Expression and regulation of NDRG2 (N-myc downstream regulated gene 2) during the differentiation of dendritic cells

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Abstract **We searched for genes with expressions specific to human monocyte-derived dendritic cells (DCs) using differential display reverse transcription-polymerase chain reaction, and found that N-myc downstream regulated gene 2 (NDRG2), a member of a new family of differentiation-related genes, was expressed in DCs. While DCs derived from CD34$^+$ progenitor cells also showed strong NDRG2 expression, the corresponding mRNA expression was absent in other cell lines including monocytes, B cells, and NK cells. The inhibition of DC differentiation by dexamethasone or vitamin D_3 treatment down-regulated the expression of the NDRG2 gene in DCs. In addition, gene expression was induced in a myelomonocytic leukemia cell line, which is capable of differentiating into DCs in cytokine-conditioned culture. The level of NDRG2 gene expression in DCs was significantly higher than that of other members of the NDRG gene family. Finally, in contrast to the stable NDRG2 expression in CD40-stimulated DCs, the induction of DC maturation by lipopolysaccharide (LPS) resulted in the down-regulation of NDRG2 gene expression. This down-regulation is likely to be due to a modification and subsequent destabilization of NDRG2 mRNA, because co-treating with actinomycin D and LPS significantly blocked this LPS effect. Taken together, our results indicate that NDRG2 is expressed during the differentiation of DCs, and that NDRG2 gene expression is differentially regulated by maturation-inducing stimuli.**

Key words: Dendritic cell; N-myc downstream regulated gene 2; Northern blot; DC differentiation; Lipopolysaccharide

1. Introduction

Dendritic cells (DCs) are highly specialized antigen-presenting cells, and are present in peripheral lymphoid and non-lymphoid organs. Their unique T-cell stimulatory capacity is due to the expression of high levels of costimulatory molecules, which include CD40, CD80, and CD86, and of major histocompatibility complex class I and II antigens that are essential for the presentation of endogenous and exogenous protein antigens and for the production of T-cell stimulatory cytokines such as interleukin-12 (IL-12), IL-15, and IL-18 [1–3]. In addition, the immune response may be regulated by the ability of T-cells and various other stimuli to enhance the survival of DCs and prevent their apoptosis [4,5].

The phenotypic and functional characteristics of DCs are intimately linked to their stage of maturation, which can be induced by CD40 stimulation or lipopolysaccharide (LPS), a component of the Gram-negative microbial cell wall product. Furthermore, cytokines such as tumor necrosis factor-α (TNF-α) or IL-1β, and reagents such as metrizamide and immune complex also induce the maturation of DCs. To better understand DC maturation at the molecular level, various basic studies have been performed upon DC surface molecules [6–8], gene expression [9–12] and signal transduction pathways [13,14]. However, the specific biochemical pathways and genes that mediate the differentiation and maturation of progenitors to DCs remain largely undefined.

The search for induced genes in the differentiation of hematopoietic precursors is the subject of intensive investigation. For example, a number of transcription factors involved in lymphoid lineage specification in progenitor cells have been identified. GATA-3 and Pax5 are essential for T- and B-cell development, respectively [15,16], whereas Notch1 is required for T-cell development but inhibits B-cell development, and therefore, determines T/B-cell diversification [17]. Moreover, the helix-loop-helix (HLH) factor Id2 is essential for development of natural killer (NK) cells [18]. Further, it has been reported that basic HLH factors are involved in development of plasmacytoid DCs (pDC), but not in the development of myeloid DCs, and that the transcription factor Spi-B is also involved in the control of pDC development and inhibits T-, B-, and NK cell development [19–21].

In this study, we searched for genes that were specifically transcribed during DC differentiation. To this end we utilized the differential display polymerase chain reaction (DD-PCR) method and analyzed the gene expression of a monocytic cell line, DCs, and mature DCs. One of the genes identified is the N-myc downstream regulated gene 2 (NDRG2), which belongs to a new family of differentiation-related genes.

2. Materials and methods

2.1. Reagents for cell culture, cytokines and antibodies

All cultures were performed in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with L-glutamine (2 mM), penicillin

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Abbreviations: DCs, dendritic cells; NDRG2, N-myc downstream regulated gene 2; PBMC, peripheral blood mononuclear cells; VD3, vitamin D_3

