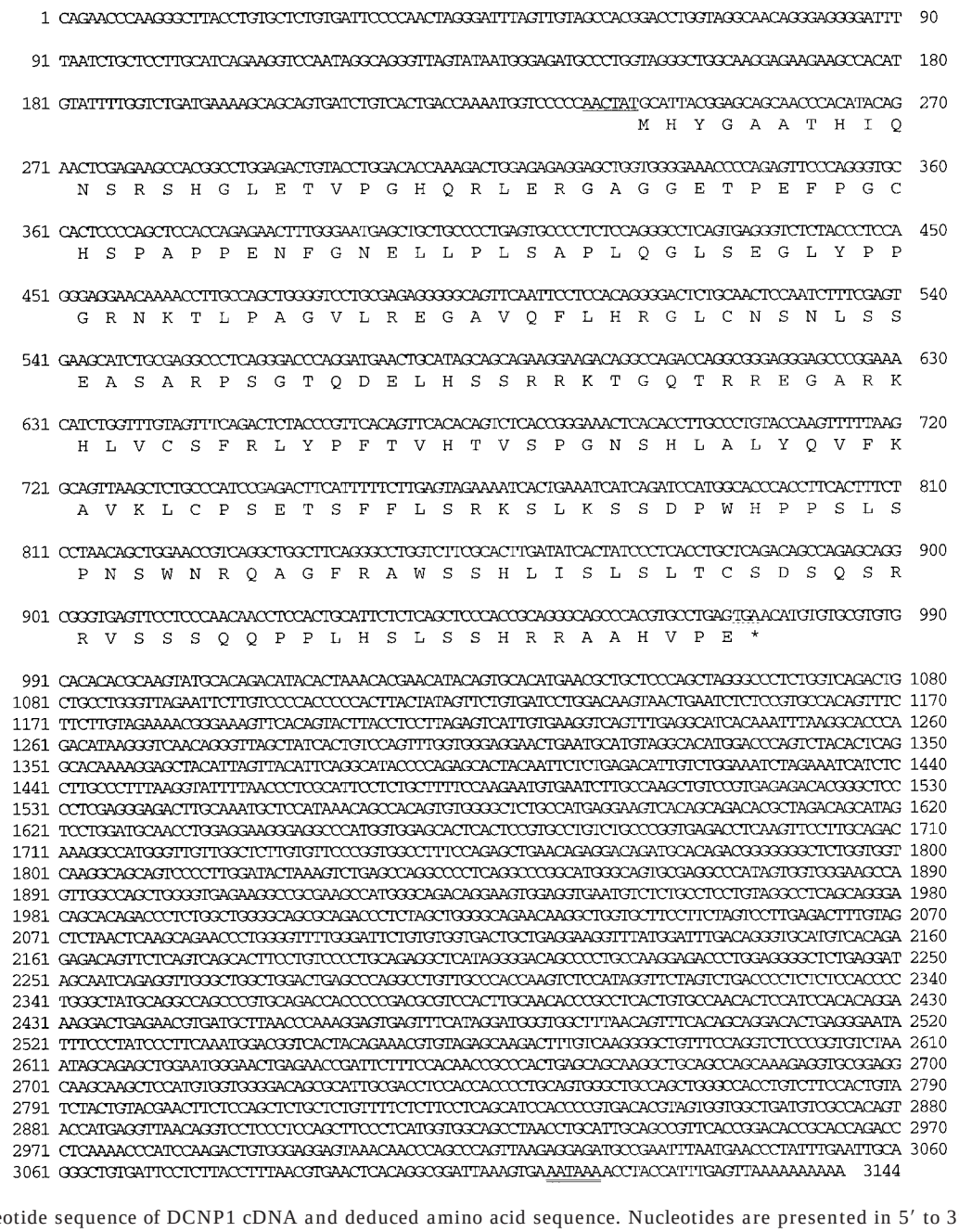

FIG. 2. Nucleotide sequence of DCNP1 cDNA and deduced amino acid sequence. Nucleotides are presented in 5′ to 3′ orientation. The amino acid sequence is displayed using the single-letter code. Kozak sequence adjacent to initiation codon is underlined. The stop codon is indicated by a dotted line. The poly(A) signal AATAAA is double underlined. The nucleotide sequence has been deposited to DDBJ (Accession No. AB074498).

