Identification of Differentially Expressed Messenger RNAs in Human Melanocytes and Melanoma Cells

Hans-Georg Simon, Barbara Risse, Monika Jost, Simone Oppenheimer, Csaba Kari, and Ulrich Rodeck


ABSTRACT

The phenotype of malignant lesions is a reflection of genetic events altering the RNA and protein expression patterns of normal cells. We have investigated RNA expression patterns distinguishing normal melanocytes (FM 902), a primary melanoma cell line (WM 793), and its variant cell line (1205-LU), selected for metastatic phenotype in athymic mice. Using mRNA differential display, we identified 42 different cDNA PCR products with cell line-specific expression patterns. Direct sequence analysis matched approximately 50% of the cDNA PCR products with gene sequences accessible in DNA databases. Among the known genes, two functionally distinct groups were recognized: (a) genes encoding ribosomal and mitochondrial proteins that were predominantly up-regulated in the malignant cells; and (b) genes encoding modulators of the immune response. Among the immunomodulators, the T-cell antigen MART-1 and the protease inhibitor a2-macroglobulin were detected in the melanocyte cell line but not in the tumor cells. By contrast, mRNAs for the complement inhibitor CD59 and the cytokine IL-1β were found to be overexpressed in the malignant melanoma cells.

RNA slot blot hybridization on a larger panel of melanocyte and melanoma cell lines confirmed differential expression of 15 of 42 genes including MART-1, a2-macroglobulin, and CD59. This molecular screening approach identified also three partially characterized and three novel sequences with differential expression patterns in normal and malignant melanocytes.

INTRODUCTION

The development of human melanoma is characterized by distinct stages of progression, ranging from clinically benign lesions to advanced primary and metastatic disease associated with high mortality. Normal melanocytes and cell lines derived from successive stages of melanoma progression can be propagated in culture, allowing the direct comparison of traits associated with malignant transformation. We and others have used this resource extensively to study melanoma-associated expression of genes related to growth regulation, adhesion, and the invasive phenotype (for review, see Refs. 1 and 2). These previous expression studies were guided largely by results in other tumor systems implicating the corresponding gene products also in melanocyte transformation.

In contrast to this earlier work, we describe here an unbiased molecular screen for differentially expressed genes in the progression of melanocytic neoplasia. We used modified protocols (3) of the recently described mRNA DD4 (4) and compared the gene expression patterns between a normal melanocyte line, a primary melanoma, and a metastatic melanoma cell line. This DD strategy, together with an efficient secondary screen based on slot blot hybridizations, enabled us to identify genes that were up- or down-regulated in malignant melanoma cells as compared to their untransformed melanocyte counterparts.

We describe the identification and evaluation of 42 distinct differentially expressed amplimers corresponding either to known genes or novel sequences.

MATERIALS AND METHODS

Cell Lines. Normal melanocytes were isolated from neonatal skin and cultured as described previously (5). Cell line WM 793 has been established from a primary lesion, and 1205-LU cells were selected from WM 793 for their ability to metastasize in nude mice as described (6). Other melanoma cell lines used in the RNA slot blot screening were obtained and cultured as previously described (7). The SW 480 colorectal carcinoma cell line was obtained from the American Tissue Type Culture Collection, and primary keratinocyte cultures were established from neonatal skin as described (8).

DD Analysis of mRNA. DDs were performed as described (3). In short, total cellular RNA was isolated following the acid-gradinidium-thiocyanate protocol (9), and 1 μg of the respective DNA-free RNAs was reverse transcribed using four different pools of oligo(dt)-anchored 3' primers (see below). For PCR (18 cycles), 1/20 volume of the RT reaction was amplified in the presence of [35S]-labeled dATP in a two-step, low-stringency/high-stringency PCR protocol and displayed on standard sequencing gels. The following 3' primers were used for RT and DD: (T₃)₅MA; (T₃)₅MC; (T₅)₅MG; and (T₅)₂MT, where M represents either G, A, or C. Randomly selected 5' primers for DD were: 161, TGTGTCACAG; 162, CTTCTAGGTC; 172, CATTCCTCTC; 177, CTGATAGAG; 178, GCCTACAAGA; 179, AAGGCTAGTG; 180, CTGACAGAG; 181, CTGACAGAGA; 183, ACACACATTC; 210, CCTTAGACAG; 211, TCTTGTCTCG; 222, ATGCTCTCAC; 223, TGTGACTGCA; 232, ATGATAGAG; 233, AGCATGACCT; 234, CTGACACCTA; 235, TGCCAGAGA; 236, CAACCTGCT. Bands representing potentially differentially expressed mRNAs were excised from the gel and eluted in H₂O. The CDNA PCR products were then reamplified incorporating M13 and RM13 sequence tags at their 5' and 3' ends, respectively.