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(100 U/ml), streptomycin (100 μg/ml), HEPES (10 mM), and 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA). The growth factors used in the primary cultures of DC precursors were recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF; kindly provided by LG LS, Iksan, South Korea) and recombinant human IL-4 (rhIL-4; Endogen, Woburn, MA, USA). Dexamethasone and vitamin D_3 (1α,25-dihyroxyvitamin D_3, VD3) were purchased from Sigma. Other chemicals were from Sigma unless noted otherwise. The cytokine-dependent human myelomonocytic leukemia cell line MUTZ-3 (DSMZ, Braunschweig, Germany) was maintained in 10 ng/ml of recombinant human stem cell factor (rhSCF; R&D Systems, Minneapolis, MN, USA) in 6-well plate (Costar, Cambridge, MA, USA), and differentiation into MUTZ-3 DCs was induced by culture with rhGM-CSF (400 ng/ml), rhIL-4 (20 ng/ml) and rhTNF-α (2.5 ng/ml; PeproTech, Rochy Hill, NJ, USA) for 4 days. Human agonistic CD40 monoclonal antibody (mAb) was affinity-purified from ascites of BALB/c mice immunized with G28.5 hybridoma (HB-9110, ATCC, Rockville, MD, USA), which secreted a mouse mAb (IgG_1) binding to the human CD40 molecule. In order to induce DC maturation, DCs were stimulated with agonistic CD40 mAb or LPS (1 μg/ml; *Escherichia coli* O127-B8, Sigma) for 16 h. Flow cytometric analysis was used to determine the antigen expression of human DCs using the following mAbs of mouse origin: fluorescein isothiocyanate (FITC)-conjugated CD1a, phycoerythrin (PE)-conjugated CD11c, PE-conjugated CD80, PE-conjugated CD83, PE-conjugated CD86, purified CD40 (PharMingen, San Diego, CA, USA), FITC-conjugated CD54 (Immunotech, Marseille, France), FITC-conjugated CD14 and FITC-conjugated HLA-DR (Becton Dickinson, Mountain View, CA, USA). FITC-conjugated goat $F(ab')_2$ anti-mouse immunoglobulin (Biosource International, Camarillo, CA, USA) was used as an isotype control or as a secondary reagent.

2.2. Generation of dendritic cells in vitro

For human monocyte-derived DC generation, we used a modification of the method described by Sallusto et al. [22]. Briefly, peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy donors (Red Cross Blood Center, Daejeon, South Korea) by density centrifugation on Histopaque 1077 (Sigma). After lysing erythrocytes, cells were resuspended in culture medium and allowed to adhere to a T-75 culture flask (Costar) for 2–3 h. Plastic-adherent cells were cultured overnight and non-adherent cells were removed. Plastic-adherent monocytes that showed high CD14 expression and were negative for CD1a were cultured in DC medium containing rhGM-CSF (400 ng/ml) and rhIL-4 (20 ng/ml). The medium was replaced with fresh DC medium after 4 days. Human monocyte-derived DCs were usually harvested after 6–7 days, counted, and used in this study. For generation of DCs from human $CD34^+$ cells, $CD34^+$ progenitor cells were isolated from umbilical cord blood (School of Medicine, Eulji University, Daejeon, South Korea) using a MACS isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Isolated $CD34^+$ progenitor cells (2×10^6 cells/2 ml) were cultured in RPMI 1640 with 10% FBS in the presence of rhGM-CSF (400 ng/ml), rhTNF-α (2.5 ng/ml) and rhSCF (20 ng/ml) until day 5. SCF was then replaced with IL-4 (20 ng/ml) over the following 7–9 days. After a total of 12–14 days of culture, $CD34^+$ cell-derived DCs were harvested and used in this study.

2.3. Flow cytometric analysis

DCs generated by in vitro culture were subjected to flow cytometric analysis using FACSCalibur (Becton Dickinson), and data were analyzed using CellQuest software (Becton Dickinson). Cells were incubated with various FITC- or PE-conjugated mAbs on ice for 30 min at 4°C. After washing twice with cold phosphate-buffered saline (pH 7), cells were analyzed by flow cytometry. Rat IgG_1-PE isotype control (Becton Dickinson) was used as an isotype control. FITC or PE fluorescence was measured using 530-nm and 575-nm filters with logarithmic mode acquisition.

2.4. Proliferation assay of allogeneic PBMC

PBMC (1×10^5) were incubated with graded numbers of irradiated (3000 rad) MUTZ-3 or MUTZ-3 DCs. Experiments were performed in each well of 96-well round-bottom plates. Cell proliferation during the last 18 h of the 5-day culture was quantified by measuring the thymidine uptake of cells incubated with 1 μCi of [methyl-^{3}H]thymidine (Amersham Pharmacia, Piscataway, NJ, USA). Cells were harvested onto glass fiber filters and radioactivity was measured using a scintillation counter. Results are presented as mean cpm of cultures performed in triplicate.

2.5. Isolation of total RNA

Total RNA was extracted from the cultured cells by the acid guanidinium thiocyanate–phenol–chloroform extraction method [23]. Total RNA was dissolved in 0.1% diethylpyrocarbonate (Sigma)-treated water. The amount of RNA was determined by measuring spectrometric absorbance at 260 nm. For determination of the exact RNA concentration, equal amounts of RNA were loaded on a 0.9% RNA gel in 1×MOPS buffer, and the 18S ribosomal RNA bands were compared.

2.6. Reverse transcriptase (RT)-PCR analysis

First-strand cDNA was synthesized from total RNA in an RNase-free condition. The reaction was performed with 10 μg of total RNA using a ProSTAR™ First-Strand RT-PCR kit (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions. PCR was performed in a GeneAmp PCR system 2700 (Perkin-Elmer/Cetus, Norwalk, CT, USA) using the first-strand cDNA and Taq polymerase (TaKaRa Shuzo, Kyoto, Japan) as follows for each primer: NDRG1, 5′-GGTTCCAGCGTCACTTCT-3′ and 3′-CTTTCTAGATGCACCAACTT-5′; NDRG2, 5′-AATGGCCCTTGTTGCCCT-3′ and 3′-TCCTTCCCCACACTCGTT-5′; NDRG3, 5′-ACCAGACCATGGAGGTGT-3′ and 3′-CTCTCTATGTAACAGGCAC-5′; NDRG4, 5′-TTGAAGCCCTTGATCCCG-3′ and 3′-TCCAGATGATTGTCACATGT-5′; tapasin, 5′-TTCAACCCTTTCAGGAGG-3′ and 3′-TACTACAGAAGCTTGGGC-5′; β-actin, 5′-GCCATGTACGTTGCTATCCAGGCTG-3′ and 3′-AGCCGTGGCCATCTCTTGCTCGAAG-5′. PCR-amplified products were separated on 1.5% agarose gels containing 0.1 μg/ml ethidium bromide and visualized under UV light.

