Loss of E-cadherin Expression in Melanoma Cells Involves Up-regulation of the Transcriptional Repressor Snail*

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Malignant transformation of melanocytes frequently coincides with loss of E-cadherin expression. Here we show that loss of E-cadherin in melanoma cell lines does not involve mutations in the E-cadherin gene, promoter methylation, or alterations in expression of AP-2 transcription factors as suggested previously. In a panel of different melanoma cell lines, E-cadherin expression was negatively regulated by up-regulation of the transcription factor Snail. In comparison with primary human melanocytes, where Snail expression was not detected by reverse transcription-polymerase chain reaction, significant expression was found in all eight melanoma cell lines. In parallel, Western blot and reverse transcription-polymerase chain reaction analysis revealed strong reduction of E-cadherin expression in the melanoma cells. Consistently, transient transfection of a Snail expression plasmid into human primary melanocytes led to significant down-regulation of E-cadherin, whereas transient and stable transfection of an antisense Snail construct induced reexpression of E-cadherin in Mel Ju and Mel Im melanomas. In summary, we conclude that activation of Snail expression plays an important role in down-regulation of E-cadherin and tumorigenesis of malignant melanomas.

The cell-cell adhesion molecule E-cadherin has been shown to execute important functions in embryogenesis and tissue architecture by forming intercellular junction complexes and establishing cell polarization (1). The extracellular domain of E-cadherin is involved in a molecular zipper mediating cell-cell adhesion, whereas the cytoplasmic tail is linked to the actin cytoskeleton via catenins (2, 3). Because of its critical function in intercellular adhesion, E-cadherin has also been assumed to act as a tumor suppressor negatively regulating several critical steps of invasion and metastasis. Loss of E-cadherin expression during tumor development was recently observed in a variety of different tumor types, including malignant melanomas (4–10). Transfection of E-cadherin cDNA into invasive carcinoma cells led to significant reduction of their invasive capacity in vitro (11, 12), and activation of E-cadherin resulted in growth retardation of tumor cell lines (13, 14). Subsequently, results obtained from in vivo tumor models provided consistent evidence for a role of E-cadherin as a potent tumor suppressor (12, 15–17). Further, the tumor suppressor gene fat in Drosophila melanogaster was revealed as a member of the cadherin gene family (18).

Immunohistochemical studies of E-cadherin expression in malignant melanomas demonstrated significant down-regulation of E-cadherin in the tumor tissue compared with benign melanocytes and melanocytic nevi (4, 5). Evidence for the functional relevance of this phenomenon was obtained by Herlyn and co-workers (19–21), who showed that down-regulation of E-cadherin leads to deregulated control of melanocyte proliferation by keratinocytes and in parallel to an invasive growth behavior. E-cadherin is involved in a signaling pathway mediated by β-catenin and lymphocyte enhancer factor and T cell factor transcription factors. Deregulation of this pathway results in constant activation of β-catenin, lymphocyte enhancer factor, and T cell factor target genes, including c-myc and cyclin D1, and occurs in many kinds of malignant tumors (22). In melanoma both loss of E-cadherin and mutations of β-catenin, leading to a more stable, nondegradable protein, have been reported (5, 19, 23–25).

The mechanism of down-regulation of E-cadherin in malignant melanoma is still unknown. One possibility involves promoter inactivation attributable to hypermethylation, which has been observed in human breast, gastric, and prostate cancers and in leukemias (8, 26, 27). Furthermore, loss of activating protein-2 transcription factor expression as a potential activator of E-cadherin has been suggested (28–31). Also, mutations in the E-cadherin gene resulting in a functionally inactive protein have been detected in some tumors, including colorectal, gastric, and breast carcinomas (10, 32–34). Only very recently, up-regulation of the transcription factor Snail was reported to mediate significant negative regulation of E-cadherin expression in bladder, colorectal, and pancreatic carcinomas (35, 36).

