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Enhanced p73 Expression during Differentiation and Complex p73 Isoforms in Myeloid Leukemia

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The p53 homologue p73 is expressed in at least six different isoforms (α, β, γ, δ, ε, and ζ), but unlike p53 it has rarely been found mutated in human cancers. However, altered expression of this gene has been reported in cancer cells. In order to understand if p73 is involved in normal and malignant development of myeloid cells, we investigated the expression pattern of the different p73 isoforms in progenitor and mature normal myeloid cells as well as in cells derived from acute and chronic myeloid leukemias. The results show that expression of p73 is markedly enhanced during differentiation of myeloid leukemic cells and that leukemic blasts from patients show an increased expression of the shorter p73 isoforms (γ, δ, ε, ζ). In particular the ε isoform is only expressed in leukemic cells and completely absent in mature myeloid cells. Altogether our data suggest that p73 is involved in myeloid differentiation and its altered expression is involved in leukemic degeneration. © 2000 Academic Press

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The tumor suppressor gene p53 is one of the most frequently mutated genes in human cancers. Recently two analogues of these genes p73 and p63 have been identified (1, 2). In contrast to p53 these two genes are expressed as multiple splice variants, most of which differ at the C-terminal part of the protein that is absent in p53. In particular six p73 splice variants (α, β, γ, δ, ε, and ζ) with different hetero- and homodimeric interaction properties as well as different transactivation capacities of known p53 target genes have been identified (1, 3–5). The high homology that these genes share with p53 (reaching 63% identity in the DNA binding domain) suggests that they may play an important role in cancer. In accordance with this hypothesis p73, like p53, can cause cell cycle arrest through transactivation of p21 and induce apoptosis. In addition this gene maps to a chromosome region (1p36.3) frequently deleted in neuroblastoma, colon carcinoma, and melanoma (6). Furthermore, genotoxic damage can activate p73 in a c-Abl dependent pathway (7–9). Unlike p53 however, p73 has rarely been found mutated in human cancers. Moreover data from p73 KO mice (10) and in vitro studies in neuroblastoma cells (11) suggest that this gene may play an important role in differentiation. Therefore it is possible that deregulated expression of this gene rather than loss of function (due to mutations or deletions) determines the de-differentiated phenotype observed in tumor cells. Indeed, this gene has been shown to be overexpressed in a number of different cancers (12) and we have previously shown a similar overexpression of p73 in acute (AML) and chronic myeloid leukemia (CML) (13).

In this study we investigated the expression pattern of the different p73 isoforms during *in vitro* induced granulocytic differentiation of HL60 and NB-4 hematopoietic cell lines as well as the pattern of expression of these isoforms in cells derived from patients with AML and CML.

MATERIALS AND METHODS

Patients, normal controls. Peripheral blood and bone marrow samples of untreated adult patients with AML or CML as well as CD34+ progenitor cells from cancer patients who underwent peripheral blood stem cell mobilization, granulocytes, and lymphocytes from healthy donors were isolated as described (19).

Abbreviations used: AML, acute myeloid leukemia; ATRA, all-*trans* retinoic acid; CML, chronic myeloid leukemia; DIG, digoxigenin; DMSO, dimethyl sulphoxide.

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Drug-induced differentiation of myeloid cell lines. Human myeloid leukemic cell lines HL60 and NB-4 were differentiated towards the granulocytic lineage with dimethyl sulphoxide (DMSO; 1.25%, Sigma, Buchs, Switzerland) and all-*trans* retinoic acid (ATRA; 2 μM, Sigma, Buchs, Switzerland). Cytospins were stained by May–Gruenwald–Giemsa for morphological evaluation of drug induced differentiation.

p73 isoform analysis by PCR. Total RNA was reverse transcribed and a p73 specific PCR with primers spanning exons 8 to 14 (sense 5′-GACCGAAAAGCTGATGAGGA-3′ and antisense 5′-CAGATGGT-CATGCGGT-ACTG-3′; Microsynth, Balgach, Switzerland) was performed. 2 μl of the PCR products was separated on 3% agarose gels and blotted to nylon membranes by capillary transfer. The membranes were hybridized at 50°C with a PCR generated; digoxigenin labeled probe (Roche Diagnostics, Rotkreuz, Switzerland) spanning exons 8–10 according to the manufacturer's protocol.