2.7. Northern blot analysis

Northern blot analysis was performed according to a previously described method [24]. Briefly, total RNA was separated in a 1% denaturing formaldehyde–agarose gel, and transferred to a nylon membrane (Boehringer Mannheim, Mannheim, Germany). After UV fixation, the membrane was hybridized at 68°C in ExpressHyb™ solution (Clontech, Palo Alto, CA, USA) with the complementary DNA (cDNA) probes labeled with [^{32}P]dCTP (Perkin-Elmer) using a random primer DNA labeling kit (Roche, Mannheim, Germany). In the case of NDRG2, a cDNA probe was prepared from the RT-PCR product using a specific primer set: 5′-ATGGCGGAGCTGCAGG-3′ and 3′-TCAACAGGAGACCTCCAT-5′. The probed membrane was then washed in 2×sodium saline citrate and 0.1% sodium dodecyl sulfate at room temperature, and exposed to autoradiographic film using an intensifying screen at −70°C for 2 to 3 days.

3. Results

3.1. Monocyte-derived DCs express the NDRG2 gene

DCs differentiated from monocytes showed high levels of CD1a, CD54, CD80, CD86 and HLA-DR expression, but did not express CD83, indicating an immature DC phenotype. However, after stimulation with anti-CD40 antibody (1 μg/ml), they showed CD83 antigen expression as well as increased levels of CD54, CD86 and HLA-DR antigen expression (data not shown). In order to search for gene expression in DCs, DD-RT-PCR was performed on the U937 cell line, monocyte-derived DCs, and agonistic anti-CD40 antibody-stimulated DCs. One of the differentially expressed genes was found to be the NDRG2 gene, which was not expressed in U937 cells, but was expressed significantly in DCs when expression levels were compared using Northern blotting (Fig. 1A). Its expression in DCs was not further increased by CD40 antibody stimulation. DCs are known to strongly express the tapasin gene that is involved in the processing of class I antigen [25,26]. As expected, DCs, but not U937 cells, expressed

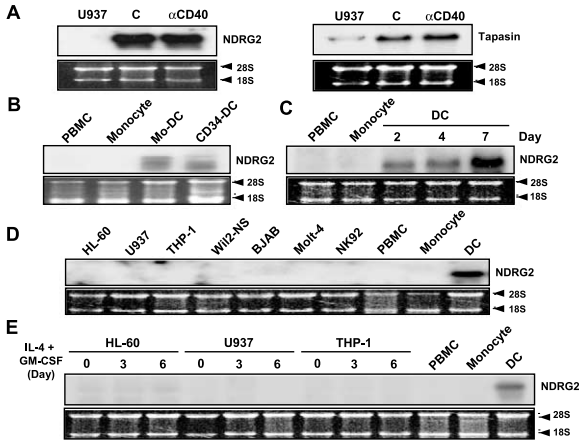

Fig. 1. Expression of NDRG2 mRNA is restricted to monocyte- and cord blood cell-derived DCs. A: Total RNA (5 μg) isolated from U937 cells, DCs (symbol: C) or anti-CD40 antibody-stimulated DCs (αCD40) was blotted and hybridized to a ^{32}P-labeled NDRG2-specific probe. The expression of tapasin in DCs was used as a positive control. B: NDRG2 mRNA expression was determined in PBMC, primary monocytes, monocyte-derived DCs and CD34-positive cord blood cell-derived DCs. C: RNA was extracted from DCs cultured for 2, 4 or 7 days, and NDRG2 expression was determined. D: Total RNA isolated from a variety of human cell lines and primary cells was hybridized to a ^{32}P-labeled NDRG2 probe. Independent experiments were repeated three times and representative autoradiographs are shown. E: Human leukemic cell lines (HL-60, U937 and THP-1) were cultured in medium containing GM-CSF (400 ng/ml) and IL-4 (20 ng/ml). After 3 and 6 days, the cultured cells were harvested and total RNA was isolated from the cultured cells.

tapasin, and this expression was enhanced after CD40-induced maturation, suggesting that the RNA prepared from cells contained representative transcripts.