Therefore, we aimed to analyze regulation of E-cadherin in melanoma cells and sequenced the E-cadherin gene in a panel of different melanoma cell lines and studied the effect of AP-2 transcription factors, promoter methylation, and transient transfection of Snail sense and antisense expression constructs.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture Conditions—The melanoma cell lines Mel Im, Mel Ei, Mel Wei, Mel Ho, Mel Juso, Mel Ju, SK-Mel-28, and HTZ19d have been described in detail previously (37, 38). The cell lines Mel Ei, Mel Wei, Mel Ho, and Mel Juso were derived from a primary cutaneous melanoma, and Mel Im, Mel Ju, SK-Mel-28, and HTZ19d
were derived from metastases of malignant melanomas. For tissue culture, the cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with penicillin (400 units/ml), streptomycin (50 μg/ml), 1-glutamine (300 μg/ml), and 10% fetal calf serum (Sigma) and split 1:5 every 3 days.

Human primary melanocytes derived from normal skin were cultivated in melanocyte medium melanocyte growth medium-3 (Life Technologies) under a humidified atmosphere of 5% CO₂ at 37 °C. Cells were used in passages 6–10 and not later than 3 days after trypsinization. Cells were detached for subcultivation or assay with 0.05% trypsin and 0.04% EDTA in phosphate-buffered saline (PBS).1

Transfection Experiments—For transient transfections, 2 × 10⁶ cells per well were seeded into six-well plates and transiently transfected with 0.5 μg of AP-2α or AP-2β cytomegalovirus promoter expression plasmids (39), E-cadherin expression plasmid, or sense- and antisense Snail cDNA in pcDNA3 (35) using the LipofectAMINE Plus method (Life Technologies) according to the manufacturer’s instructions. The cells were harvested 48 h after transfection and E-cadherin expression was evaluated using fluorescence-activated cell sorting, Western blotting, and reverse transcription-polymerase chain reaction (RT-PCR). All transfections experiments were repeated three times.

A panel of Mel Im cell clones was established by stable transfection with an antisense Snail expression plasmid under the control of a cytomegalovirus promoter and cotransfected with the neo-selectable marker expression plasmids (39), E-cadherin expression plasmid, or sense- and antisense Snail cDNA in pcDNA3 (35) using the LipofectAMINE Plus method (Life Technologies) according to the manufacturer’s instructions. One day after transfection, cells were placed into the selection medium containing 50 μg/ml G418 (Sigma). Twenty-five days after selection, individual G418-resistant colonies were subcloned.

Western Blots—Cells (3 × 10⁶) were lysed in 200 μl of radioimmunoprecipitation assay buffer (Roche Molecular Biochemicals) and incubated for 15 min at 4 °C. Insoluble fragments were removed by centrifugation at 15,000 rpm for 10 min, and the supernatant lysate was immediately shock frozen and stored at −80 °C. Eight micrograms of radioimmunoprecipitation assay cell lysate were loaded per lane and separated on SDS-polyacrylamide gel electrophoresis gradient gels (Invitrogen) and subsequently blotted onto a polyvinyllidine difluoride membrane. After blocking for 1 h with 3% bovine serum albumin and PBS, the membrane was incubated for 1 h with a 1:50 dilution of a mouse monoclonal E-cadherin antibody (clone M106, 0.2 μg/ml; Takara, Shiga, Japan). Then the membrane was washed three times in PBS, incubated for 1 h with a 1:300 dilution of an alkaline phosphatase-coupled isotype-matched mouse IgG instead of specific primary antibody are displayed as dotted profiles. A. Mock-transfected control. B. AP-2α-transfected cells. C. AP-2β-transfected cells. D. E-cadherin-transfected cells. E. E-cadherin antibody were displayed as bold profiles. Stainings with the specific E-cadherin antibody were displayed as bold profiles; stainings with non-specific mouse IgG instead of specific primary antibody are displayed as dotted profiles.

