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J Clin Invest. 1996;**97**(3):852-857. <https://doi.org/10.1172/JCI118486>.

Research Article

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DCC Protein Expression in Hematopoietic Cell Populations and Its Relation to Leukemogenesis

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Abstract

Using flow cytometry and immunoprecipitation (IP), we have investigated the deleted in colon cancer (DCC) protein expression on the bone marrow (BM) and peripheral blood (PB) cells of 16 normal subjects, 17 myelodysplastic syndrome (MDS) patients, and 10 acute myelogenous leukemia (AML) patients.

With regard to the BM mononuclear cells (BM-MNCs) of normal subjects, the DCC protein expression ranged from 6.6 to 57.0%. Two-color flow cytometry revealed that among the BM-MNCs the DCC protein was clearly expressed on the CD14⁺, CD13⁺, and factor 8⁺ cells, whereas it was low on the CD19⁺ and CD7⁺ cells and did not express on the CD34⁺, CD8⁺, and the glycoporphin A⁺ cells. Further, the DCC protein expression was not seen on the PB CD11b⁺ and CD13⁺ cells. The IP results revealed that the 180-kD DCC protein was detected on the MNCs of both the BM and PB cells by the antibodies AF5, specific for the DCC extracellular domain, and G97-449, specific for the cytoplasmic domain.

In contrast, flow cytometry did not detect the DCC protein on any BM-MNC MDS lineages (0.1–1.5%) or on AML leukemic cells (0.1–0.9%). The IP results indicated that the AF5 antibody did not detect the DCC protein on BM-MNCs of three of five MDS patients and four of five AML patients; however, the G97-449 antibody detected the 180-kD DCC protein in two MDS patients in whom AF5 had detected greatly reduced DCC band.

These findings suggest that the DCC protein presence appears to be associated with normal hematopoiesis, and that its absence on the surfaces of the BM-MNCs and AML cells may contribute to the MDS and AML pathogenesis. (*J. Clin. Invest.* 1996. 97:852–857.) Key words: myelodysplastic syndromes • acute myelogenous leukemia • hematopoiesis

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Received for publication 6 July 1995 and accepted in revised form 13 November 1995.

J. Clin. Invest.

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0021-9738/96/02/0852/06 \$2.00

Volume 97, Number 3, February 1996, 852–857

Introduction

The deleted in colorectal carcinoma (DCC)¹ gene has been identified as a possible tumor-suppressor gene (1). Consistent with its possible function as a tumor-suppressor gene, DCC gene expression has been shown to be markedly decreased or absent in many cancers (2), including leukemia (3, 4) and myelodysplastic syndromes (MDS) (5, 6). The predicted nucleic acid sequence of human DCC cDNA from fetal brain tissue indicates that the DCC gene encodes a 1,447-amino acid transmembrane protein having extensive similarity to neural cell adhesion molecules and other cell-surface glycoproteins (7, 8). The DCC protein is composed of four immunoglobulin-like and six fibronectin type III-like extracellular domains, and a cytoplasmic domain (1).

So far, DCC expression studies have focused on DCC gene expression. Expression studies of the DCC protein have been limited for colon and neural tissues particularly at the cellular level (8, 9). Although important roles of the DCC protein in control of growth of the normal intestine and colon carcinoma have been emphasized, little is known about the DCC protein's function and antioncogenicity. Thus, we investigated DCC protein expression of mononuclear cells (MNCs) in normal subjects and patients with MDS and acute myelogenous leukemia (AML).

Methods

Patients for DCC protein expression. 10 untreated AML patients and 17 untreated MDS patients, consisting of 6 with refractory anemia (RA), 2 with RA with ringed sideroblasts, 5 with RA with an excess of blasts (RAEB), and 4 with RAEB in transformation, were investigated after obtaining informed consent. Diagnosis was made according to the French-American-British classification (10). 16 healthy volunteers were also investigated for DCC protein expression.