bridization was done in MicroHyb solution (Research Genetics) at 42°C overnight. Hybridization signals were detected autoradiographically, and the intensity of signals for DC and monocyte probes was compared. DNA sequencing reaction was done using BigDye Terminator Cycle Sequencing FS kits (Perkin–Elmer Applied Biosystems), and sequencing samples were analyzed on an ABI PRISM 310 DNA sequencer (Perkin–Elmer Applied Biosystems).

Screening of an immature DC cDNA library. A cDNA library of human monocyte-derived immature DC was constructed using ZAP Express cDNA synthesis kits (Stratagene) following the manufacturer's instruction, then were screened by plaque hybridization under high-stringency conditions in accordance with standard procedures. cDNA clones in phagemid vector, pBK-CMV (Stratagene) were excised from phage clones, using ExAssist helper phage (Stratagene), and subsequently subjected to DNA sequence analysis.

Northern blot analysis. Total cellular RNA was extracted from immature and mature DCs, monocytes, and B cells prepared as mentioned above. RNA was electrophoresed in a 1.0% agarose gel containing formaldehyde and transferred onto nylon membrane (Hybond N^+, Amersham). Poly(A)$^+$ RNA blots of human tissues (Human 12-Lane MTN Blot, Clontech) was also used. Membranes were hybridized with DNA probes labeled with [^{32}P]dCTP.