RT-PCR—Expression of Snail and E-cadherin mRNA was measured by RT-PCR. First-strand cDNA was synthesized using 2 μg of total cellular RNA as template, 1 μg of a random primer (Amersham Pharmacia Biotech), 4 μl of 5 × first-strand buffer (Life Technologies), 2 μl of 10 mM dithiothreitol, 1 μl of 10 mM dNTPs, and 1 μl of Superscript II (Life Technologies) in a volume of 20 μl. Snail amplification, a nested PCR was performed using 2 μl of the cDNA preparation and the following program: 15 cycles of 45 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C and a final extension of 5 min at 68 ° C. Two microliters of the PCR product were applied to the same reaction profile but with 25 cycles using nested primers (hsnail forward, 5’-TGG CCG AAT CGG CGA; hsnail reverse, 5’-ACT ACA GCG AGC TGC AGG; and hsnail reverse, 5’-CTT GCC GAA TCC CCC GCA G; hsnail forward nested, 5’-ACT ACA GCG AGC TGG AGG; and hsnail reverse nested, 5’-GGG TGG TTG CCT GGA ATG TGC). PCR products were separated on a 1.8% agarose gel and stained with ethidium bromide. For E-cadherin, both conventional RT-PCR and quantitative real time PCR were performed on a LightCycler (Roche Molecular Biochemicals). Two microliters of cDNA template, 2 μl of 25 mM MgCl₂, 0.5 μM forward and reverse primers (CLONTECH), and 2 μl of SybrGreen LightCycler mix in a total of 20 μl were applied to the following PCR program: 30 s at 95 °C (initial denaturation); 20 °C/s temperature transition rate up to 95 °C for 15 s, 10 s at 58 °C, 22 s at 72 °C, and 10 s at 82 °C acquisition temperature and 7 s at 72 °C for 30 cycles. E-cadherin expression was measured using conventional RT-PCR.

Quantitation of E-cadherin expression in melanoma cell lines. Displayed from left to right are melanoma cell lines (Mel Wei, SK-Mel 28, Mel Ju, Mel Ei, Mel Juso, Mel Ho, Mel Im, and HTZ19d), primary human skin melanocytes (PHM), and the LoVo colon cancer cell line.
mode single, repeated for 40 times (amplification). The PCR reaction was evaluated by melting curve analysis following the manufacturer’s instructions and checking the PCR products on 1.8% agarose gels.

**E-cadherin Sequencing**—Genomic DNA was isolated from melanoma cell lines as described previously (40). Exons and intron-exon boundaries of E-cadherin were amplified using the primers described by Berx et al. (10). The PCR products were purified, and direct cycle sequencing reactions were performed using a PRISM dye primer cycle sequencing kit with AmpliTaq FS (Perkin-Elmer) and analyzed by capillary electrophoresis (Applied Biosystems ABI 310).

**Analysis of E-cadherin Promoter Methylation Status**—This method is based on the selective conversion of unmethylated cytosine to uracil by the effect of bisulfite (41). Genomic DNA (1 μg) was denaturalized with NaOH and treated with 2.5% sodium bisulfite in the presence of 0.5 mM hydroquinone for 16 h at 50 °C. DNA was purified from the excess of salts, and 100 ng of modified DNA was used as a template in the PCR reaction. This reaction was performed under the following conditions: 67 mM Tris HCl, pH 8.8, 16.6 mM ammonium sulfate, 6.7 mM MgCl₂, 10 mM β-mercaptoethanol, and 1.25 mM dNTPs using as primers oligonucleotides 5′-TTAAGTTAGAGCTTCATCCGG-3′ (sense) and 5′-TAACTAAAAATTACCTACCCG-3′ (antisense) for the methylated DNA and 5′-TAAATTTTAGTTAGGGTTAT-TGGT-3′ (sense) and 5′-CACACAACATCACAACACAC-3′ (antisense) for unmethylated DNA. The nucleotides in bold correspond to the uracils generated from cytosine by effect of bisulfite; the nucleotides underlined correspond to the cytosines protected by methylation. These four oligonucleotides correspond to sequences –177/–156 (sense) and –84/–62 (antisense) (methylated) and –182/–158 (sense) and –104/–85 (antisense) (unmethylated) from the human E-cadherin promoter. The conditions of amplification used were 37 cycles composed of 20 s at 96 °C, 30 s at 53°C (unmethylated reaction) or 57 °C (methylated reaction), and 30 s at 72 °C. The presence of amplification products (116 base pairs bp for methylated and 97 base pairs, respectively. The lowest DNA band, also seen in the − DNA control, is caused by primer dimerization.