Reverse transcriptase (RT)-PCR for detection of DCC mRNA. 48 patients with MDS and 53 with AML were examined by RT-PCR. The total RNA of bone marrow (BM) MNCs was extracted by the CsCl method (11). RT-PCR was performed as described previously (3). Briefly, cDNA synthesis was performed first using an oligonucleotide primer (DCC2: 5'-AGCCTCATTTTCAGCCACACA-3', antisense strand). PCR was performed with 10 μ l of cDNA reaction mixture by using 0.0125 OD₂₆₀ (optical density) units of each oligo-

1. *Abbreviations used in this paper:* AML, acute myelogenous leukemia; BM, bone marrow; DCC, deleted in colorectal carcinoma; IP, immunoprecipitation; MDS, myelodysplastic syndromes; MNC, mononuclear cell; PB, peripheral blood; PE, phycoerythrin; RA, refractory anemia; RAEB, RA with an excess of blasts; RT, reverse transcriptase; SBA, soybean agglutinin.

nucleotide primer (DCC1: 5'-TTCCGCCATGGTTTTTAAATCA-3', coding strand; and DCC2: antisense strand). These oligonucleotide primers are the same ones used by Fearon et al. (1). PCR of 35 cycles was performed, consisting of 30 s at 94°C, 30 s at 55°C, and 1 min at 75°C (12). 20 μ l of the PCR products was phenol-extracted, ethanol-precipitated, and electrophoresed through a 3.5% agarose gel (NuScience 3:1 agarose; FMC Corp. BioProducts, Rockland, ME). The PCR products were visualized directly in ethidium bromide-stained gels and photographed.

Separation of CD13+, CD14+, and CD34+ for RT-PCR analysis. For RT-PCR analysis of DCC gene expression in the CD13+ and CD14+ populations, these populations were sorted with a FACStar Plus[®] (Becton Dickinson, San Jose, CA) using FITC-conjugated MY4 (CD14) or MY7 (CD13) (Becton Dickinson) from BM-MNCs. The purity of these cells obtained by sorting was 97 and 96%, respectively.

For CD34+ isolation, CD34+ cells were first enriched from MNCs using an anti-CD34 antibody-coated MicroCELLector flask (Applied Immune Sciences, Inc., Santa Clara, CA) and then sorted using FITC-conjugated HPCA1 (CD13; Becton Dickinson) (13, 14).

BM-MNCs were suspended in Dulbecco's phosphate-buffered Ca/Mg-free saline containing 1 mmol/liter EDTA (DPBSE) at a concentration of 5×10^6 cells/ml. The cells were loaded into AIS MicroCELLector SBA (Applied Immune Sciences, Inc.) containing covalently immobilized soybean agglutinin (SBA) and incubated for 45 min at room temperature. The nonadherent (SBA-) cells were removed, and 2×10^7 cells were subsequently loaded into AIS MicroCELLector CD34- flasks, which contained a covalently immobilized anti-CD34 monoclonal antibody, ICH3. After incubation for 1 h at room temperature, the nonadherent cells (CD34-) were removed, and the adherent cells (CD34+) were collected after physical agitation in DPBSE containing 10% FBS. The enriched cells (45% positivity for CD34) were collected and washed with Dulbecco's PBS containing 0.1% sodium azide. FITC-conjugated HPCA1 antibody was added to the cells, followed by incubation for 30 min at 4°C. The cells were washed twice with DPBS-azide and sorted with a FACStar Plus[®]. The purity of the obtained CD34+ was 97%. Total RNA extraction from each population was performed with RNazol[™] solvent (Cinna/Biotech Laboratories, Houston, TX).

