CEACAM1-3S Drives Melanoma Cells into NK Cell-Mediated Cytolysis and Enhances Patient Survival

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Abstract

CEACAM1 is a widely expressed multifunctional cell–cell adhesion protein reported to serve as a poor prognosis marker in melanoma patients. In this study, we examine the functional and clinical contributions of the four splice isoforms of CEACAM1. Specifically, we present in vitro and in vivo evidence that they affect melanoma progression and immune surveillance in a negative or positive manner that is isoform specific in action. In contrast with isoforms CEACAM1-4S and CEACAM1-4L, expression of isoforms CEACAM1-3S and CEACAM1-3L is induced during disease progression shown to correlate with clinical stage. Unexpectedly, overall survival was prolonged in patients with advanced melanomas expressing CEACAM1-3S. The favorable effects of CEACAM1-3S related to enhanced immunogenicity, which was mediated by cell surface upregulation of NKG2D receptor ligands, thereby sensitizing melanoma cells to lysis by natural killer cells. Conversely, CEACAM1-4L downregulated cell surface levels of the NKG2D ligands MICA and ULBP2 by enhanced shedding, thereby promoting malignant character. Overall, our results define the splice isoform-specific immunomodulatory and cell biologic functions of CEACAM1 in melanoma pathogenesis.

Introduction

Melanoma is one of the most aggressive types of cancer, and its prevalence has risen faster than any other malignant disease in the western world. Tumor progression starts very early, resulting in a median survival of 6 to 12 months in patients with advanced melanoma (1). Melanoma progression is a complex multistep process orchestrated by a variety of cellular factors, including dysregulation of cell adhesion molecules (2). Evidence has amassed that expression of the multifunctional carcinoembryonic antigen (CEA)-related cell adhesion molecule 1 (CEACAM1) may be involved during this process (3). CEACAM1 belongs to the CEA family within the immunoglobulin superfamily (4) and is expressed in human epithelial (5, 6), endothelial (7), and hematopoietic cells (8, 9). Downregulation of CEACAM1 has been reported in colon (10), prostate (11), and breast cancer (12). Melanocytes are CEACAM negative (13), while high expression levels of CEACAM1 have been detected in melanoma (14) and adenocarcinoma (15). CEACAM1 was reported to inhibit cell proliferation in several tumor entities, excepting melanoma (13, 16, 17). On the tumor cell surface, the CEACAM1 protein has been shown to interact directly with CEACAM1 on immune cells, leading to functional inhibition of those cells (18–21), or to indirectly modulate cancer cell immunogenicity by downregulating their cell surface expression of MHC class I-related molecule A and B (MICA/B) and UL-16-binding protein1 (ULBP; ref. 22), both ligands for the natural killer (NK) gene complex group 2 member D (NKG2D) receptor expressed on malignant cells (23, 24).

Although 12 different splice variants of the human CEACAM1 gene have been reported, only four are shown to be expressed at mRNA level (25, 26). The CEACAM1-4 variants consist of four, CEACAM1-3 of three heavily glycosylated extracellular domains. Both isoforms are trans-membrane anchored and carry either the long (L, 73 aa) or short (S, 10 aa) cytoplasmic domain (27).

CEACAM1 has been controversially discussed as tumor suppressor and driver of invasion (28, 29). Most studies contributing to this discussion were focused on total CEACAM1 or CEACAM1-4L/S. In 2002, Thies and colleagues showed that the CEACAM1 protein expression in primary cutaneous melanoma predicts the development of metastatic disease (30). Furthermore, levels of soluble CEACAM1 in sera from melanoma patients have been shown to inversely correlate with overall survival (31, 32). This spurred discussion of CEACAM1 as a more specific and sensitive biomarker than those currently used, including melan-A, S100β, and HMB45, and has implicated CEACAM1 as a potential novel therapeutic target (14). Nevertheless, none of these studies addressed the impact of the four CEACAM1 splice variants.
Furthermore, the clinical impact and the precise mechanism by which the four CEACAM1 variants modulate melanoma progression are completely unknown. Our present study analyzed the biologic function and clinical relevance of the individual CEACAM1 splice variants in human melanoma biopsies of increasing disease stages and in cell lines, established from patient’s metastases. We show for the first time that CEACAM1-3S, CEACAM1-3L, CEACAM1-4S, and CEACAM1-4L differentially affects cellular function and melanoma progression. Furthermore, we demonstrate that the expression of CEACAM1-3S correlates significantly with the clinical stage and strikingly with a prolonged patient overall survival. Finally, we provide strong evidences that CEACAM1-3S triggers melanoma cells for NK cell-mediated cytolysis by upregulating cell surface expression of MICA and ULBP2, whereas CEACAM1-4L causes the contrary effect due to enhanced shedding of both NKG2D ligands (NKG2DLs).