**RESULTS**

Previously, down-regulation of E-cadherin has been described as an important molecular event in tumorigenesis of melanomas. Therefore, we first aimed to verify the deregulated E-cadherin expression pattern in a panel of different melanoma cell lines and then addressed potential molecular mechanisms involved in down-regulation of the gene. Western blotting with radiolabeled antibody assay lysates derived from eight different melanoma cell lines and from primary human melanocytes revealed significant E-cadherin down-regulation in all melanoma cell lines. As depicted in Fig. 1A, an ~120-kDa E-cadherin signal was present in the skin melanocyte culture (primary human melanocytes) and in the colon carcinoma cell line LoVo, which was not detected in any of the melanoma cell lines. In parallel to the results obtained by Western blots, we observed significantly reduced mRNA expression levels by RT-PCR analyses. After 26 PCR cycles, E-cadherin amplification products were clearly detected in primary human melanocytes and LoVo cells but not in the melanoma cell lines. Consistently, PCR reactions prepared with RNA template from human skin fibroblasts failed to amplify the E-cadherin cDNA fragment (Fig. 1B). Because down-regulation of E-cadherin protein was closely paralleled by reduction of mRNA in all eight different melanoma cell lines, we hypothesized that defective E-cadherin expression in these cell lines involved transcriptional but not translational mechanisms.

To exclude the possibility that structural gene mutations lead to failures in processing mature mRNA, we sequenced the entire 16 exons and flanking intron regions of the E-cadherin gene in all eight melanoma cell lines. No mutations were detected either in the coding region or in the flanking intron-exon boundaries (data not shown). Three intron polymorphisms were detected, none of which affected a region critical for exon-intron processing.

It has been speculated previously that loss of E-cadherin possibly involves absence of AP-2 transcription factors, hypermethylation of the promoter region, or up-regulation of the transcription factor Snail (26, 28, 35). To analyze the effect of AP-2 on E-cadherin expression in melanoma cells, we re-introduced the AP-2 protein by transfecting AP-2 cDNA expression vectors. Results shown in Fig. 2 revealed that transient transfections of both AP-2α and AP-2β expression plasmids did not significantly activate E-cadherin expression. Fluorescence-activated cell sorting analyses of AP-2α-transfected (Fig. 2B) and AP-2β-transfected (Fig. 2C) Mel Im provided only minimally enhanced E-cadherin profiles compared with mock-transfected control cells (Fig. 2A). Identical results were obtained from AP-2-transfected Mel Ju cells, and RT-PCR analyses consistently failed to result in amplification of an E-cadherin cDNA product (data not shown). In contrast, transient transfection of an E-cadherin expression plasmid led to significant E-cadherin immunostaining in a large portion of transfected Mel Im cells (Fig. 2D).

To determine the effect of promoter hypermethylation on E-cadherin expression, the methylation status of all melanoma cell lines was analyzed. In none of the cell lines was promoter methylation detectable (Fig. 3).

Encouraged by recent studies of colon cancers revealing repression of E-cadherin expression by up-regulation of the transcription factor Snail (31, 36), we further analyzed Snail expression patterns and the effect of transfected Snail in melanoma cells. RT-PCR experiments demonstrated significant Snail mRNA expression in all eight different melanoma cell lines and human skin fibroblasts but not in primary human melanocytes and the colon cancer cell line LoVo (Fig. 4). From these results we concluded that significant expression of Snail
Regulation of E-cadherin Expression in Melanoma

A variety of phenotypical alterations distinguish tumor cells from normal cells, the most prominent of which include changes in cellular morphology (referred to as “dedifferentiation”), differences in adhesion, and acquisition of an invasive and metastatic phenotype. In many tissues E-cadherin was identified as a critically important cell-cell adhesion molecule, being a potential molecular target involved in these alterations. Several groups provided clear evidence that E-cadherin is frequently down-regulated in a variety of different human tumors, correlating with dedifferentiation and increased invasiveness in vitro. Disturbance of E-cadherin function by inhibitory antibodies led to induction of invasiveness and vice versa, and enhanced expression of E-cadherin in tumor cells by cDNA transfection experiments inhibited invasion (11, 12, 42). Recent studies have offered even further evidence for a causal tumor suppressor function of E-cadherin in vivo. Germ line-inactivating mutations in the E-cadherin gene have been detected in families with inherited predisposition to gastric and breast carcinoma (43–45).