Flow cytometric analysis. MNCs were obtained by Ficoll-Hypaque centrifugation (Lymphoprep, Neegard, Norway) of BM cells or peripheral blood (PB) collected from the patients and normal volunteers. The DCC protein expression was examined by an indirect immunofluorescence technique. Briefly MNCs were dispensed at 2×10^5 cells/tube and incubated with 100 μ l of PBS containing 100 nM of an anti-DCC mAb, AF5 (Oncogene Science, Inc., Manhasset, NY) specific for the extracellular domain, and 0.1% azide for 60 min on ice. After washing with PBS, the cells were incubated with 50 μ l of phycoerythrin (PE)- or FITC-conjugated goat anti-mouse IgG1 antibody at 4°C for 30 min. The samples were then washed, fixed, and analyzed with a FACScan[®] (Becton Dickinson). FITC- or PE-conjugated MY4, MY7, HPCA1, Leu9 (CD7), Leu12 (CD19), Leu2a (CD8), Leu 15 (CD11b), anti-factor 8, and anti-glycophorin A were used for staining surface membrane antigens, as described elsewhere (15). CD34-rich cells (mean: 40.8%, $n = 4$) prepared with an anti-CD34 antibody-coated CELLector flask (Applied Immune Sciences, Inc.) (13) were subjected to two-color FACS[®] analysis of CD34 and AF5. Each two-color analysis experiment was performed at least four times. When two-color FACS[®] analysis of Leu 15 and AF5 was performed, cells in the granulocyte gate of PB were the test materials.

Immunoprecipitation (IP) analysis. 2×10^7 MNC were metabolically labeled with 10 μ Ci/ml of [³⁵S]methionine and [³⁵S]cysteine for 3.5 h in methionine- and cysteine-free RPMI 1640 medium containing 10% dialyzed FBS. After labeling, the cells were washed with PBS and lysed in 500 μ l of RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS with protease inhibitors (1 mM PMSF, 50 μ g/ml antipain, 5 μ g/ml aprotinin, and 2 μ g/ml leupeptin). Cell debris was removed by cen-

trifugation. The supernatant was precleared by incubation with rabbit serum and protein A-Sepharose on ice for 30 min. The precleared lysates were assayed for incorporation, and $1-3 \times 10^7$ cpm was used for each IP. The supernatant was then incubated with an mAb, AF5 (20 μ g/ml) or G97-449 (specific for the cytoplasmic domain, 20 mg/ml; Pharmingen, San Diego, CA) for 1 h on ice. DCC-specific immunoprecipitates were recovered with 30 μ l of a 50% suspension of protein A-Sepharose and washed two times with NP-40 buffer (50 mM Tris, pH 8.0, 1.0% NP-40, 50 mM NaCl) and once with 10 mM Tris (pH 8.0). The proteins released from the immunoprecipitates by Laemmli's sample buffer were subsequently analyzed by electrophoresis on 5-25% SDS-PAGE (16). After drying, the gels were exposed to films. The protein concentration was determined by Bio-Rad protein assay (Bio Rad Laboratories, Hercules, CA).

Results

DCC protein is expressed on cell surface of specific lineages of normal BM and PB cells. Fig. 1 B and Table I show the results of a normal volunteer by single-color flow cytometric analysis. The DCC protein was detected at rates of 6.6-57.0% ($n = 16$; mean: 31.4%). To clarify which cell lineages of MNCs express the DCC protein, two-color flow cytometric analysis was performed. Fig. 2 shows a typical example of a normal individual, and Table II summarizes the data. The DCC protein was expressed in high positivity on CD14+, CD13+, and factor 8+ cells among BM-MNCs. CD19+ and CD7+ cells were also positive, whereas CD34+ and glycophorin A+ cells were negative for the DCC protein. CD11b+, CD13+, and CD8+ cells