Materials and Methods

Some Materials and Methods are detailed in the Supplementary Data.

Tissues and cell culture

Malignant melanoma cell lines and biopsies were obtained from the Skin Cancer Biobank of the Dermatology Department, University Hospital Essen, Germany. Informed patient consent and the appropriate Institutional Review Board approval were obtained for all patients. Clinical information including age, gender, stage of disease, tumor load, and survival time was documented and retrieved from the electronic database (Achiver Anyware Medical, Achiver Software). Disease staging was performed according to the staging criteria of the American Joint Committee on Cancer (AJCC; ref. 33). Cell lines were established from malignant melanoma as described before (34, 35). RPMI-1640 supplemented with 10% FCS, 1% penicillin/streptomycin, and 1% l-glutamine (all from PAA Laboratories) was used as culture medium. All cell lines were maintained at 37°C and 5% CO₂ and regularly tested for mycoplasma infection.

Plasmid constructs and transfection

The coding sequences for CEACAM1-3L (NM_001184816.1), CEACAM1-3S (NM_001184816.1), CEACAM1-4S (NM_001712.4), and CEACAM1-4L (NM_001024912.2) were cloned into the pcDNA3.1(-) Neo plasmid (Invitrogen) and verified by sequencing. Constructs were transfected into the Ma-Mel-86a cell line using Metafectene (Biontex) according to the manufacturer’s protocol, and single clones selectively grown in medium containing 1 mg/mL G418 (Carl Roth).

xCELLigence System

The Real-Time Cell Analyzer System was used to analyze cellular functions (36) and cytotoxicity (37). The experiments were performed as described by the manufacturer’s instructions. In short, the half-maximum cell index (Cl₅₀) between 0 hour and 70 hours (Cl₇₀) was used for determination of statistical differences calculated by the Student t test. Cytotoxicity results are presented as percentages of cytolyis determined from normalized cell index (nCI): % of specific lysis = [nCI (no effector) – nCI (effector)]/nCI (no effector) × 100. Experiments were performed in duplicates.

Please refer to the detailed Supplementary Experimental Procedures.

Immunofluorescence

Malignant melanoma cells on coverslips were grown to 90% confluency. Cells were fixed either with methanol or 4% PFA, blocked with 3% BSA/PBS, then incubated at 4°C overnight with primary antibodies in 1.5% BSA/PBS. Primary antibodies were visualized by fluorescent labeled secondary antibodies. Nuclei were counterstained with DAPI (Carl Roth), and fluorescence microscopy was conducted on a Leica SP8 confocal microscope (Leica), Zeiss AxioObserver.Z1 with Apotome and Zeiss ELYRA PS.1 using SIM technology (Zeiss). Antibody information is provided in Supplementary Materials and Methods section.

Flow cytometry

Surface expressions were analyzed using a FACScalibur flow cytometer and the CellQuest Pro software (BD Biosciences), Gallios system (Beckman Coulter) equipped with FlowJo software and Accuri C6 flow cytometer (BD Biosciences). Cells were harvested, incubated with primary antibodies and fluorescence-conjugated secondary antibodies. Isotype matched control antibody staining served as negative control. Dead cells were excluded from measurements by propidium iodide staining. Antibody information is provided in Supplementary Materials and Methods section.