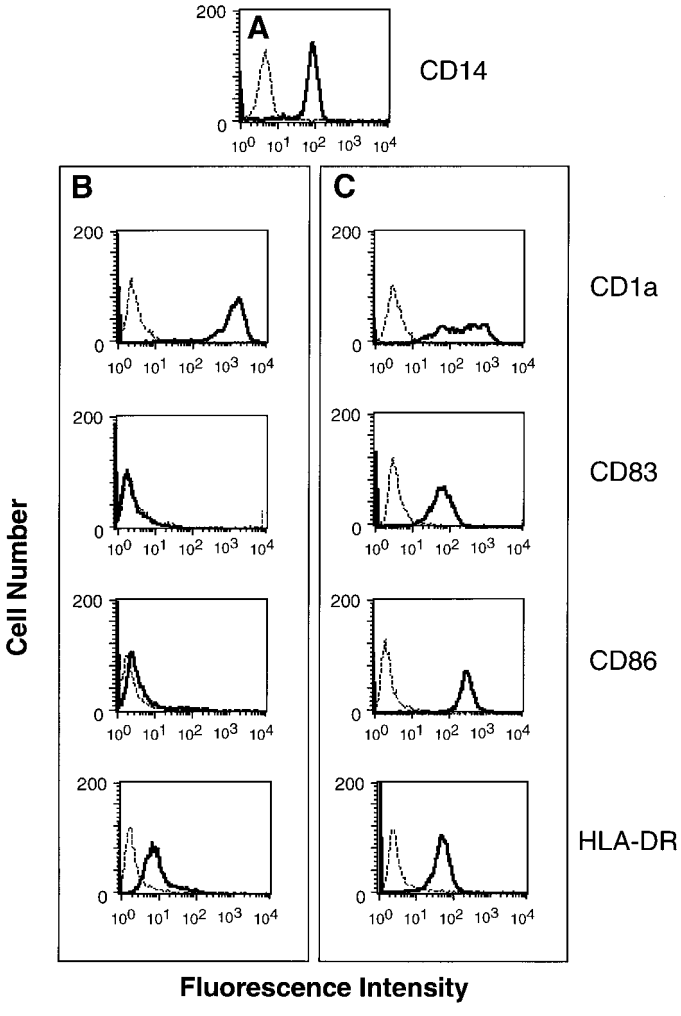

FIG. 3. Surface phenotype of monocyte-derived DCs and monocytes used for northern blot analysis. $CD14^+$ monocytes were purified from PBMC using a magnetic cell sorter system (A). $CD14^+$ monocytes were cultured in 10% fetal bovine serum/RPMI 1640 containing GM-CSF and IL-4 for 7 days (B, immature DC), and mature DCs were obtained by an additional 2-day culture in the presence of TNF-α (C, mature DC). Thick lines represent staining with specific antibodies, and the dotted lines represent staining with the isotype-matched control.

Preparation of anti-DCNP1 antibody. A polyclonal antibody recognizing the DCNP1 protein was raised by immunizing rabbits with a keyhole limpet hemocyanin (KLH)-conjugated synthetic oligo peptide, CQNSRSHGLETVPGHQR, corresponding to amino acid residues 10 to 25 of DCNP1 added with a cysteine residue at the amino terminus for KLH conjugation. Antibody was purified from immune sera, using peptide affinity chromatography. For pre-absorption experiments, the antibody was treated with the peptide in 100-fold molar excess.

Immunocytochemical analysis of DCs. Mature DCs derived from monocytes cultured on a poly-L-lysine-coated slide glass were washed twice with PBS and treated with fixative solution (PBS containing 4% paraformaldehyde) for 10 min at room temperature. After washing three times with PBS, cells were treated with ethanol for 2 min, with permeabilizing buffer (PBS containing 0.1% Triton X-100) for 2 min, and with blocking buffer (PBS containing 2% bovine serum albumin and 2% FCS) for 30 min. The cells were stained with primary antibody, anti-DCNP1, for 1 h, washed three times, and then stained with Cy3-labeled anti-rabbit IgG (Amersham) for 30 min. A confocal microscope (Fluoview FV300, Olympus) was used for observation.

Immunohistochemical analysis of tissue sections. Human normal tissues (liver, spleen, lymph node, and brain) were obtained at autopsy or at surgery. Following the fixation with 2% periodate-lysine-paraformaldehyde fixative the tissues were placed in liquid nitrogen, and stored at −80°C. Six-micrometer-thick cryostat sections were prepared for the indirect immunoperoxidase method. These sections were first incubated in 0.3% hydrogen peroxide/methanol for 30 min to block endogenous peroxidase activity, then treated with PBS containing 5% normal sheep or donkey serum for 15 min. Subsequently, the sections were incubated overnight with the primary antibody, anti-DCNP1 or KP1 (mAb specific for human CD68, Dako), then incubated in donkey anti-rabbit Ig (DCNP1) or sheep anti-mouse Ig (CD68), both conjugated with peroxidase (Amersham). Peroxidase activity was visualized using 3,3′-diaminobenzidine as substrate. Finally the sections were stained with hematoxylin and examined for light microscopic analysis. In double immunofluorescence staining, non-fixed fresh frozen sections were used. The sections were treated with PBS containing 5% normal goat serum for 10 min and incubated with primary antibodies for 1 h, washed three times, and stained with secondary antibodies (Alexa 546-labeled goat anti-rabbit IgG and Alexa 488-labeled goat anti-mouse IgG, Molecular Probes) for 1 h. The sections were then stained with TOTO3-iodide (Molecular Probes) for nuclear staining. The sections were examined using a confocal microscope (TCS SP2, Leika).