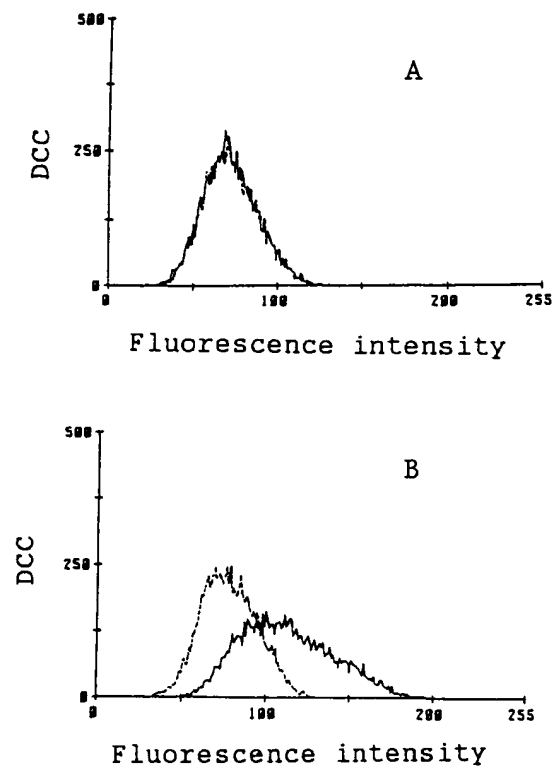


Figure 1. Single-color flow cytometric analysis of DCC expression on MNCs from an MDS case (A) and a normal volunteer (B). Cells were incubated with AF5 mAb, followed by FITC-labeled goat anti-mouse IgG. Cells were analyzed by single-color fluorescence using a FACScan[®] flow cytometer. The percentage of positive cells was 0.2 and 55.4%, respectively. Solid lines, AF5; dashed lines, control mouse IgG.