MICA ELISA

Soluble MICA (sMICA) was quantified, using the DuoSet ELISA Development System (R&D Systems) according to the manufacturer’s protocol. Melanoma cells were starved for 24 hours. Supernatant was collected and centrifuged to remove cellular debris. Levels of sMICA were normalized to cell number at time of harvesting.

Enrichment of primary polyclonal NK cells

CD3–CD56⁺ NK cells were enriched from PBMCs of healthy donors using MACS technology (Miltenyi Biotec) according to the manufacturer’s protocol. Enrichment of CD3–CD56⁺ NK cells was confirmed by flow cytometry and ranged between 90% and 95%. NK cells were cultured in the presence of 200 IU/ml IL2 (Chiron) before analyses.

FACS-based cytotoxicity assay

To determine specific lysis of melanoma cells by NK cells, flow-cytometric analysis was performed (38). In brief, melanoma cells (5 × 10⁶ cells/mL) were labeled with 2 nmol/L CFSE for 10 minutes (Invitrogen). Then antibody pretreated NK cells were added to 5 × 10⁴ of CFSE-labeled melanoma cells at various effector to target ratios for 3 hours. 7-AAD (Cayman Chemicals) was added to each sample according to the manufacturer’s protocol. Probes were measured directly in a Gallios flow cytometer (Beckman Coulter). Gating strategy was used as described in Heinemann and colleagues (38).

Statistical analysis

Quantitative expression values between two groups were compared using Wilcoxon-Mann–Whitney tests. In the case that comparisons were to a group with no observable
expression values (stage I/II of CEACAM1-3S), we applied the one-sided t test (against an expected value of 0). We used the Student t test to compare two experimental conditions in functional experiments. Spearman correlations (ρ) were used to assess associations between gene expression levels and tumor stages. Time-to-event data (from melanoma diagnoses until death or the last observation in case of censoring) were displayed using the method of Kaplan–Meier. Given the problems of selecting an appropriate cutoff/cut point value, which is often data driven, we decided to display our results for the "presence of any transcripts" in contrast to "no transcripts." Subgroups in Kaplan–Meier plots were compared using log-rank statistics; in addition, we used Cox regression to the related estimate HR. All reported P values are nominal and two sided. We applied a significance level of 5% and did not adjust for multiple testing.

Results

Analysis of CEACAM1 variants in malignant melanoma

To examine the presence of CEACAM1-3S, CEACAM1-3L, CEACAM1-4S, and CEACAM1-4L in melanoma, we analyzed 46 cell lines established from human melanoma metastases using RT-PCR. We detected CEACAM1 variant expression in 33 of 46 cell lines (72%, Supplementary Table S1). Remarkably, all CEACAM1-positive cell lines expressed CEACAM1-4L in combination with other variants. We assessed relative variant expression by densitometric quantification. For each case (both CEACAM1-4 and CEACAM1-3), the longer (L)-variant was expressed at a higher level in the melanoma cell lines (Supplementary Table S1).

Next we assessed the impact of CEACAM1 splice variants during melanoma progression, by analyzing variant expression in 51 biopsies from melanoma patients that spanned stages I–IV according to the AJCC. First, in 45 of 51 (88%) biopsies, we observed CEACAM1 variant expression (Table 1 and Supplementary Table S2). Surprisingly, both CEACAM1-3 variants were absent in stage I/II melanomas, with only one exception that expressed CEACAM1-3L (Fig. 1A and Supplementary Table S2). CEACAM1-3S and CEACAM1-3L expression was induced during melanoma progression, and significantly positively correlated with clinical stage, reaching the highest expression levels in stage IV tumors (CEACAM1-3S stage III: P = 0.031, stage IV: P = 0.002; CEACAM1-3L stage III: P = 0.002, stage IV: P < 0.0001 vs. stage I/II; Fig. 1A). Furthermore, expression of both CEACAM1-3 isoforms was even higher in late-stage melanoma (stage IV) compared with stage III biopsies (CEACAM1-3S P = 0.004; CEACAM1-3L P = 0.015; Fig. 1A). CEACAM1-4S expression was detected in 40 of 51 (78%) biopsies but did not vary according to stage (Fig. 1A and Supplementary Table S2). CEACAM1-4L expression was detected in all CEACAM1-positive patient biopsies (88%; Supplementary Table S2) and was significantly induced during early disease progression from stages I/II to stage III (P = 0.030, Fig. 1A). Moreover, CEACAM1-4L was either expressed alone or in combination with other isoforms, while no other isoform was expressed without CEACAM1-4L (Supplementary Table S2). In correspondence to our in vitro observations, CEACAM1-3S exhibits the lowest expression intensities, whereas CEACAM1-4L was predominantly expressed (Fig. 1A and Supplementary Table S1).