We wondered whether blood mononuclear cells and monocytes similarly express NDRG2, but found no NDRG2 expression (Fig. 1B). Interestingly, NDRG2 was also expressed in DCs obtained by culture of CD34$^+$ cells derived from cord blood for 2 weeks. Its expression in monocyte-derived DCs was evident after 2 days and significantly increased thereafter (Fig. 1C). In addition, NDRG2 was not detected in leukemia, lymphoma, B-lymphocyte and NK cell lines (Fig. 1D). These findings suggest that NDRG2 may be induced during DC differentiation from precursor cells, because NDRG2 was not detected in blood mononuclear cells or in several lymphoid/myeloid cell lines. In order to examine the possibility that cytokines are responsible for NDRG2 gene expression, leukemic cell lines with hematopoietic or monocytic lineages, which were previously described to be further differentiated in response to GM-CSF [27], were cultured under the same condition as DCs, and tested for NDRG2 mRNA expression. As shown in Fig. 1E, no induction of NDRG2 gene expression was observed, even after treatment with cytokines for 6 days. Thus, the absence of NDRG2 expression in primary blood cells and in several other cell lines and the inability of cytokines to induce NDRG2 suggest that NDRG2 expression is related to the differentiation of DCs.

3.2. Induction of NDRG2 expression during DC differentiation

In order to investigate NDRG2 expression in CD34$^+$ cell-derived DCs, DCs were generated from cord blood cells by in vitro culture for 12–13 days in the presence of cytokine combinations. DCs differentiated from CD34$^+$ progenitor cells expressed several DC-related marker antigens on their surfaces at high levels, including CD1a, CD11c, CD40, CD54, CD80 and HLA-DR, but were negative for CD8, CD14, CD83 and CD86 antigen expression (Fig. 2A). During the differentiation process, dendrite formation increased after 1 week of culture, and the morphological features of the

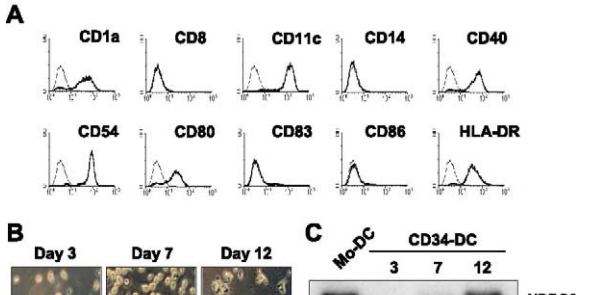

Fig. 2. Expression of NDRG2 mRNA in CD34$^+$ cell-derived DCs. A: Isolated CD34-positive cells were cultured in the presence of GM-CSF, TNF-α and SCF followed by IL-4 over the following 7–9 days, and induced to differentiate into DCs. After 12 days surface antigen expression was measured by immunostaining with the respective antibodies by flow cytometry (thin line, antibody or non-stained control; bold line, staining with respective antibodies). B: Light microscopic images of cultured cells during the differentiation process in the presence of the cytokine combination. C: RNA was extracted from monocyte-derived DCs or cord blood CD34-positive cells cultured for 3, 7 or 12 days, and NDRG2 expression was analyzed by Northern blotting.

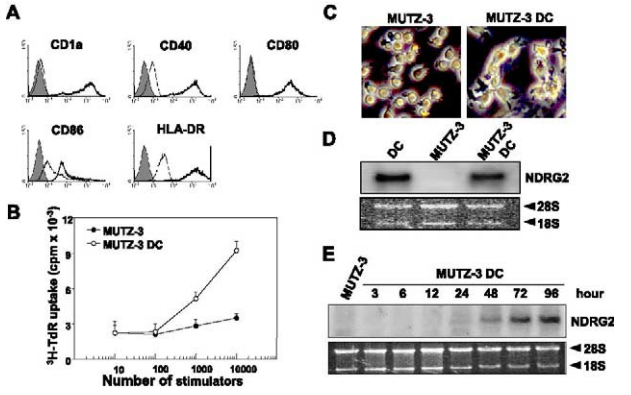

Fig. 3. Dendritic cell differentiation of MUTZ-3 cells leads to the expression of NDRG2. A: MUTZ-3 cells were induced to differentiate by adding cytokine-conditioned medium. After 4 days, surface antigen expression on differentiated cells (bold line) was compared with the undifferentiated parent cells (thin line) by flow cytometry. Shaded histograms show the staining of cells with control antibody. B: Allogeneic PBMC (1×10^5) were incubated with the indicated numbers of parent MUTZ-3 or MUTZ-3 DCs differentiated with the cytokine combination for 5 days. Cell proliferation was measured by the incorporation of radioactive thymidine in a scintillation counter. C: Phase contrast microscopy of undifferentiated MUTZ-3 cells and MUTZ-3 DCs after 4 days of differentiation. D: MUTZ-3 cell line did not express NDRG2, whereas MUTZ-3 DCs strongly expressed the NDRG2 gene. Northern blot using a NDRG2-specific probe was performed with RNA obtained from primary DCs, parent MUTZ-3 and MUTZ-3 DCs. E: NDRG2 expression during the DC differentiation of MUTZ-3 cells. Total RNA was isolated from MUTZ-3 cells at the indicated times during differentiation into MUTZ-3 DCs.

DC population were evident on day 12 (Fig. 2B). In parallel with this observation, cells that had been negative for NDRG2 gene expression at the beginning of the cultures progressively expressed the NDRG2 gene (Fig. 2C).