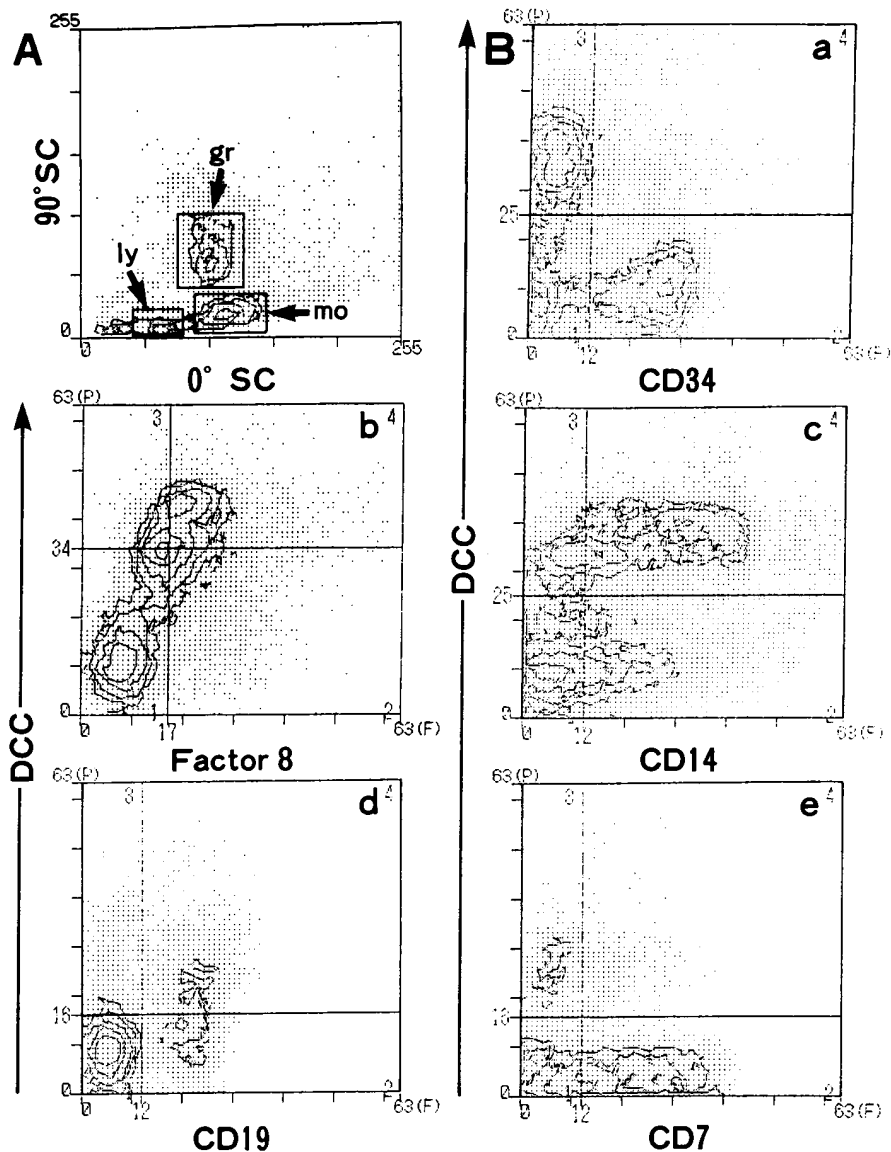


Figure 2. Example of analysis of DCC protein expression on surface MNCs from normal BM and PB. A depicts the light scattering of BM-MNCs. Viable MNCs were gated with forward scatter (x-axis) against side-ward scatter (y-axis). Lymphocyte (ly), monocyte (mo), and granulocyte (gr) gates were created. (B) x-axis: a, CD34-FITC intensity in all gates; b, factor 8-FITC intensity in ly gate; c, CD14-FITC intensity in mo gate; d, CD19-FITC intensity in ly gate; e, CD7-FITC intensity in ly gate. y-axis: a-e, PE-labeled DCC intensity.

of PB were also negative. Thus, two-color flow cytometric analysis revealed that the monocyte lineage was consistently DCC positive and that the granulocyte lineage was transiently DCC positive in the immature stage. B cell lineages were weakly positive, although T cell lineages were low positive or negative for DCC. After fractionating BM-MNC of normal volunteers, expression of the DCC gene in the CD13+, CD14+, and CD34+ populations was investigated by RT-PCR analysis (Fig. 3). The RT-PCR data paralleled the abundance of the immunoreactive DCC protein on BM-MNC. No PCR product of the DCC transcript, or the DCC protein, could be detected from CD34+ cells.

DCC protein is absent from cell surface of BM-MNCs of MDS/AML patients. Although the DCC protein was expressed on MNCs from normal volunteers, it was not detected (0.1–1.5%) in any of the 17 MDS or 10 AML patients, as shown in Fig. 1 A and Table I. Two-color flow cytometric analysis also showed that the DCC protein was not expressed in the MDS patients. BM-MNCs of the MDS contain the same populations as that of normal subjects. Thus, in the MDS pa-

tients, the CD14+, CD13+, and factor 8+ populations of BM-MNCs were all negative for the DCC protein (data not shown), whereas these cell lineages from normal volunteers were positive.

Loss of antigenicity of the DCC protein on cell surface of BM-MNC from MDS/AML patients. Because the DCC transcript was detected by RT-PCR analysis in 80 of 101 MDS/AML patients, as shown in Fig. 4 and summarized in Table III, we speculated that the antigenicity of the DCC protein must have been reduced or lost even in cases having a DCC-specific RT-PCR product. To confirm this speculation, IP analysis was performed of BM-MNC from normal volunteers and MDS/AML patients. As shown in Fig. 5, A and B, the DCC protein from normal MNCs migrated at the region of a molecular mass of ~180 kD. On the other hand, the DCC band corresponding to 180 kD could not be detected in an AML (M0) patient (lane 5) or an MDS (RAEB) patient (lane 7) (Fig. 5 B). A reduced amount of DCC protein was detected in an RA patient (Fig. 5 B, lane 6). These three AML/MDS patients have a DCC-specific RT-PCR product. Two antibodies specific for either the

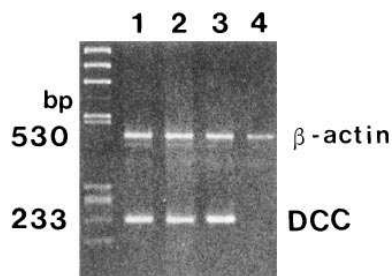


Figure 3. RT-PCR analysis of expression of the DCC of purified CD34+, CD14+, and CD13+ cells from normal BM-MNCs. Isolated CD34+ cells were prepared in an anti-CD34 antibody-coated CELLector flask followed by cell sorting as

described in Methods. CD14+ and CD13+ cells were prepared by cell sorting. cDNA was synthesized from 0.0125 OD₂₆₀ units of total RNA. After cDNA was synthesized using a DCC2 primer, PCR was performed using DCC1 and DCC2 primers. The PCR products (233 bp) were size fractionated by 3% agarose gel electrophoresis. Expression of the β -actin gene is shown at 530 bp as an internal control. Lane 1 shows BM-MNCs from healthy volunteers; lane 2, CD13+ cells; lane 3, CD14+ cells; lane 4, CD34+ cells. The left lane shows markers of λ DNA fragments digested by HindIII and ϕ x 174 DNA fragments digested by HaeIII.