RESULTS

Generation of a cDNA Library Derived from DCs

DCs were obtained by culturing $CD34^+$ cells from umbilical cord blood, using GM-CSF and TNF-α. On days 11∼14 of culture, large cells of polygonal, fusiform, or dendritic shape had formed clusters. Flow cytometric analysis showed that 20∼70% of cells expressed CD1a, CD83, and CD86, and most cells highly expressed HLA-DR (Fig. 1). On the contrary, expression of CD14 was nil (Fig. 1). Cells were harvested on days 11∼14 of culture, and $CD1a^+$ DCs were purified. mRNA extracted from DCs and cDNA was synthesized. cDNA of $CD1a^+$ DCs was subtracted with that of

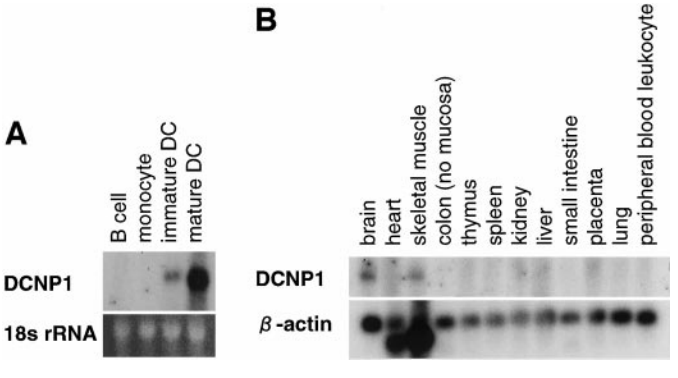

FIG. 4. Northern hybridization of DCNP1 gene in blood cells (A), and human normal tissues (B). Loading control is ethidium-bromide staining of 18S ribosomal RNA in the agarose gel before blotting (A) and blotting with aβ-actin probe (B).