Then we applied the CEACAM1 isoforms data to analyze the prognostic power. Surprisingly, patients with advanced melanomas expressing CEACAM1-3S showed significantly (P = 0.039) prolonged overall survival compared with patients with melanomas lacking CEACAM1-3S expression (HR, 0.43; 95% confidence interval, 0.19–0.98; Fig. 1B), whereas expression of CEACAM1-

### Table 1. Characteristics of melanoma patients

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Abbreviations: y, years; mm, millimeter; N, number of biopsies.

*According to AJCC criteria 2010.
CEACAM1 variants localize to different intracellular compartments in melanoma cells

To explore CEACAM1 isoform functionality, we stably transfected CEACAM1-3S, CEACAM1-3L, CEACAM1-4S, or CEACAM1-4L, respectively, into the CEACAM-negative cell line Ma-Mel-86a, established from a stage III metastases (Fig. 2). Empty vector Ma-Mel-86a-transfection served as a negative control. Expression of exogenous CEACAM1 isoforms was identified by RT-PCR (Fig. 2A), Western blot analysis (Fig. 2B), and flow cytometry (Fig. 2C).

Next, we investigated the subcellular localization of each CEACAM1 isoform using IHC. Beside their surface expression, the CEACAM1-3S and CEACAM1-3L isoforms were primarily detected in vesicle-like structures that accumulated around the nucleus (Fig. 3A, I and II), whereas the CEACAM1-4S and CEACAM1-4L isoforms were predominantly localized to sites of cell–cell contact on the cell surface (Fig. 3A, III and IV, arrowheads). No CEACAM1 staining was detectable in control cells transfected with empty vector (data not shown). Interestingly, CEACAM1-3S- and CEACAM1-3L-positive vesicle-like structures were arranged like a “string of pearls” orientated toward sites of cell–cell contact (Fig. 3A, I and II, arrows), pointing to a recruitment of both variants to areas of cellular interactions. To analyze how this linear orientation is achieved, CEACAM1-3L and CEACAM1-3S transfectants were immunostained for total CEACAM1 and F-actin. Costaining of CEACAM1-3S transfectants revealed that CEACAM1-positive vesicular structures associated with F-actin fibers in cell protrusions extending toward contact points with adjacent cells (Fig. 3B). Similar observations were made in CEACAM1-3L transfectants (data not shown). Taken together, all CEACAM1 isoforms were predominantly expressed at cell–cell contacts, but cellular localization of the CEACAM1 isoforms varied and was dependent on the presence of the extracellular A2 domain.
CEACAM1 isoforms differently affect migration and invasion of melanoma cells