extracellular or cytoplasmic domain were used to examine for the DCC protein by IP analysis in another MDS patient having a DCC-specific RT-PCR product. An \sim 180-kD DCC protein was obviously detected by the G97-449 antibody (specific for the cytoplasmic domain of the DCC protein), whereas a band with greatly reduced intensity and the same mobility, as shown in lane 2 of Fig. 5 C, was detected by the AF5 antibody (specific for the extracellular domain). Table IV compiles the qualitative levels of DCC expression by various assays in selected MDS/AML patients. DCC-RNA was detectable by the more sensitive RT-PCR assay in many patients. The normal-sized DCC protein was detected by G97-449 and/or AF5 antibody in some RT-PCR-positive patients (MDS-1, -2, AML-1, and -2 in Table IV).

Discussion

The DCC gene, which has been postulated to be a tumor-suppressor gene, was first identified in a colon cancer study by Fearon et al., in which they found that the tissue of 71% of their colon cancer patients showed an allelic DCC deletion and that 88% of these patients showed a reduced DCC expression, in contrast to the DCC expression seen in most normal tissues (1). Further, the DCC expression has been found to be low or lost in patients with pancreatic (17), esophageal (18), prostatic (19), and other carcinomas (2, 20). Also, in leukemia

Table I. Positivity for the DCC Protein

Sources of BM-MNC		DCC positivity (%)
Normal	(n = 16)	6.6–57.0 (mean: 31.4)
MDS	(n = 17)	0.1–1.5 (mean: 0.9)
AML	(n = 10)	0.1–0.9 (mean: 0.5)

Single-color flow cytometry analysis of BM-MNC was performed by indirect immunofluorescence assay. An anti-DCC mAb, AF5, specific for the extracellular domain of DCC, was used. The detailed patients with MDS or AML are presented in Methods.

cases, we and other investigators have found that DCC gene inactivation in some instances appears to contribute to leukemogenesis (3–6). However, while these findings suggest that a loss in the DCC expression is critical to cancer development, little is known about DCC mechanisms that may mediate tumoral suppression.

Similarly, the role that the DCC protein plays in cellular development and differentiation also remains unclear. It has been reported that the DCC protein was found in the axons of the central and peripheral nervous system and in differentiated cell types of the intestine (9). Further, a recent study suggests that the DCC protein may stimulate the PC12 neuritic outgrowth in a manner similar to neural cell adhesion molecules and N-cadherin (7). Cell-cell interaction through the DCC protein was required to stimulate PC12 to differentiate morphologically. Given these considerations, we thus have speculated that the participation of the DCC protein in both cellular development and differentiation occurs in a hematopoietic manner.

This study found that the DCC protein expression in CD34+ cells is very low, whereas in mature cells, i.e., the CD14+, CD13+, CD19+, and the factor 8+ cells, the DCC protein expression is high. These findings are consistent with a previous study in which the DCC protein was found in differentiated cell types of the normal intestine (9); of great interest is that the DCC protein content was very low in the CD8+ (T cells and NK cells) subsets, the erythrocyte lineage, and the CD11b subset of the mature granulocyte lineage. An uneven DCC protein distribution in normal hematopoiesis strongly supports the hypothesis that the DCC protein may act on differentiation pathways and that a cell's fate is determined by the cell-to-cell interaction (7).

With regard to oncogenesis, the first direct evidence that the DCC gene is functionally a tumor suppressor was obtained from an experimental animal study (21), wherein it was found

Table II. DCC Protein Positivity by Two-Color Flow Cytometric Analysis of Normal BM- and PB-MNC

CD+ cells	CD34	CD14	CD13	CD11b	Fact.8	Gly.A	CD19	CD7	CD8
BM	+/-/ (4)	++ (11)	+++/ (12)		+++/ (6)	- (10)	+ (5)	+/-/ (5)	
PB		+++/ (6)	- (8)	- (4)			+/- (6)	+/- (7)	- (5)

+++, > 50%; +, 20–50%; +/-, 5–10%; -, 0–5%. Fact.8, Factor 8; Gly.A, glycophorin A. Numbers in parentheses represent numbers of study, respectively.