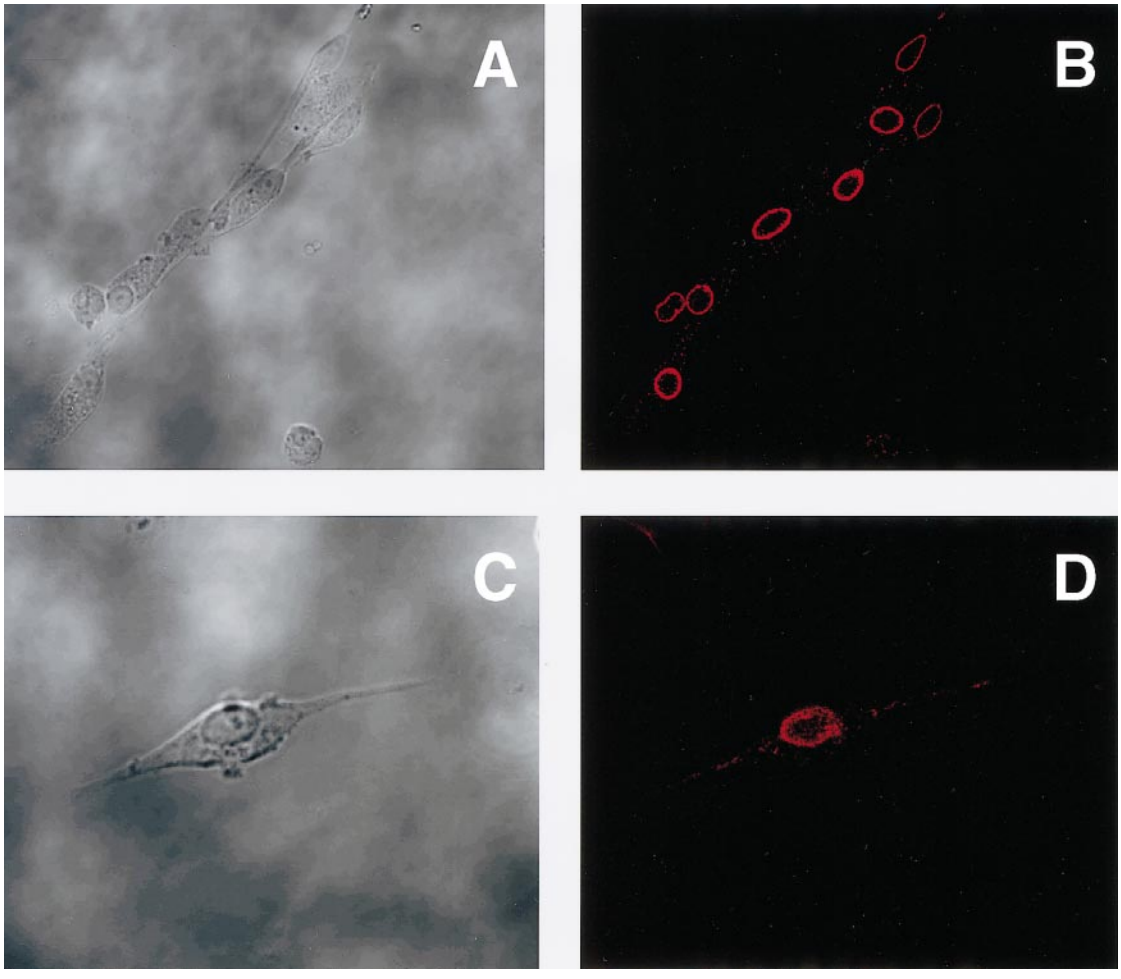

FIG. 5. Immunofluorescence analysis of monocyte-derived mature DCs with an anti-DCNP1 antibody. Mature DCs were differentiated from monocytes and stained with an anti-DCNP1 polyclonal antibody raised in a rabbit. Staining signal was visualized by using Cy3-labeled anti-rabbit IgG as a secondary antibody and analyzed using a confocal microscope (A, C). In B and D, the morphology of cells was shown by transillumination. In each A and B and C and D, same visual fields are shown, respectively. No fluorescence signal was observed when the anti-DCNP1 antibody was preabsorbed by the peptide used to raise the antibody, or normal rabbit IgG at the same concentration as anti-DCNP1 antibody was used as the primary antibody.

$CD14^+$ monocytes, using PCR-based cDNA subtraction. This subtraction was checked by PCR analysis of gelsolin and DC-LAMP, of which the expression is known to be higher in DCs than in monocytes (14, 17). As expected, these genes were enriched in the cDNA pool after subtraction (data not shown). Subsequently, PCR products were cloned into plasmid vector to generate the subtracted cDNA library. We picked up fifteen hundred clones from the library and made two copies of DNA dot-blots. Differential hybridization was done using as probes radiolabeled cDNA of DCs and monocytes. A visual comparison of the signal for each clone in the two replicas produced two hundred positive clones with a higher intensity signal for DC probes than for monocyte probes. We sequenced the positive clones and did similar searches against a DNA sequence database.

Identification of DCNP1

Among the cDNA clones, clone 13-75 is identical to several expressed sequence tag sequences (ESTs, GenBank Accession Nos. AI141949, AI673027, AW136257, and AA342611). To obtain the full-length cDNA sequence, we screened a human immature DC cDNA library using as a probe an EST clone IMAGE; 2345401 (Accession No. AI673027). Three overlapping cDNA fragments were isolated, and the full sequence of the cDNA was determined (Fig. 2). We named this gene DCNP1. The full-length cDNA contained an open reading frame (ORF) of 732 bp with an in-frame stop codon in the 5′ untranslated region, encoding a protein composed of 244 amino acids with a predicted molecular mass of 26,703 daltons (Fig. 2). DCNP1 has no significant sequence similarity with known genes of human and other species and is devoid of functionally indica-