CEACAM1-4L expression has been shown to enhance the migratory capacity and invasive potential of melanoma cells without affecting cell proliferation (13). We found that expression of the different CEACAM1 isoforms transfected into Ma-Mel-86a did also not alter proliferation (data not shown). To investigate the specific contributions of each CEACAM1 isoform to cellular motility, we monitored the CEACAM1 transfectants in real time by using xCELLigence impedance measurement. Expression of CEACAM1-4S (P = 0.009) and CEACAM1-4L (P = 0.025) had the strongest migration enhancing influence on melanoma cells, compared with control cells (Fig. 4A and B). Also, CEACAM1-3L significantly increased migratory capacity, but to a lesser extent (P = 0.023, Fig. 4A and B). In accordance to this finding, expression of CEACAM1-4S (P = 0.006) and CEACAM1-4L (P = 0.005) resulted in enhanced invasive behavior, which was only slightly increased by CEACAM1-3L (Fig. 4C and D). Interestingly, CEACAM1-3S expression significantly decreased both cell migration (P = 0.031, Fig. 4A and B) and invasion (P = 0.020, Fig. 4C and D) compared with control cells. Analyses of a second set of independently generated CEACAM1 transfectants confirmed these results (data not shown). Now we hypothesized that activity of matrix metalloproteinases (MMP), enzymes known to be key regulators of invasive potential in melanoma cells (40, 41), could be involved in modulating the cellular function of the individual CEACAM1 isoforms. Consequently, we treated our CEACAM1 transfectants with the MMP inhibitor marimastat (42–44), and reassessed invasive capacity after inhibition of MMP activity. In accordance to our hypothesis, marimastat treatment impaired the invasive promoting effect of CEACAM1-4S or CEACAM1-4L (Fig. 5).

CEACAM1-3S and CEACAM1-4L direct tumor immunogenicity by deregulating MICA and ULBP2 expression

It has been reported that the CEACAM1 expression influences the immunogenicity of cancer cells by modulating the surface expression of NKG2D ligands. These data prompted us to analyze the impact of the four CEACAM1 isoforms on the expression of NKG2D ligand MICA and ULBP2 by flow cytometry. Although expression of CEACAM1-3L or CEACAM1-4S did not modulate MICA and ULBP2 expression, significant upregulation of MICA (P = 0.006) and ULBP2 (P = 0.011) on CEACAM1-3S transfectants was detected compared with control cells (Fig. 6A and B). Remarkably, expression of CEACAM1-4L resulted in damped cell surface expression of MICA (P = 0.020) without affecting ULBP2 levels (Fig. 6A and B).

Figure 2.
Transfectants of the human melanoma cell line Ma-Mel-86a show specific expression of CEACAM1 splice variants. A, expression of CEACAM1-4L, CEACAM1-4S, CEACAM1-3L, and CEACAM1-3S transfected into Ma-Mel-86a was analyzed by RT-PCR. Multiple PCR products result from the lack of the A2 domain and the different length of the cytoplasmic domains (CEACAM1-4L = 779 bp; CEACAM1-4S = 726 bp; CEACAM1-3L = 491 bp; CEACAM1-3S = 438 bp). Cells transfected with empty vector served as controls. B, Western blot analyses of the CEACAM1 transfectants. β-actin served as loading control. C, flow-cytometric analyses of CEACAM1 cell surface expression. Cells were stained for CEACAM1 (thick line) and for isotype-matched control (thin line). Numbers indicate mean fluorescence intensity values. For A–C, one representative experiment out of three is shown.
CEACAM1 splice variants localize to different cellular compartments. A, immunofluorescence-based assessment for cellular localization of CEACAM1 (green) in the indicated CEACAM1 isoform transfectants. Nuclei were counterstained using DAPI (blue). White arrowheads, CEACAM1 staining at cell–cell contact sites; white arrow, CEACAM1-positive vesicles arranged in a line toward cell–cell contacts. B, double staining for total CEACAM1 (mAb 4/3/17, green) and F-actin (phalloidin, red) in CEACAM1-3S transfectants; nuclei were counterstained using DAPI (blue). Similar results were observed in CEACAM1-3L cells (data not shown). Representative images show maximum z-projection of confocal optical sections. Analyses were repeated three times by using different passages of each transfectant. Scale bar, 10 μm.