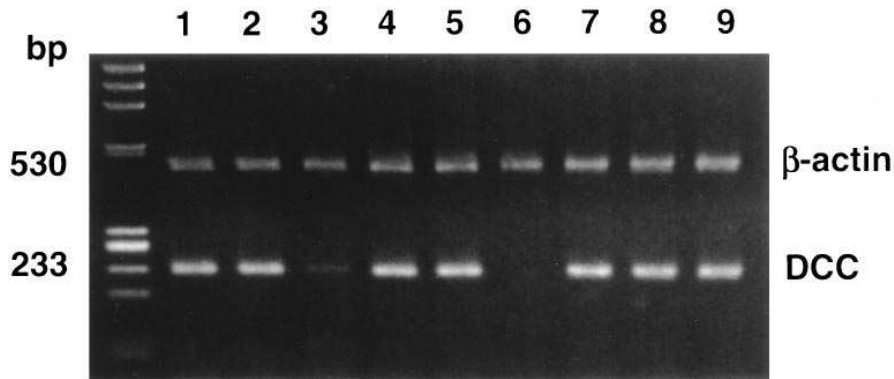


Figure 4. RT-PCR analysis of expression of the DCC gene in MDS/AML patients. After cDNA was produced using a DCC2 primer, PCR was performed using DCC1 and DCC2 primers. The PCR products (233 bp) were size fractionated by 3% agarose gel electrophoresis. Expression of the β -actin gene is shown at 530 bp as an internal control. Lanes 1 and 2 are control samples from BM-MNCs from healthy volunteers. Lanes 3–5 are BM-MNCs from MDS patients. Lanes 6–9 are BM-MNCs from AML patients. Expression of the DCC gene was reduced in lane 3 and absent in lane 6. The left lane shows marker of λ DNA/HindIII fragments and ϕ x 174 DNA/HaeIII fragments.

that the wild-type, full-length DCC gene expression inhibited tumor growth in human epithelial cells (1811-NMU-T1) in subcutaneous tissue of nude, athymic mice. In contrast, a truncated DCC gene is unable to suppress tumorigenesis, since it lacks the major portion of the cytoplasmic carboxy domain of the DCC protein.

Our present observation that there is very low expression or absent expression of DCC on MNC from AML and MDS

patients supports previous DCC gene expression studies. Thus, it may be that some BM-MNCs of MDS or leukemic cells that show an apparent RNA expression of the DCC gene are expressing an aberrant protein after a reduction in their antigenicity.

Although the results shown in Fig. 5 C appear to suggest that the AF5 antibody may be less efficient at immunoprecipitating DCC than the G97-449 antibody, based on the summary provided in Table IV, two other possibilities that may account for poor IP may be a loss in the specific antigenicity of the extracellular (or cytoplasmic) domain or a disturbance in the DCC protein transport to the cell surface due to an unknown disorder. In this regard, since the AF5 antibody was suffi-