Total p73 expression and protein analysis. p73 real-time quantitative RT-PCR and p73 Western blot analysis were performed as described (13).

Statistical analysis. For differences between p73 isoform numbers in six independent groups we used the Mann–Whitney *U* test. Bonferroni correction was performed and the *P* value was set at 0.0033.

RESULTS AND DISCUSSION

In order to understand whether p73 may be associated to myeloid differentiation we induced the promyelocytic cell lines, NB-4 and HL60 to differentiate towards neutrophils using dimethylsulphoxide (DMSO) and all-trans retinoic acid (ATRA). Our results show that DMSO induced a transient induction of p73 mRNA (measured by real time RT-PCR), both in HL60 and NB-4 cells. However no difference in the expression pattern of the different splice variants (assessed by specific RT-PCR) was observed in either cell line and with either treatment (data not shown). In HL60 cells a peak of expression is observed after 48 h of treatment that returned to basal levels after 120 h of treatment (Fig. 1A). Western blot analysis confirmed these findings at the protein level (Fig. 1B, upper panel). The induction of p73 expression preceded the morphological differentiation of these cells assessed by Giemsa staining. The granulocytic population in the cultures increased from less than 5% in untreated cultures to 75% after 7 days of treatment. A similar induction was observed in DMSO treated NB-4 cells, both at the mRNA and the protein level (Fig. 1B, lower panel).

Treatment with ATRA also resulted in differentiation of these cells, but in this case, differentiation was not accompanied by p73 mRNA induction in either cell lines. Nevertheless p73 protein levels are increased in ATRA treated NB-4 cells with a kinetic different from DMSO treated cells with highest expression at day 5 of treatment (Fig. 1B, lower panel) preceding morphological differentiation. These results suggest that ATRA treatment is capable of increasing p73 protein levels by a post-translational mechanism that stabilizes the protein. A similar effect in p73 protein levels has been