To verify whether CEACAM1-3S indeed affected cell surface expression of NK2G2 ligands, MICA and ULBP2 expression was determined by confocal microscopy. Expression of CEACAM1-3S resulted in increased recruitment of MICA and ULBP2 to the M-Mel-86a-cell surface (Fig. 6C). Parallel we analyzed ligand expression levels in the CEACAM1 transfectants using Western blot analysis. MICA and ULBP2 expression in whole-cell lysates of CEACAM1-3L, CEACAM1-3S, and CEACAM1-4S transfectants was consistent with the flow-cytometric data (Fig. 6D). Furthermore, we confirmed that CEACAM1-3S-transfected cells upregulated MICA and ULBP2 expression (Fig. 6D). Unexpectedly, CEACAM1-4L expression did not alter the total protein expression of MICA and enhanced ULBP2 protein expression compared with control cells (Fig. 6D). These data did not correlate with our flow-cytometric findings, unless the reduced MICA and ULBP2 surface levels in CEACAM1-4L-positive cells would appear due to shedding. Accordingly, we examined the levels of soluble MICA (sMICA) and ULBP2 (sULBP2) in media conditioned by the CEACAM1 transfectants using Western blot analysis, and exclusively detected enhanced amounts of sMICA and sULBP2 in conditioned medium obtained from CEACAM1-4L-expressing cells (Fig. 6E). Quantification of sMICA by ELISA revealed an approximately 2-fold elevation \( (P = 0.033) \) in conditioned medium from CEACAM1-4L–expressing cells compared with control cells (Fig. 6F). Noteworthy, marimastat treatment reduced the level of soluble MICA \( (P = 0.0034) \) to the control level (Fig. 6G).

Next we asked, whether the approximately 2-fold induction of MICA and ULBP2 on the surface of CEACAM1-3S–expressing cells would affect melanoma cell sensitivity to NK cell-mediated recognition and cytolysis. Thus, the cytolytic activity of NK cells was measured by a CFSE/7-AAD cytoxicity assay. As shown in Fig. 6H and I, the sensitivity of CEACAM1-3S–expressing melanoma cells to NK cell-mediated cytolysis was proportional to MICA and ULBP2 cell surface expression. Cytolysis of CEACAM1-3S cells were significantly enhanced compared with control cells at an effector to target ratio of 10:1 \( (P = 0.031, \text{Fig. 6I}) \) with a corresponding outcome using a 5:1 ratio \( (\text{data not shown}) \). Blocking the NK2G2 receptor by antibody significantly reduced NK cell-mediated cytolysis \( (\text{control}, P = 0.0113; \text{CEACAM1-3S, } P = 0.0085) \) compared with cultures to which isotype control antibody was added (Fig. 6I).

To analyze whether the reduced MICA-surface level on CEACAM-4L transfectants could dampen the NK cell-mediated killing, we performed xCELLigence-based cytoxicity assays. Expression of CEACAM1-3S resulted in significant upregulation of NK cell-mediated cytolysis compared with control transfectants (Supplementary Fig. S1A, P = 0.0016). The specific lysis of control \( (P = 0.0275) \) and CEACAM1-3S transfectants \( (P = 0.0137) \) was reduced by blockage of NK2G2 (Supplementary Fig. S1A). The expression of CEACAM1-4L revealed no significant modulatory effect although blockage of NK2G2 significantly reduced cytolysis \( (P = 0.0141, \text{Supplementary Fig. S1A}) \). Nevertheless, data presented in Supplementary Fig. S1A are averaged values of five independent experiments. Therefore, NK cells of five different donors have been used. Notably, two experiments showed reduced killing, two enhanced killing, and one no alteration of the NK cell-mediated killing if CEACAM1-4L was expressed.