DNA Sequencing. The tagged CDNA PCR products were directly sequenced, using M13 and RM13 sequencing primers (M13, TGTAAAGACGAGGCGCCATTG; RM13, CAGGAAACAGCTATGACC), with an ABI 373A DNA sequencing system (Applied Biosystems, subdivision of Perkin-Elmer Corp., Foster City, CA). Sequence data were analyzed using the sequence analysis software package (version 7.1), Genetics Computer Group, Inc. (University Research Park, Madison, WI) and the MacDNASIS sequence analysis software system (Hitachi, San Bruno, CA).

RNA Slot Blot Hybridization. Two sets of either six or seven different RNA slot blots were prepared. Two μg total RNA per slot were denatured for 15 min at 65°C in 1× SSC (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 50% deionized formamide, and 6% formaldehyde; applied to GeneScreenPlus membranes (DuPont NEN); and UV cross-linked. Slot blot membranes were prehybridized in 0.1 ml/cm² membrane 5× SSC, 5× Denhardt’s solution, 50 mM sodium phosphate (pH 6.5). 0.1% SDS, 50% formamide, and 0.5 μg/ml sheared heat-denatured salmon-sperm DNA (Sigma Chemical Co., St. Louis, MO) at 42°C for 4–24 h. Hybridization was performed in 0.1 ml/cm² membrane 5× SSC, 1× Denhardt’s solution, 0.02 μM sodium phosphate, pH 6.5, 0.1% SDS, 50% formamide, 10% Dextran sulfate, 100 μg/ml sheared heat-denatured salmon-sperm DNA, and heat-denatured [32P]-dCTP-labeled cDNA PCR fragments (10⁶ cpm/ml hybridization solution) at 42°C for 18–24 h. After hybridization, membranes were washed in 2× SSC at room temperature for 15 min and twice in 2× SSC, 0.1% SDS at room temperature for 30 min each. The final two washes were in 0.2× SSC, 0.1% SDS at 65°C for 30 min. Hybridization signals were analyzed using the PhosphorImager 445 SI.
scanning system and Image Quant software (Molecular Dynamics, Sunnyvale, CA).

**Northern Blot Analysis.** Northern blot analyses were performed as described previously (10).

**RESULTS AND DISCUSSION**

In this study, we describe differential patterns of gene expression in normal versus malignant melanocytes as detected by mRNA DD with 18 different 5 primer sets in combination with four sets of 3 primer pools. A representative example of DD results is shown in Fig. 1. Comparison of cDNA PCR products from the normal melanocyte line FM 902, the primary melanoma cell line WM 793, and its metastatic variant cell line 1205-LU revealed that most amplimers were shared between normal and malignant melanocytes. However, we identified 44 cDNA PCR fragments that were expressed preferentially in either melanocytes only or in melanocytes and primary melanoma cells, but not in metastatic melanoma cells (n = 21), or expressed in primary and/or metastatic melanoma cells but not in melanocytes (n = 23). Direct DNA sequencing of these PCR products yielded sequence data for 37 (84%) of 44 PCR fragments identifying four redundant isolates. DNA database searches revealed sequence homologies with previously published sequences in 20 (57%) of 35 fragments (Tables 1 and 2). Homologies to published sequences ranged between 72 and 96%. The relatively low percentage of sequence homology obtained for some amplimers (e.g., ribosomal protein L3 in Table 1) is most probably due to RT-PCR or sequencing artifacts, although expression of hitherto unidentified genes or isoforms of known genes cannot be excluded. Nine of the amplimers corresponding to known genes were previously not reported to be expressed in human cells of the melanocytic lineage. In addition, 15 cDNA PCR products did not reveal homologies to recorded sequences, indicating novel genes.