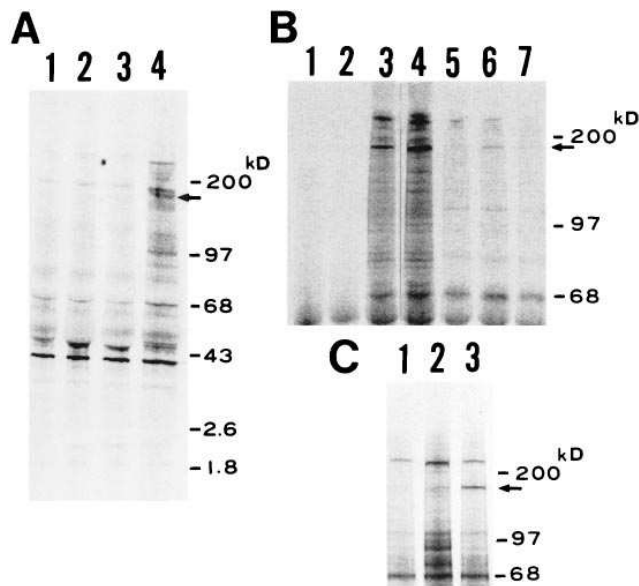


Figure 5. Characterization of DCC molecule identified by anti-DCC mAbs. IP analysis of normal PB (A). Lanes 1–3 show bands immunoprecipitated by three types of control mouse IgG1, IgG1, and IgG2a, (Becton Dickinson and DAKO, Copenhagen, Denmark). Lane 4 shows a DCC-specific 180-kD band identified by AF5 mAb. IP analysis of BM-MNCs from a normal volunteer and from two MDS patients and one AML patient using AF5 and G97-449 mAbs (B). Lanes 1 and 2 show two types of IgG1 (Becton Dickinson and DAKO) immunoprecipitates of normal BM-MNCs, and lanes 3 and 4 show G97-449 and AF5 immunoprecipitates of the same normal BM. Lane 5 shows an AF5 immunoprecipitate of an AML (M0) patient. Lanes 6 and 7 show AF5 immunoprecipitates of two MDS (RA and RAEB) patients. IP analysis of BM-MNCs of an MDS (RA) patient using AF5 and G97-449 mAbs (C). Lane 1 is control IgG1 immunoprecipitate; lane 2, AF5 immunoprecipitate; lane 3, G97-449 immunoprecipitate. Arrows indicate DCC-specific bands. The relative mobilities of molecular mass markers are indicated at the right side of each figure.

Table III. Expression of the DCC Gene in MDS/AML Patients

Disease	No. of patients examined	No. of patients with reduced DCC expression	No. of patients with absent DCC expression
MDS			
RA	16	1	0
RARS	3	0	0
RAEB	14	2	1
RAEB-t	15	2	1
Total	48	5	2
AML			
M0	3	1	1
M1	12	1	3
M2	16	3	2
M3	7	0	1
M4	10	1	0
M5	2	0	0
M6	3	0	1
Total	53	6	8
Normal sample	24	0	0

Expression of the DCC gene was analyzed by RT-PCR as described in Methods and reference 3. 48 untreated MDS patients and 53 AML patients were analyzed by RT-PCR assay. 24 healthy volunteers were also investigated. AML and MDS were diagnosed according to the French-American-British criteria (10). Compared with the density of the DCC product of the normal sample, no band for the product was surmised to represent absent DCC expression, whereas reduced density was interpreted as reduced DCC expression. RARS, RA with ringed sideroblasts; RAEB-t, RAEB in transformation.

Table IV. DCC mRNA and Protein Expression in Selected MDS/AML Patients

Patients	RT-PCR	FACS®	IP	
			AF5	G97-449
Normal	+	+	+	+
MDS				
MDS-1	+	-	+-	+
MDS-2	+	-	+-	+
MDS-3	+	-	-	ND
MDS-4	+-	-	-	-
MDS-5	-	-	-	-
AML				
AML-1	+	-	-	+-
AML-2	+	-	+	-
AML-3	+-	-	-	ND
AML-4	-	-	-	-
AML-5	-	-	-	ND

The qualitative levels of DCC expression were determined by various assays in normal subjects and MDS/AML patients. The assays for DCC protein include FACScan® flow cytometric analysis and IP using AF5 or G97-449 mAbs. mRNA was assayed by RT-PCR. Three normal samples were investigated. ND, assay not done for the individual patient. +, results of DCC assay were positive; +-, result of DCC assay was reduced; -, result of DCC assay was negative.

ciently efficient at immunoprecipitating the DCC of normal BM-MNCs (Table IV and Fig. 5 B) and, unlike the G97-449 antibody, it detected a sufficient intensity in the DCC band of an AML patient (AML-2; Table IV), it may be that either or both of these two possibilities may be the reason(s).

The DCC role in normal hematopoiesis and leukemogenesis is certain to be better understood once its ligand and effector molecules are more clearly characterized.

Acknowledgments

We thank the many physicians who referred their patients for study. We thank Drs. K.R. Cho, L. Hedrick, and B. Vogelstein for providing DCC plasmids. We also thank S. Inokuchi and H. Hanawa for preparing the manuscript.

This study was supported in part by a Grand-in-Aid for Scientific Research on Priority Areas (No. 06671116) from the Ministry of Education, Science and Culture of Japan.

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