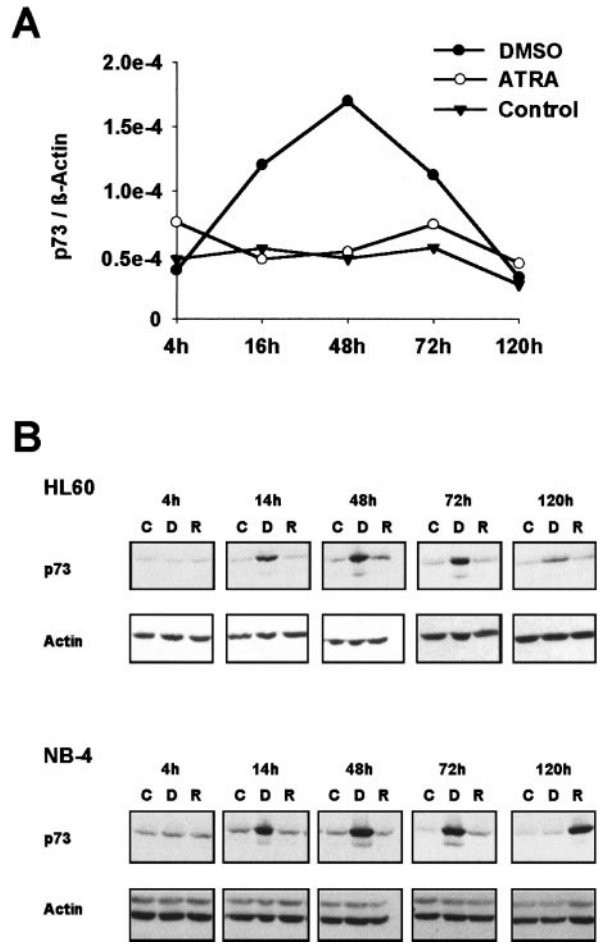

FIG. 1. Analysis of p73 expression upon chemical differentiation of the myeloid leukemic cell lines HL60 and NB-4. (A) p73 real-time RT-PCR results. The total p73 mRNA of DMSO, ATRA, and non-treated HL60 cells at different time points is shown as p73/β-actin ratio to correct for differences in RNA quantity and quality. (B) p73 Western blot analysis of control untreated (C), DMSO (D), and ATRA (R) treated HL60 cells harvested after 4, 14, 48, 72, and 120 h. The assay was performed using a polyclonal antibody raised against the C-terminal part of p73α. Equal loading and transfer of protein was confirmed by reprobing the membranes with anti-actin antibody.

observed in cisplatinum treated cells and it has been shown to be mediated by c-Abl (7). More studies are required to evaluate whether a similar stabilization mechanism involving c-Abl activity is involved in ATRA induced differentiation.

The involvement of p73 in granulocytic differentiation suggests that an altered expression of p73 may be involved in the differentiation block typical of immature leukemic blasts. To this end we studied the expression pattern of the different p73 splice variants in patients with acute and chronic myeloid leukemia (AML and CML). Using a very sensitive Southern technique with a digoxigenin (DIG)-labeled cDNA probe (Fig. 2A), we analyzed the different p73 mRNA splicing variants in primary samples from 65 AML and 22 CML patients, in 18 hematopoietic and 5 nonhematopoietic