To assess whether the mRNAs corresponding to the DD amplifiers were expressed differentially in a larger panel of melanocyte and melanoma cell lines, the DD fragments were used as probes in RNA slot blot hybridizations. In this second screen, we used total RNA of either 12 or 13 melanoma cell lines and either 4 or 5 different neonatal melanocyte lines, including the two melanoma (WM 793; 1205-LU) and the melanocyte (FM 902) lines used in the DD. Moreover, we included two independent RNA preparations of WM 793 and 1205-LU cells to control for reproducibility. The other melanoma cell lines used originated either from primary (WM 115, 983-A, 902-B, 35, 1366, and 1341-D) or metastatic lesions (WM 164, 9, 983-C, 1617B, and 451-LU). To test for cell lineage-specific gene expression, we included RNA from normal human keratinocytes and from SW 480 colorectal carcinoma cells. In line with previous findings (4, 11), several DD fragments did not reveal hybridization signals with total RNA, most likely because of low mRNA abundance, whereas the majority (36 of 42) of nonredundant DD amplimers yielded detectable signals consistent with relative abundant expression of the corresponding genes. Of those, approximately 42% (15 of 36) revealed hybridization patterns suggesting up- or down-regulation of expression in malignant melanocytes as compared to normal melanocytes. Overall, we could confirm differential gene expression with a higher success rate as reported previously (12-14), indicating a robust performance of our mRNA DD with two-step PCRs and low number of amplification cycles (3). With few exceptions, the majority of mRNAs in this group appeared to be regulated differentially in a quantitative manner; i.e., hybridization signals were detectable in both normal melanocyte and melanoma RNAs, but the signal intensity varied between those two groups. By contrast, 21 fragments showed heterogeneous expression patterns in the RNA slot blot hybridizations. Few cDNA PCR products were identified as differentially expressed between primary WM 793 and metastatic 1205-LU cells. Generally, the slot blot hybridization with the larger panel of melanocyte and melanoma RNA samples did not confirm correlations between the corresponding RNA expression patterns and these two melanoma progression stages. Representative examples for genes either over- or underexpressed in melanoma cells or expressed in a heterogeneous fashion are shown in Figs. 1–3 and are discussed in more detail below.

**Genes Associated with Metabolic Processes.** A substantial fraction of amplifiers [7 (20%) of 35] showed sequence homology to genes encoding ribosomal and mitochondrial proteins (Table 1, group I). With few exceptions, the corresponding mRNAs were expressed at higher levels by melanoma cells as compared to normal melanocytes. Interestingly, using subtractive hybridization, Hutchins et al. (15) have shown that human melanoma M-1 cells overexpress certain mitochondrial genes when compared to a squamous carcinoma cell line of the lung. Additional support for the up-regulation of selected mitochondrial (16) and ribosomal (17, 18) genes in tumors comes...
from recent studies in SV40-transformed human fibroblasts and gastrointestinal tumor cell lines, respectively. Overexpression of different ribosomal proteins in various tumors (Ref. 18 and the present study) may suggest specific functions related to the transformation process. Alternatively, the up-regulation of mitochondrial and ribosomal gene expression may reflect the higher level of metabolic activity in melanoma cells as compared to normal melanocytes.

**Genes Associated with Proliferation.** p18, also termed phosphoprotein 18 or stathmin, was found at high levels in the cytoplasm of fast-proliferating leukemia cells but not in resting normal lymphocytes (19). Although believed to be expressed ubiquitously, expression of p18 has thus far not been described for melanocytic cells. The DD screen and the slot blot hybridizations revealed that p18 expression is up-regulated in melanoma cells (Fig. 2). A role for p18 in cell-cycle progression has been suggested by the findings that p18 is a substrate of p34cdc2 (20) and of mitogen-activated protein kinase (21), and that inhibition of p18 translation with antisense oligodeoxynucleotides delays S-phase progression of activated lymphocytes (22). Thus, overexpression of p18 in melanoma cells may reflect the higher rate of proliferation of cultured melanoma cells when compared to normal melanocytes (2). However, p18 expression has also been observed in some postmitotic cells in vivo, including neurons and glial cells (23) suggesting functions not related to the proliferative state. It needs to be determined whether overexpression of p18 by melanoma cells is related to proliferation or, rather, to their state of differentiation.

**Genes Described Previously in Melanoma.** The DD screen identified five partial cDNAs of genes that were described previously to be expressed in human melanoma cells. These include genes encoding from recent studies in SV40-transformed human fibroblasts and gastrointestinal tumor cell lines, respectively. Overexpression of different ribosomal proteins in various tumors (Ref. 18 and the present study) may suggest specific functions related to the transformation process. Alternatively, the up-regulation of mitochondrial and ribosomal gene expression may reflect the higher level of metabolic activity in melanoma cells as compared to normal melanocytes.