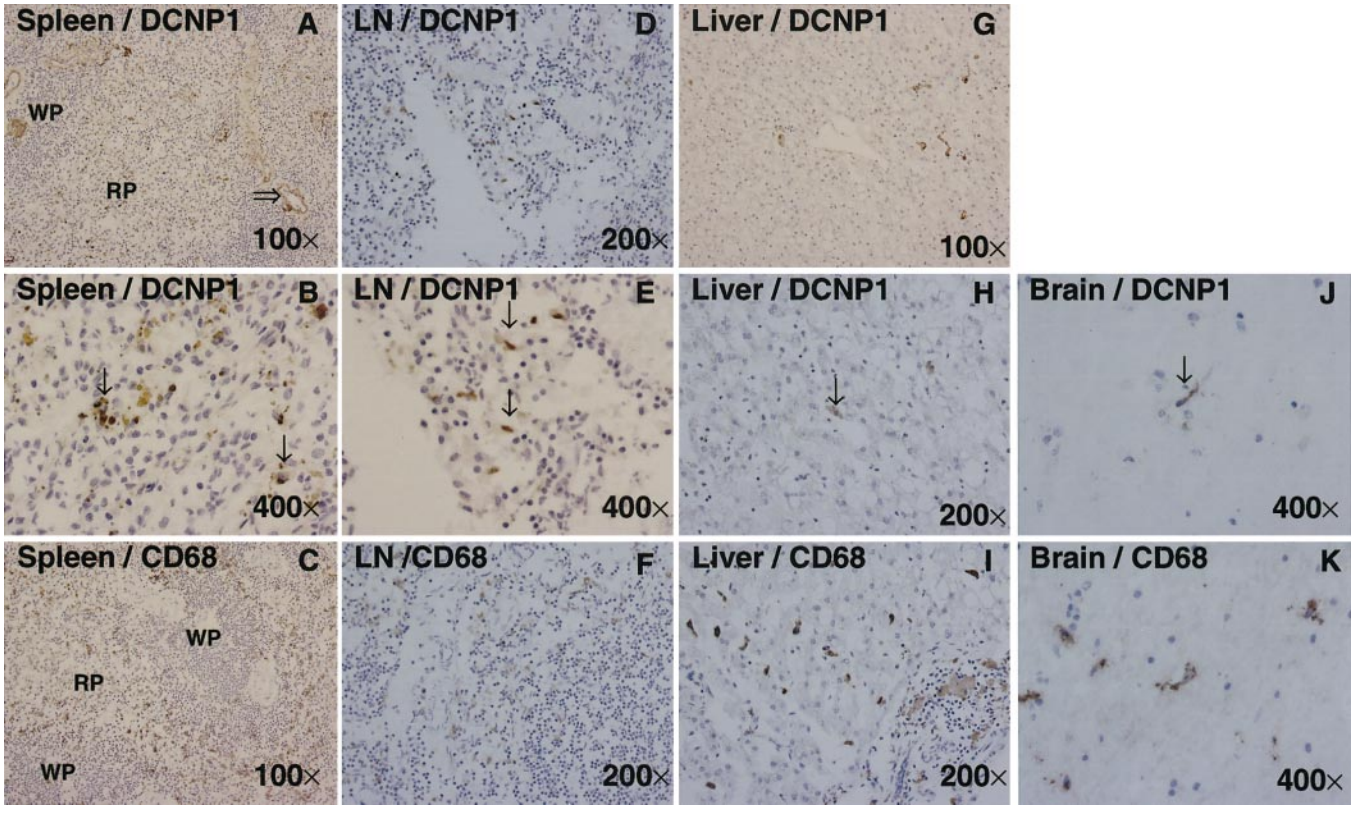

FIG. 6. Immunohistochemical analysis of DCNP1 and CD68. Cryostat sections of spleen (A–C), lymph node (D–F), liver (G–I), and brain (J, K) were analyzed. RP, red pulp; WP, white pulp. DCNP1 positive cells (indicated by arrows, →) exist in splenic red pulp (A, B), medulla of lymph node (D, E), and hepatic sinusoids (G, H). Some microglias in the brain were also stained (J). CD68-positive cells (most are macrophages) included DCNP1 positive cells (C, F, I, K). Tunica media of arteries (A, ⇒) were also stained with the anti-DCNP1 antibody. Sections were counter-stained with hematoxylin to indicate nuclei. Magnifications were indicated in the panels.

tive motifs. Sequence alignment with the human genome database revealed that DCNP1 is a one exon gene localized on chromosome 5.