We next examined whether NDRG2 expression could be induced during the DC differentiation of the myelomonocytic leukemia cell line MUTZ-3 [28]. When these cells were incubated in the presence of GM-CSF, IL-4 and TNF-α for 4 days, CD1a and CD80 antigen expressions were newly induced in addition to the increased expressions of CD40, CD86 and HLA-DR (Fig. 3A). Morphologic features including the development of characteristic dendritic cell processes clearly showed the gaining of the DC phenotype (Fig. 3C). Moreover, when MUTZ-3 DCs were incubated with allogeneic PBMC, significant T-cell stimulatory activity was observed, whereas parent MUTZ-3 cells only stimulated very weakly (Fig. 3B). These results suggest that MUTZ-3 leukemia cells in cytokine-conditioned media have the unique ability to acquire the DC phenotype. Importantly, it was notable that similar to primary DCs, high levels of NDRG2 expression were observed after DC phenotype induction (Fig. 3D). Northern blot analysis using the RNA of cells harvested at several time points demonstrated that gene expression, which began to appear 48 h after culture, gradually increased in parallel with the development of DC morphology (Fig. 3E). These results, therefore, provide evidence that the modulation of NDRG2 expression may be correlated with the acquisition of the characteristic DC phenotype.

3.3. *Down-regulation of NDRG2 gene expression by the inhibition of DC differentiation*

Dexamethasone and VD3 have been described to affect the function of DCs by inhibiting NF-κB-induced transcription and NF-κB activation, respectively, and to inhibit DC differentiation [29,30]. When DCs were cultured in the presence of dexamethasone and VD3, they, in fact, showed low levels of CD1a expression and significantly increased levels of CD14 expression, as compared with controls (Fig. 4A). Importantly, NDRG2 mRNA levels were reduced in dexamethasone- or VD3-treated DCs (Fig. 4B), indicating that the inhibition of DC differentiation affects the induction of NDRG2 gene expression. However, this result does not rule out the possibility

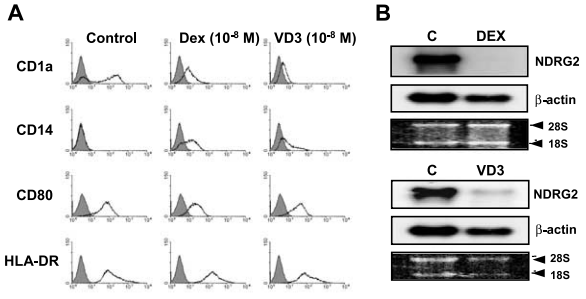

Fig. 4. NDRG2 expression is down-regulated by the inhibition of DC differentiation. A: Monocyte-derived DCs were generated in the absence or presence of 10^{-8} M dexamethasone or VD3 and surface antigen expression on day 7 was determined by flow cytometry. The shaded histogram shows control antibody-stained or non-stained cells. B: Total RNA was obtained from DCs cultured in the absence (symbol: C) or presence of 10^{-8} M dexamethasone or VD3. NDRG2 mRNA expression was detected by Northern blotting. β-Actin was used as a loading control.

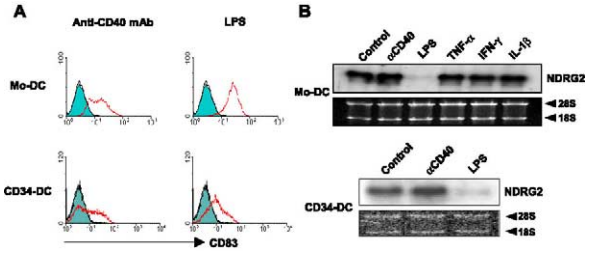

Fig. 5. LPS treatment decreases NDRG2 expression. Monocyte- or $CD34^+$ cell-derived DCs were stimulated with anti-CD40 mAb (1 μg/ml) or LPS (1 μg/ml) for 16 h. A: The surface expression of CD83 was compared on control cells by immunostaining and flow cytometric analysis (filled histogram, unstimulated DCs; open histogram, stimulated DCs). B: DCs were stimulated with anti-CD40 mAb (1 μg/ml), LPS (1 μg/ml), TNF-α (100 ng/ml), IFN-γ (1000 U/ml) or IL-1β (10 ng/ml) for 16 h. Total RNA was isolated from unstimulated or stimulated DCs and NDRG2 mRNA expression was detected by Northern blotting.

that dexamethasone or VD3 directly affects NDRG2 gene expression in DCs.

3.4. *Effect of maturation-inducing stimuli on NDRG2 gene expression*

We examined whether the NDRG2 expression induced during DC differentiation is affected by culturing DCs with anti-CD40 mAb or LPS for 16 h. These conditions led to the induction of CD83 expression, although it was relatively low, in $CD34^+$ cell-derived DCs (Fig. 5A), and increased the expressions of costimulatory molecules (data not shown). Importantly, CD40-mediated stimulation did not affect elevated NDRG2 gene expression, whereas LPS treatment strongly inhibited NDRG2 gene expression (Fig. 5B). In contrast, DCs treated with TNF-α or IL-1β, which also induces DC maturation, displayed high NDRG2 expression, at the same level as the untreated controls, indicating that the LPS effect on NDRG2 expression was not due to the production of TNF-α or IL-1β by LPS treatment.