In malignant melanomas, loss or down-regulation of E-cadherin was first described in vitro (23, 46, 47) and later substantiated by immunohistochemical studies of tumor specimens (4, 5) in vivo. Functional consequences resulting from loss of E-cadherin expression were elicited by Herlyn and co-workers (19–21). Several studies revealed that down-regulation of E-cadherin leads to defects in control of melanocyte proliferation by keratinocytes and to acquisition of an invasive tumor cell phenotype (19–21).

Results from our present study confirm that significant down-regulation of E-cadherin is a frequent event in melanoma cell lines but does not involve mutations in the E-cadherin gene. These data agree with other reports indicating that loss of E-cadherin expression because of structurally inactivating gene mutations occurs very infrequently (48). Mutational inactivation of the gene resulting in uniform loss of E-cadherin expression has been detected in a few gynecological cancers, lobular carcinomas of the breast, and gastric carcinomas (10, 33, 43, 49). In all of these cases, uniform E-cadherin loss correlated with typical scattered, highly dissociated tumor cell growth (10, 27). However, in the vast majority of tumors, loss of E-cadherin is heterogeneous (50) and can be modulated by the tumor microenvironment (51, 52), suggesting that irreversible genetic alterations are not common.

Furthermore, loss of expression of the transcription factor AP-2 as a potential positive regulator of E-cadherin expression was described in other tumors (28). Because loss of AP-2 expression in melanoma was shown by several groups (30, 31, 53), the same mechanism was speculated to be involved in loss of E-cadherin in melanoma but until now was not experimentally proven (29). In this study we could clearly show that expression of AP-2, either α or β, in melanoma cell lines by transient transfection did not lead to expression of E-cadherin. This is in contrast to c-kit (53), in which AP-2 plays an important role in regulating gene expression. Also, melanoma cell adhesion molecule/mucin (31) was first demonstrated to be up-regulated because of loss of AP-2, but recent studies indicated that loss of AP-2 in melanoma is not required for MCAM expression in melanoma (54).

Enhanced methylation of CpG islands in the E-cadherin gene promoter has been proposed as a possible mechanism for E-cadherin inactivation in carcinomas by several reports (26, 27, 55, 56). Our results indicate clearly that hypermethylation of the promoter region is not molecularly linked to down-regulation of E-cadherin expression in melanoma cells. Also Tamura et al. (27) demonstrated that loss of E-cadherin expression in vitro.
vivo was not uniformly associated with promoter hypermethylation and therefore concluded that alterations in promoter methylation patterns are unlikely the only mechanisms involved in switching off E-cadherin expression. Possibly, promoter hypermethylation may result from long-term promoter inactivation by other transcriptional mechanisms rather than representing the primary cause of gene inactivation. In addition, Graff et al. (56) recently showed that hypermethylation may be dynamic, unstable, and changing in relation to signals provided by the tumor microenvironment.

We then analyzed the expression of the transcription factor Snail, which has recently been identified as a potent inhibitor of E-cadherin expression in colon, gastric, and pancreas carcinomas (35, 36).

Significant levels of Snail mRNA expression were detected in all melanoma cell lines that we analyzed but not in primary human melanocytes, indicating that Snail up-regulation is a very frequent event in melanoma cells. Snail expression in these cells is clearly causally involved in regulating E-cadherin expression, because we observed up-regulation of E-cadherin mRNA and protein levels both after transient and stable antisense Snail expression plasmids and, in addition, down-regulation of Snail mRNA expression with antisense Snail-transfected Mel Im cells (Mel Im) and six stably antisense Snail-transfected Mel Im cell clones (MI as1–MI as6) immunoprobed with an E-cadherin antibody. B, quantification of E-cadherin expression on the surface of Mel Im cells versus MI as4 and MI as5 by flow cytometry. Stainings with the specific E-cadherin antibody are displayed as bold profiles; stainings with nonspecific mouse IgG instead of specific primary antibody are displayed as dotted profiles. A, isotype control. B, control transfected Mel Im cells. C, MI as4 cells. D, MI as5 cells. FL1-H, intensity of fluorescence.
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In summary, our results provide further insights into the regulation of E-cadherin expression in malignant melanoma cells and reveal that Snail up-regulation plays significant roles in switching off E-cadherin expression in malignant melanoma cells.

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