Furthermore, we validated the expression of the NK2G2 ligand ULBP1 and ULBP3, the DNAM-1 ligands CD112, CD155 and ligands for NKp30 and NKp46 receptors on CEACAM1 transfectants and control cells by flow cytometry (Supplementary Fig. S2). We found enhanced expression for CD155 in Ma-Mel-86a-CEACAM1-3S \( (P = 0.0124) \), whereas expression of CEACAM1-4S \( (P = 0.0442) \) and CEACAM1-4L \( (P = 0.0117) \) resulted in decreased expression (Supplementary Fig. S2A, I). Also, CD112 was found to be reduced in CEACAM1-4S \( (P = 0.0169) \) and CEACAM1-4L \( (P = 0.0424) \) transfectants (Supplementary Fig. S2A, II). NKp30 and NKp46 did not show altered expression (Supplementary Fig. S2A, III and IV). On the basis of these findings, we focused the cytotoxicity assays on CD155, blocking the NK2G2 receptor, the DNAM-1 receptor, or both on NK cells before coculture with the CEACAM1-3S transfectant and control cells, respectively (Supplementary Fig. S2B). First, 2 hours after NK cell addition \( (E/T = 1:1) \), anti-NK2G2 decreased the lysis of CEACAM1-3S transfectant \( (6.6\%) \), and to lesser extend the vector control \( (1.7\%) \). This confirmed our prior observation \( (\text{Fig. 6I}) \). We observed similar effects utilizing anti-DNAM-1, resulting in reduced cytolysis of CEACAM1-3S \( (15\%) \) and control \( (5.1\%) \) transfectants under corresponding conditions. Nevertheless, simultaneous blockage of NK2G2 and DNAM-1 resulted in...
synergistic effects and reduced the NK cell-mediated lysis of both
target cell lines to a comparable level (CEACAM1-3S: 26%;
control: 21.6%, Supplementary Fig. S2B, 2 hours after NK cell
addition, E/T $\frac{1}{1}$).

Discussion

Tumor progression is a complex multistep process orchestrated by
a variety of cellular factors, such as cell adhesion molecules, but
also influenced by host-derived microenvironmental cell popula-
tions including cells of the immune system. Most recent studies
discussed CEACAM1 as a novel promising target for immuno-
therapy of malignant melanoma patients (3, 45).

Here, we report CEACAM1-3S, CEACAM1-3L, CEACAM1-4S,
and CEACAM1-4L expression in melanoma cell lines and biop-
sies. The isoform expression pattern differed during malignant
progression. Early in tumor establishment (stage I/II), CEACAM1-
4L and CEACAM1-4S were exclusively expressed in 50% of
melanoma biopsies, whereas CEACAM1-4L was expressed to
a higher extend in metastatic melanoma compared with pri-
mary tumors. Interestingly, CEACAM1-3L was predominately
and CEACAM1-3S was solely expressed in progressed

![Figure 4. CEACAM1 splice variants differentially affects cell motility. A–D, functional analysis of CEACAM1 transfectants using the Real-Time Cell Analyzer System (xCELLigence). Verification of migration (A and B) and invasiveness (C and D) of each CEACAM1 variant. xCELLigence analyses were repeated three times, each variant performed in triplicates per assay with corresponding results. A representative experiment is shown. B and D, statistical analyses were performed by comparing the half maximum CI values (CI50) in the range from 0 to 70 hour of each transfectant with control cells by Student t test. The inverted mean values of three independent experiments are shown. *, P < 0.05; **, P < 0.01; error bars, mean ± SEM.](cancerres.aacrjournals.org/article-fig4.png)

![Figure 5. Enhanced invasiveness of CEACAM1-4S and CEACAM1-4L is mediated by MMPs. The xCELLigence system was used to analyze invasive potential of CEACAM1-4L, CEACAM1-4S transfectants in the presence or absence of the MMP inhibitor marimastat compared with CEACAM1-negative control cells in real time. Monitoring was conducted in triplicates and experiments were repeated three times. One representative experiment out of three is shown.](cancerres.aacrjournals.org/article-fig5.png)
Figure 6. CEACAM1-3S enhances the immunogenicity of melanoma cells by upregulating cell surface expression of NKG2D ligands. A, MICA and ULBP2 cell surface expression of control cells compared with CEACAM1 transfectants by flow cytometry. Representative histograms are shown: background fluorescence of control cells (thin black line) or of indicated CEACAM1 transfectants (thin gray line), expression for NKG2D ligands of control cells (thick black line), or of CEACAM1 isoform transfectants (dashed black line). (Continued on the following page.)
melanoma (stage III/IV). Previously, Gambichler and colleagues reported elevated CEACAM1 expression in melanoma compared with benign nevi (39). The data we present suggest that the enhanced CEACAM1 expression levels observed by Gambichler and colleagues in primary melanomas were due to changes in the expression of CEACAM1-4S/L. Thus, they seemed to have underestimated the impact of individual biologic functions of the four CEACAM1 isoforms in melanoma progression and metastasis whose importance has been implicated by our study. The lack of CEACAM1-3S expression in early melanoma (stage I/II) followed by the dramatic increase in de novo expression for both CEACAM1-3 variants (86%) in later stages (stage III/IV) identified CEACAM1-3 and, in particular, CEACAM1-3S as potential novel biomarker for disease progression. This finding, however, requires a prospectively planned longitudinal evaluation.