3.5. *Expression of NDRG family genes in DCs and the differential effect of CD40 or LPS stimulation on NDRG2 mRNA expression*

The NDRG gene family currently consists of four members and is highly conserved in plants, invertebrates and mammals, so underlining the importance of this gene family. When the expression levels of the NDRG family in U937 cells and monocyte-derived DCs were compared by Northern blotting, it was observed that while NDRG1 and NDRG3 were moderately expressed in both cell types, NDRG4 transcripts could not be detected in U937, DCs or anti-CD40 mAb-stimulated DCs (Fig. 6A). The expressions of NDRG1, 3, and 4 genes were unaffected by CD40 or LPS stimulation (Fig. 6B). In addition, the expression of NDRG genes, including NDRG2, was unaffected by treatment with TNF-α, IL-1β or interferon-γ (IFN-γ).

The time course of NDRG2 down-regulation after inducing the maturation of monocyte-derived DCs with LPS was followed to investigate the mechanism further and to provide information on mRNA stability. Decreased amounts of NDRG2 mRNA were detected within 6 h of LPS treatment, and the down-regulation of expression was more significant after 12 h (Fig. 6C). We examined whether transcription was

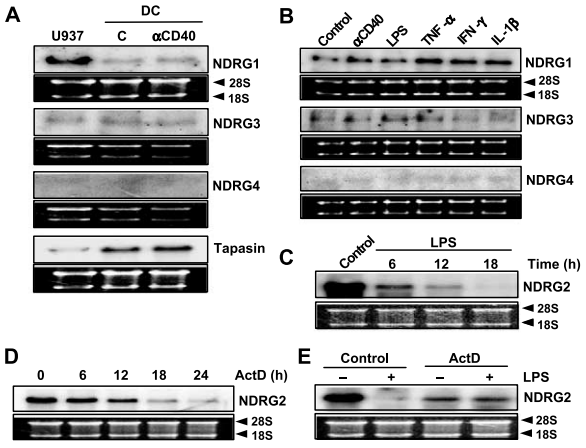

Fig. 6. Effects of several maturation-inducing stimuli on NDRG gene expression. A: After overnight stimulation of DCs, total RNA (5 μg) isolated from cells was hybridized to a ^{32}P-labeled NDRG1-, NDRG3- or NDRG4-specific probe. The expression of tapasin in DCs was used as a positive control (C; control DCs). B: Monocyte-derived DCs were stimulated with anti-CD40 mAb (1 μg/ml), LPS (1 μg/ml), TNF-α (100 ng/ml), IFN-γ (1000 U/ml) or IL-1β (10 ng/ml) for 16 h. Total RNA isolated from unstimulated or stimulated DCs was hybridized to ^{32}P-labeled NDRG1, NDRG3 or NDRG4 probe. C: DCs were treated for the indicated times with LPS (1 μg/ml). Total RNA was prepared and Northern blotting was performed with the NDRG2-specific probe. D: DCs were treated for the indicated times with actinomycin D (0.1 μg/ml). E: DCs were cultured with LPS (1 μg/ml) alone or with actinomycin D (0.1 μg/ml) pretreatment (1 h). Cultures were harvested after 12 h and total RNA was prepared and analyzed by Northern blot hybridization for NDRG2 mRNA levels.

required in the NDRG2 response to LPS by treating cells with actinomycin D. In the presence of actinomycin D, NDRG2 mRNA decreased in a time-dependent manner, but it was detectable for at least 12 h (Fig. 6D). However, actinomycin D treatment before LPS addition prevented the NDRG2 mRNA attenuation observed in the presence of LPS alone and gene expression was still maintained at high levels 12 h after LPS treatment (Fig. 6E). Therefore, this result demonstrated that the inhibition of NDRG2 mRNA expression by LPS could be regulated at the transcriptional level.

4. Discussion

In this report, we demonstrate that NDRG2 mRNA is strongly expressed in primary DCs, including monocyte- and CD34$^+$ cell-derived DCs, and in leukemic DCs originating from a myelomonocytic leukemia cell line. NDRG2 mRNA was not detected in DC precursor cells (e.g. monocytes), CD34$^+$ precursor cells, or the MUTZ-3 leukemia cell line. However, NDRG2 mRNA was gradually induced in DCs that were cultured to differentiate in vitro. In other cell lines including leukemia, lymphoma, B lymphocyte and NK cell lines, NDRG2 was not detected, which is consistent with a study which found that NDRG2 is essentially expressed in the brain, heart, skeletal muscle, and kidney [31]. To our knowledge, the present study is the first to indicate that NDRG2 gene expression can be induced in cancer cells and in normal cells of hematopoietic origin.

NDRG2 belongs to the NDRG family, a new family of differentiation-related genes. This family comprises four recently identified members: NDRG1–4 [32]. NDRG1 was first observed in various tissues in different species [33–36]. Moreover, human NDRG members are highly homologous, except for their C- and N-terminal regions, and exhibit distinct patterns of expression during development and adult life. Whereas NDRG1 is a widely expressed gene, NDRG2 and NDRG3 are essentially expressed in the brain, heart, skeletal muscle, and kidney, and NDRG4 expression is restricted to the brain and heart [31]. In fact, in the present study, we found that the cells examined did not express NDRG4 mRNA at a detectable level, unlike the other NDRG family members. The distinct expression patterns of the four members of the NDRG family suggest that they have different but related functions in different tissues and organs. However, the exact functional role of the NDRG genes remains to be identified. Accumulated data suggest that NDRG1 plays a role in growth arrest and cell differentiation, and that it could act as a signaling protein by shuttling between the cytoplasm and the nucleus. Indeed, NDRG1 expression has been found to be up-regulated during cell differentiation [34,35,37,38] and repressed during cell transformation [36] and by N-myc or c-myc [39], known to inhibit terminal differentiation and to stimulate cell proliferation. In terms of the other members of the NDRG family, no information exists on the functions of NDRG2, NDRG3, and NDRG4, though their expression patterns appear to be under spatiotemporal regulation, which differentiate them from NDRG1, and suggests that they have specific functions.