Expression of DCNP1 mRNA in Cells and Tissues

The expression of DCNP1 mRNA in several types of blood cells was analyzed using northern blot hybridization. Cell surface phenotypes of monocyte ($CD14^{+}$) (Fig. 3A), immature DCs ($CD1a^{++}$, $CD83^{-}$, $CD86^{low/-}$, $HLA\text{-}DR^{+}$) (Fig. 3B) and mature DC ($CD1a^{+/++}$, $CD83^{+}$, $CD86^{++}$, $HLA\text{-}DR^{++}$) (Fig. 3C) used for RNA source are shown. DCNP1 mRNA is abundantly expressed in mature DCs and was detected at a lower level in immature DCs (Fig. 4A). In contrast, no DCNP1 mRNA

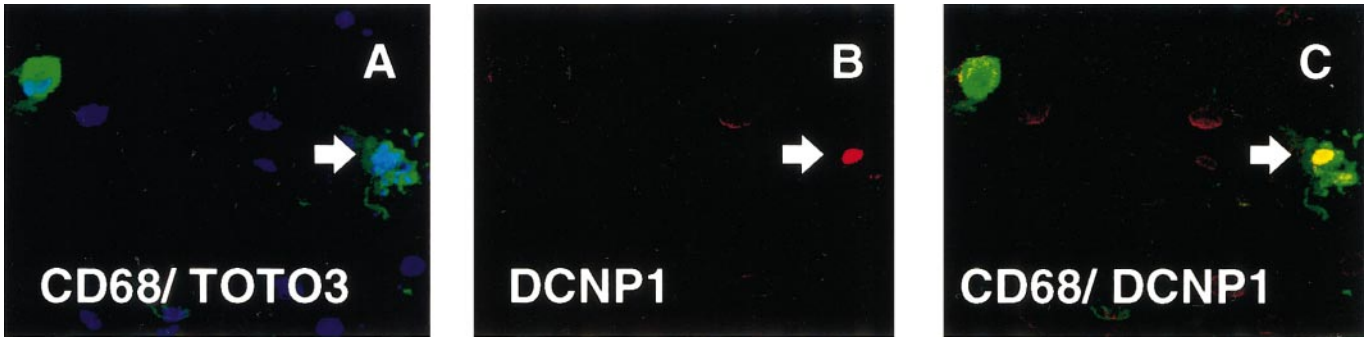

FIG. 7. Double immunofluorescence staining analysis of a human liver cryostat section using anti-DCNP1 and anti-CD68 antibodies. Staining signals were visualized by Alexa 488-labeled goat anti-mouse IgG (CD68, green, shown in A and C) and Alexa 546-labeled goat anti-rabbit IgG (DCNP1, red, shown in B and C). One of the two CD68 positive cells in the field was also positive for DCNP1 (indicated by arrows). The section was counterstained with TOTO3 to indicate nuclei (blue, shown in A).

expression was detected in monocytes and B cells. On multiple human tissue northern blots, DCNP1 mRNA was detected in the brain and skeletal muscle (Fig. 4B).

Subcellular Localization of DCNP1

We generated a polyclonal anti-DCNP1 antibody by immunizing rabbits with a synthetic peptide corresponding to the amino-terminal region of the protein. To determine the subcellular localization of DCNP1, we used the antibody for indirect immunofluorescence staining analysis. In the confocal microscopic analysis of mature DCs derived from monocytes, the staining signal was mainly observed in the nucleus, with intense fluorescence in periphery of the nuclei (Fig. 5). The cytoplasm was also slightly stained.