**Table 1: Amplimers isolated from primary and metastatic melanoma cells**

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- Group I, metabolism/growth associated; group II, genes identified previously in melanoma cells; group III, partially sequenced genes not identified previously in melanocytic cells or with unknown function.
- O, strongly expressed in melanoma cells; H, heterogeneously expressed in normal and malignant melanocytes; NI, no hybridization signal identified.

**Table 2: Amplimers isolated from normal melanocytes**

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- Group II, genes identified previously in melanoma cells; group III, partially sequenced genes previously not identified in melanocytic cells or with unknown function.
- O, strongly expressed in melanocytes; H, heterogeneously expressed in normal and malignant melanocytes; NI, no hybridization signal identified.

**Fig. 2.** Comparison of differential expression patterns in melanocytes versus melanoma cells as determined by mRNA DD and RNA slot blot analysis. A. scheme of slot blot loading of the RNAs isolated from various cell lines as described in "Materials and Methods." The normal melanocytes are boxed with a hatched line, and the melanoma cell lines are boxed with a solid line. B. slot blot hybridization with a ubiquitously expressed DD fragment to control for equal loading of RNA. C. DD with RNA from normal melanocytes (WM 902: Lane 1), primary (WM 793: Lane 2), and metastatic (1205-LU: Lane 3) melanoma cells using the primer pair 179 and (T)G-MC. Arrow, differentially expressed PCR product 179MC320, which was characterized by sequencing as p18 specific. D. matching slot blot hybridization with the labeled DD fragment 179MC320 (p18) on a panel of melanocyte and melanoma cell lines.

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the complement inhibitor CD59, the cytokine IL-1β, the recently cloned melanoma-associated T cell antigen (MART-1); the protease inhibitor α2-macroglobulin (α2M); and a putative serine protease, FAP-α.

The complement inhibitor CD59 has been found recently to be expressed by melanoma cells in vitro and in vivo (24). Our results suggest that CD59 is expressed by both normal melanocytes and melanoma cells, although melanoma cells express overall higher levels of CD59 (Fig. 3). Brasoveanu et al. (24) demonstrated that CD59 expression can protect melanoma cells from antibody-dependent, complement-mediated lysis in vitro. Thus, overexpression of CD59 by melanoma cells as observed here may relate to suppression of the antitumor immune response in vivo.

IL-1β is known to be up-regulated in melanoma cells as compared to normal melanocytes (10, 25). The mRNA DD results confirm these earlier observations (Fig. 1). Melanoma-derived IL-1 may play a role in the metastatic process, because exogenous IL-1 augments the metastatic ability of human melanoma cells (26), and treatment with IL-1 receptor antagonist reduces melanoma metastasis formation in athymic mice (27). Consistent with a role of IL-1β in the metastatic process, the mRNA DD indicated highest expression of IL-1β in 1205-LU cells, which were selected from WM 793 cells for metastatic ability in athymic mice (Fig. 1).

The human melanoma-associated T cell antigen MART-1 or Melan-A was identified initially by immunoselection as an antigen that induces lysis of tumor cells by tumor-infiltrating lymphocytes (28, 29). The MART-1 gene encodes a protein of unknown function whose expression is confined to the melanocytic lineage (28). In line with this earlier observation, we demonstrate expression of MART-1 in melanocytic cells but not in epithelial cells, such as SW 480 colorectal carcinoma cells and normal keratinocytes. An earlier study with normal and malignant melanocytic cells revealed a lack of MART-1 expression in some melanoma cell lines (28). Our results confirm this observation, because all melanocyte lines but only 6 of 13 melanoma cell lines express MART-1 (Fig. 3). Down-regulation of this lineage-specific T-cell antigen may represent a mechanism by which melanoma cells escape immune surveillance.