Here we report that the DC differentiation of precursor cells strongly induces NDRG2 gene expression. The results of the in vitro experiments suggest that gene expression might be involved in the regulation of the acquisition of DC morphological characteristics such as dendrite development or a myeloid DC specification. In fact, a recent finding suggests that the NDRG4 protein may participate in regulating processes that lead to cellular differentiation and neurite formation in PC12 cells [40]. The present study shows that cytokines present in DC culture are not responsible for NDRG2 gene induction, as cytokine-responsive myelocytic cell lines stimulated with cytokines do not show increased NDRG2 expression.

The inhibition of DC differentiation by the synthetic glucocorticoid dexamethasone or VD3 made it possible to investigate NDRG2 expression during the DC differentiation process. Previous studies have demonstrated that NF-κB is involved in the generation and maturation of DCs [41] and the inhibition of NF-κB activation down-regulates cytokine production by DCs and their surface antigen expression both in the basal state and after LPS activation [13,42]. Interestingly, the inhibition of DC differentiation was found to be well correlated with reduced NDRG2 expression. Therefore, it is evident that NDRG2 mRNA is specifically expressed during the DC differentiation of monocytes and of purified CD34$^+$ cells, although it remains to be seen whether the molecule is crucial for the development or the progression of DC differentiation. In the present study, we were not able to rule out the possibility that NDRG2 expression is directly modulated by NF-κB. However, we found no significant differences in the expression level of NDRG2 in mature DCs stimulated with CD40 antibody or TNF-α, both of which can activate NF-κB [43]. In addition, it was recently shown that the glucocorticoid dexamethasone is ineffective at inducing NDRG2 expression in a rat kidney model [44].

Interestingly, LPS, unlike other stimuli that induce DC maturation, predominately down-regulated NDRG2 mRNA. This down-regulation by LPS and the gradual induction of NDRG2 expression during DC differentiation make it unlikely that NDRG2 expression is regulated by NF-κB activation, which is known to be involved in DC differentiation and maturation. We hypothesized that the decreased amounts of NDRG2 mRNA observed after LPS treatment might be due to the decrease of mRNA stability resulting in the down-regulation of NDRG2 mRNA. In fact, actinomycin D pretreatment was found to prevent NDRG2 mRNA degradation, as compared to DCs treated with LPS alone. Thus, the down-regulation of NDRG2 mRNA by LPS is probably due to the decrease of mRNA stability induced by LPS treatment. Furthermore, we observed that LPS primarily affected NDRG2 gene expression, but not NDRG1 or NDRG3 gene expression, in DCs.

In conclusion, we demonstrate in this report that though the exact function of the NDRG2 gene remains to be established, it might be involved in the process of DC differentiation of monocytes, $CD34^+$ precursor and leukemia cells. Strong NDRG2 gene expression was observed in DCs and was associated with the cellular differentiation of precursor cells. This expression pattern was not observed in other members of the NDRG family gene. Moreover, NDRG2 gene expression was differentially regulated by maturation-inducing stimuli. Therefore, we speculate that understanding the exact functions of NDRG2 may produce a deeper understanding of the DC differentiation process and DC-specific functions.