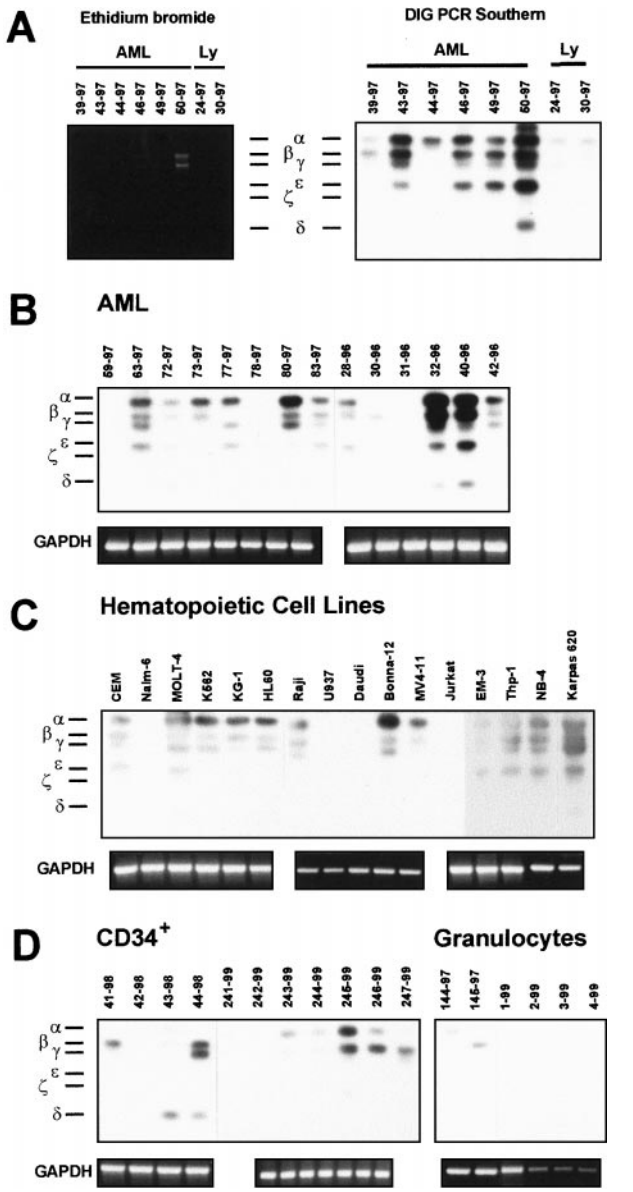

FIG. 2. DIG PCR Southern analysis of the p73 isoform expression pattern. (A) Comparison of ethidium bromide stained p73 PCR products with the newly developed Southern technique, using a digoxigenin(DIG)-labeled p73 cDNA probe against a constant N-terminal part of p73. Primers were designed to amplify this constant N-terminal part as well as the variable C-terminal region of p73. The PCR products were blotted to nylon membranes and detected with the p73 specific probe. (B–D) DIG PCR Southern analysis of p73 alternative splicing products in a representative panel of primary acute myeloid leukemia samples (B), hematopoietic cell lines (C), normal CD34+ progenitor cells and differentiated normal granulocytes (D).

cell lines and compared them with 19 CD34+ progenitor, 13 lymphocytic and 13 granulocytic cell samples. We found p73 mRNA isoform expression in 75% (49/65) AML, in all 22 CML, in 94% (17/18) of the hematopoietic cell lines, in all 5 nonhematopoietic cell lines, and in 90% (17/19) CD34+ progenitor cells. In contrast, only 46% (6/13) lymphocytic and 31% (4/13) granulocytic cell samples showed a very weak expression of p73 mRNA of any type. AML (median 4 isoforms, range 0–6) and CML (median 4, range 1–6) patient samples as well as the cell lines (median 4, range 0–6) expressed significantly more low-molecular-weight isoforms (i.e., β, γ, δ, ϵ, ζ) than normal granulocytes (median 2, range 0–3; Mann–Whitney U, all $P < 0.001$) and normal lymphocytes (median 2, range 0–2; Mann–Whitney U, $P = 0.003$, $P < 0.001$, and $P < 0.001$, respectively) (representative samples shown in Fig. 2B–D). CML samples also expressed significantly more splice variants than CD34+ cells (median 2, range 0–5; Mann–Whitney U, $P = 0.001$). In particular, there was a striking difference in the expression of p73ϵ in leukemic versus normal myeloid cells. This isoform was present in 40/65 AML, 17/22 CML, and 16/23 cell lines but only in 3/19 CD34+ cell samples and not in normal granulocytes or lymphocytes.

It has been argued that p73 may have an oncogenic potential (rather than a tumor suppressor function) as it is highly expressed in tumors versus normal tissues (14) and it may inhibit the normal p53 pathway (15). We previously reported particularly high expression of total p73 mRNA in AML and CML (13) measured by real-time RT-PCR. We now show that such high expression is associated with the expression of multiple p73 isoforms, i.e., a particularly complex isoform pattern. A recent report shows that p73 competes with p53 for its DNA binding sites in ovarian cancer cell lines (16). Since most AML display wild-type p53 (17), the strong p73 expression and/or the shift towards the shorter splice variants may contribute to p53 inactivation. These findings are also interesting as the shorter p73 variants γ, δ, and ϵ (compared to p73α) are weak suppressors of colony formation in human Saos2 osteosarcoma cells and show only little induction of p21CIP1 or cellular promoters that respond to (3, 18). Furthermore, it has been shown that p73 isoforms can strongly interact with one another indicating that shorter p73 isoforms may act as dominant negative binding partner for p73α (12). Thus the shift towards the shorter splice variants in AML may result in a decreased p73 tumor suppressor gene activity, since these isoforms are less potent transactivation factors than the larger subtypes which in turn are more prevalent in normal hematopoietic cells. A complex p73 isoform profile and in particular the selective expression of p73ϵ could therefore participate in leukemic transformation.

Altogether, our work indicates p73 upregulation in differentiating leukemic cell lines as well as deregulated expression of p73 isoforms in leukemic cells compared to normal mature myeloid cells, suggesting that altered p73 expression may be implicated in leukemia.

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