Fig. 4. Differential expression of the PCR product and cDNA 181MC530 in melanocytes versus melanoma cells as determined by mRNA DD (A), RNA slot blot (B), and Northern blot analysis (C). A, DD with RNA from FM 902 (Lane 1), WM 793 (Lane 2), and 1205-LU (Lane 3) cells using the primer pair 181(TC)5_M. The differentially expressed cDNA PCR product 181MC530 is indicated by an arrow. B, RNA slot blot analysis of total RNA. The loading scheme of the various RNAs and the control hybridization are shown in Fig. 3. The filter was hybridized with the labeled DD fragment 181MC530. C, Northern blot analysis of total RNA (15 μg/lane; top). The blot was probed in a manner identical to that shown in B, stripped, and rehybridized with a human β-actin probe (middle). Bottom, ethidium bromide-stained gel before transfer with the 28S and 18S rRNAs. Lane 1, human keratinocytes; Lanes 2-5, normal melanocytes FM 1094, FM 1070, FM 1020, and FM 1036; Lane 6-8, early primary melanomas WM 35, WM 1789, and WM 793; Lane 9, advanced primary melanoma WM 983-A; Lanes 10 and 11, metastatic melanomas WM 983-C and 1205-LU. Size markers (in kb) are given on the right.
Similar to MART-1, a2M was found to be expressed by all melanocyte lines tested, but the message was absent in 3 of 13 melanoma lines (Fig. 3). a2M is a wide-spectrum protease inhibitor but can also bind covalently to nonproteolytic proteins, notably certain growth factors, thereby inactivating them. Many of those are known to be produced by melanoma cells, including transforming growth factor-β, IL-1β, platelet-derived growth factor, IL-6, and vascular endothelial growth factor (for review, see Ref. 1). The level of a2M produced by clonal variants of HMB-2 melanoma cells has been shown to be inversely correlated with the in vitro growth rate of those cells (30). These data suggest that lack of a2M production provides a growth advantage to melanoma cells. However, the in vivo relevance of these findings is unclear, because no difference in tumorigenicity was found between low and high a2M-expressing HMB-2 clones. Independent of its growth-regulatory role, a2M has been found to increase the efficiency of antigen presentation to T cells presumably by targeting antigens for uptake by macrophages (31, 32). Lack of a2M expression may thus also be beneficial to melanoma cells by interfering with effective antigen presentation.

FAP-α is a protein identified initially in sarcomas but also expressed by stromal fibroblasts of epithelial cancers and granulation tissue of healing wounds (33, 34). It belongs to the family of serine proteases that may thus also be beneficial to melanoma cells by interfering with effective antigen presentation.

Genes Not Recognized Previously in Human Melanocytic Cells

A total of nine amplimers was isolated by mRNA DD that correspond to genes not reported previously to be expressed in human melanocytic cells. Of these, five amplimers were found to be expressed preferentially in normal melanocytes and four amplimers expressed preferentially in melanoma cells. As shown in Tables 1 and 2, three of the corresponding mRNAs were confirmed by RNA slot blot hybridization to be up-regulated by the respective cell type, whereas four were expressed heterogeneously, and two did not reveal any hybridization signals.

Two of these, the tumor suppressor protein APC and the protease cathepsin B, have been characterized in other systems. Interestingly, the DD analysis indicated down-regulation of APC mRNA expression in the melanoma cells. Because of low steady-state APC mRNA levels, we could not verify differential expression by slot blot hybridization. However, preliminary data of semiquantitative RT-PCR suggest heterogeneous expression of APC in different melanoma cell lines.4

Cathepsin B is a lysosomal thiol protease with multiple extralysosomal functions related to antigen presentation and the invasive phenotype of tumor cells (37, 38). Up-regulation of cathepsin B mRNA and/or protein expression has been shown to correlate with metastatic ability in human colorectal cancer (39) and glioma (40, 41). Similarly, transcriptional activation of the murine cathepsin B gene has been linked to the metastatic phenotype in mouse B16 melanoma cells (42) but has not been reported in human melanomas to date. Interestingly, neither WM 793 nor 1205-LU cells expressed cathepsin B at levels detectable by slot blot and Northern blot analyses (data not shown). These results confirmed the expression pattern indicated by DD, i.e., preferential expression of cathepsin B by FM 902 melanocytes as compared to WM 793 and 1205-LU melanoma cells. However, in the larger cell panel, we found cathepsin B to be expressed heterogeneously, irrespective of tumor progression stage.

Novel Sequences. A group of 15 amplimers represented as-yet-unknown sequences with no significant homology to DNA databases. The hybridization patterns in the RNA slot blot analysis demonstrated differential expression of two RNAs that were found to be up-regulated in melanomas and three RNAs that appeared to be down-regulated in melanomas. Fig. 4 shows an example of a novel gene (181MC530), which was identified as overexpressed in melanoma cells as compared to normal melanocytes.

In summary, the results demonstrate the efficient use of DD as a molecular screen for differentially regulated mRNAs in the human melanocytic system. Experiments are under way to understand the contribution of these genes to melanoma development by studying the in situ expression patterns of the corresponding genes in melanocytic lesions.

ACKNOWLEDGMENTS

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DIFFERENTIAL GENE EXPRESSION IN MELANOCYTIC CELLS
Differential gene expression in melanocytic cells


Identification of Differentially Expressed Messenger RNAs in Human Melanocytes and Melanoma Cells


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