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References

[1] Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y.J., Pulendran, B. and Palucka, K. (2000) Annu. Rev. Immunol. 18, 767–811.
[2] Hart, D.N. (1997) Blood 90, 3245–3287.
[3] Ardavin, C. (2003) Nat. Rev. Immunol. 3, 582–590.
[4] De Smedt, T., Pajak, B., Klaus, G.G., Noelle, R.J., Urbain, J., Leo, O. and Moser, M. (1998) J. Immunol. 161, 4476–4479.
[5] Manome, H., Aiba, S. and Tagami, H. (1999) Immunology 98, 481–490.
[6] Inaba, K. et al. (1994) J. Exp. Med. 180, 1849–1860.
[7] Ebner, S., Lenz, A., Reider, D., Fritsch, P., Schuler, G. and Romani, N. (1998) Immunobiology 198, 568–587.
[8] Palucka, K.A., Taquet, N., Sanchez-Chapuis, F. and Gluckman, J.C. (1998) J. Immunol. 160, 4587–4595.
[9] Hashimoto, S.I., Suzuki, T., Nagai, S., Yamashita, T., Toyoda, N. and Matsushima, K. (2000) Blood 96, 2206–2214.
[10] Dietz, A.B., Bulur, P.A., Knutson, G.J., Matasic, R. and Vuk-Pavlovic, S. (2000) Biochem. Biophys. Res. Commun. 275, 731–738.
[11] Granucci, F. et al. (2001) Nat. Immunol. 2, 882–888.
[12] Huang, Q., Liu, D., Majewski, P., Schulte, L.C., Korn, J.M., Young, R.A., Lander, E.S. and Hacohen, N. (2001) Science 294, 870–875.
[13] Ardeshna, K.M., Pizzey, A.R., Devereux, S. and Khwaja, A. (2000) Blood 96, 1039–1046.
[14] Andreakos, E., Smith, C., Monaco, C., Brennan, F.M., Foxwell, B.M. and Feldmann, M. (2003) Blood 101, 983–991.
[15] Ting, C.N., Olson, M.C., Barton, K.P. and Leiden, J.M. (1996) Nature 384, 474–478.
[16] Nutt, S.L., Heavey, B., Rolink, A.G. and Busslinger, M. (1999) Nature 401, 556–562.
[17] Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meerwijk, J., MacDonald, H.R. and Aguet, M. (1999) Immunity 10, 547–558.
[18] Yokota, Y., Mansouri, A., Mori, S., Sugawara, S., Adachi, S., Nishikawa, S. and Gruss, P. (1999) Nature 397, 702–706.
[19] Hacker, C. et al. (2003) Nat. Immunol. 4, 380–386.
[20] Spits, H., Couwenberg, F., Bakker, A.Q., Weijer, K. and Uittenbogaart, C.H. (2000) J. Exp. Med. 192, 1775–1784.
[21] Schotte, R., Rissoan, M.C., Bendriss-Vermare, N., Bridon, J.M., Duhen, T., Weijer, K., Briere, F. and Spits, H. (2003) Blood 101, 1015–1023.
[22] Sallusto, F. and Lanzavecchia, A. (1994) J. Exp. Med. 179, 1109–1118.
[23] Chomczynski, P. and Mackey, K. (1995) Anal. Biochem. 225, 163–164.
[24] Choi, S. et al. (2000) Clin. Exp. Metastasis 18, 45–50.
[25] Li, J., Schuler-Thurner, B., Schuler, G., Huber, C. and Seliger, B. (2001) Int. Immunol. 13, 1515–1523.
[26] MacAry, P.A., Lindsay, M., Scott, M.A., Craig, J.I., Luzio, J.P. and Lehner, P.J. (2001) Proc. Natl. Acad. Sci. USA 98, 3982–3987.
[27] Sayani, F. et al. (2000) Blood 95, 461–469.
[28] Masterson, A.J. et al. (2002) Blood 100, 701–703.
[29] Piemonti, L., Monti, P., Sironi, M., Fraticelli, P., Leone, B.E., Dal Cin, E., Allavena, P. and Di Carlo, V. (2000) J. Immunol. 164, 4443–4451.
[30] Xing, N., Maldorado, M.L., Bachman, L.A., McKean, D.J., Kumar, R. and Griffin, M.D. (2002) Biochem. Biophys. Res. Commun. 297, 645–652.
[31] Qu, X., Zhai, Y., Wei, H., Zhang, C., Xing, G., Yu, Y. and He, F. (2002) Mol. Cell. Biochem. 229, 35–44.
[32] Zhou, R.H., Kokame, K., Tsukamoto, Y., Yutani, C., Kato, H. and Miyata, T. (2001) Genomics 73, 86–97.
[33] Kokame, K., Kato, H. and Miyata, T. (1996) J. Biol. Chem. 271, 29659–29665.
[34] Ulrix, W., Swinnen, J.V., Heyns, W. and Verhoeven, G. (1999) FEBS Lett. 455, 23–26.
[35] Piquemal, D., Joulia, D., Balaguer, P., Basset, A., Marti, J. and Commes, T. (1999) Biochim. Biophys. Acta 1450, 364–373.
[36] Kurdistani, S.K., Arizti, P., Reimer, C.L., Sugrue, M.M., Aaronson, S.A. and Lee, S.W. (1998) Cancer Res. 58, 4439–4444.
[37] Salnikow, K., Su, W., Blagosklonny, M.V. and Costa, M. (2000) Cancer Res. 60, 3375–3378.
[38] Salnikow, K., Kluz, T., Costa, M., Piquemal, D., Demidenko, Z.N., Xie, K. and Blagosklonny, M.V. (2002) Mol. Cell. Biol. 22, 1734–1741.
[39] Shimono, A., Okuda, T. and Kondoh, H. (1999) Mech. Dev. 83, 39–52.
[40] Ohki, T., Hongo, S., Nakada, N., Maeda, A. and Takeda, M. (2002) Dev. Brain Res. 135, 55–63.
[41] Neumann, M., Fries, H., Scheicher, C., Keikavoussi, P., Kolb-Maurer, A., Brocker, E., Serfling, E. and Kampgen, E. (2000) Blood 95, 277–285.
[42] Verhasselt, V., Vanden Berghe, W., Vanderheyde, N., Willems, F., Haegeman, G. and Goldman, M. (1999) J. Immunol. 162, 2569–2574.
[43] O'Sullivan, B.J. and Thomas, R. (2002) J. Immunol. 168, 5491–5498.
[44] Boulkroun, S., Fay, M., Zennaro, M.C., Escoubet, B., Jaisser, F., Blot-Chabaud, M., Farman, N. and Courtois-Coutry, N. (2002) J. Biol. Chem. 277, 31506–31515.