Immunohistochemical Analysis of DCNP1

To examine the expression of DCNP1 protein in human tissues, we immunostained the spleen (Figs. 6A and 6B), lymph nodes (Figs. 6D and 6E), liver (Figs. 6G and 6H) and brain (Fig. 6J) by indirect immunoperoxidase staining. For a comparison with the distribution of DCNP1-positive cells, CD68, a marker for DCs and macrophages (18) was also stained (Figs. 6C, 6F, 6I, and 6K). In the spleen, DCNP1-positive cells existed in red pulp regions (Figs. 6A and 6B), where also there were many CD68-positive macrophages (Fig. 6C). In white pulp regions, there were few CD68 or DCNP1-positive cells. In the lymph node, DCNP1-positive cells were fewer than that in the spleen (Figs. 6D and 6E). DCNP1-positive cells in the lymph node were observed mainly in the medulla. In the liver, a few cells in hepatic sinusoids showed DCNP1 reactivity, but few cells stained in portal area (Figs. 6G and 6H). In these tissues, DCNP1-positive cells showed a similar localization to CD68-positive cells with a lower frequency of DCNP1-positive cells. Numerous microglia in the brain were stained by anti-CD68 (Fig. 6K) and a small fraction of microglial cells reacted with anti-DCNP1 (Fig. 6J). We also performed double immunofluorescence staining with anti-DCNP1 and anti-CD68 antibodies of a liver section (Fig. 7). Among CD68-positive macrophages and dendritic cells, a small fraction of cells was stained with anti-DCNP1 (Fig. 7C). DCNP1 staining was also detected in the tunica media of splenic arteries (Fig. 6A).

DISCUSSION

To identify genes associated with special functions of DCs, we carried out PCR-based cDNA subtraction, an effective and relatively simple method to identify less abundantly and differentially expressed genes. The presence of many known DC-specific genes (e.g., CD1c, S100B, data not shown.) at high frequency in the subtracted cDNA library suggests that subtraction processes are indeed effective. We identified a gene, we termed DCNP1. Similarity searches revealed several ESTs corresponding to DCNP1 but with no homologue in known genes. We found no known protein sequence motif suggesting functions of DCNP1.

The expression of the gene was detected in DCs but not in monocytes and B cells, and a much higher level of DCNP1 mRNA expression was detected in mature than in immature DCs. These results indicate an up-regulation of the transcription of this gene during differentiation and maturation processes of DCs. In the immunohistochemical analysis, DCNP1 was detected in red pulp in the spleen, the medulla in lymph nodes and sinusoids in the liver. Indeed, cells stained with anti-CD68, a marker for macrophages and DCs, were mostly localized in these regions (Fig. 6). However, the number of DCNP1-positive cells was much fewer than in CD68-positive cells. These results are consistent with the idea that DCNP1 is expressed specifically in DCs, which exist in lower numbers than do macrophages in tissues, although we did not exclude the possibility that DCNP1 is expressed in some macrophages. In the brain, where precursor cells of DC have been reported to exist (19), DCNP1-positive cells also showed a tissue distribution similar to that seen in CD68-positive cells.

Although many DC-associated genes have been identified, few DC-associated nuclear proteins have been documented. Le Naour *et al.* have reported the profiling of gene expression in monocyte-derived DCs and identified several nuclear proteins which were up-regulated in monocyte-derived DCs during differentiation and maturation, such as DHP-2, MINOR, PPAR-γ, LXR-α, and interferon regulatory factor-4 (15). These molecules except for DHP-2, are nuclear receptors which were reported to be related to inflammation (20), or CD40-dependent IL-12 production by DCs (21). DCNP1 does not contain any structural motif conserved in DNA-binding proteins. In confocal microscopic analysis, DCNP1 was localized in periphery of nuclei. The ring-like intense fluorescent signal suggests that DCNP1 is localized in the nuclear membrane. DCNP1 is presumably a nuclear membrane protein involved in dynamic changes in structure and function of DCs during the maturation processes.

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