Cellular functions of CEACAM1 require its localization at the cell surface and CEACAM1 has been shown to be recruited to sites of cell–cell contacts (46). Interestingly, by selective expression of individual CEACAM1 isoforms in melanoma cells, we identified variant-specific cellular localizations that are determined by the extracellular domain. However, CEACAM1-4 variants were primarily membrane associated, whereas CEACAM1-3 isoforms were predominantly localized in vesicular-like structures that accumulated around the nucleus. Schumann and colleagues (47) has previously reported that CEACAM1-S binds to F-actin. Together with our data, this leads to the speculation that CEACAM1-3L and CEACAM1-3S–positive vesicular structures move toward sites of cell–cell contact via association with actin fibers. No evidence currently exists in the literature to identify which specific motor proteins would mediate this process. Sadokova and colleagues described localization of CEACAM1 in lamellipodia, suggesting a potential role in cell motility (48). However, it is still not understood if and how the extracellular domains of CEACAM1 affect downstream effectors, which modulate subsequently the localization of CEACAM1.

Early studies reported enhanced cell migration and invasion after transfection of CEACAM1-L into human melanoma cells, whereas overexpression of the CEACAM1-S variant had no effect (13). Contrary to this report, our data precisely show that CEACAM1-4L and CEACAM1-4S trigger the migratory capacity and invasive potential of melanoma cells, whereas CEACAM1-3S diminishes these cellular properties. In this context, Ebrahimnejad and colleagues has proposed that Tyr-488 within the ITIM domain of the CEACAM1 long cytoplasmic domain is essential for the invasive and migratory effect in CEACAM1-transfected melanoma cells (13). The fact that our study show that the expression of CEACAM1-3L (also containing Tyr-488) influenced migratory and invasive potential less strongly than CEACAM1-4L and CEACAM1-4S, argues against the hypothesis. Moreover, the here shown contradictory effects of CEACAM1-4S and CEACAM1-3S on cell motility, implicate that the modification of function occurs independent of the long cytoplasmic domain. To our knowledge, this is the first report showing that the extracellular domain of CEACAM1 has the potential to modulate cellular functions. The migratory behavior and invasive potential of cancer cells are, of course, multifaceted and strongly influenced by the tumor microenvironment. In this context, our in vitro experiments implicated the involvement of MMPs in regulating CEACAM1-4L and CEACAM1-4S–mediated invasion.

Recently, it was shown that CEACAM1-3L and CEACAM1-3S dampen antitumor immunity by downregulating the surface expression of ligands for the activating NK cell receptor NKG2D in colon cancer cells (22). Contrastingly, our data clearly point to a differential isoform-specific function of CEACAM1 in melanoma cells. In our experiments, CEACAM1-3S expression triggered enhanced expression of the NKG2D ligands MICA, ULBP2, and DNAM-1 ligand CD155 on the melanoma cell surface, which drove these cells to NK cell-mediated cytolysis. In contrast, expression of CEACAM1-4L resulted in reduced cell surface expression of MICA, ULBP2, CD155, and CD112, whereas the lower surface expression of NKG2D ligands was mediated by enhanced shedding of these ligands.

Our differing data on the impact of CEACAM1-4L for NK cell-mediated tumor cell cytolysis could be caused by donor-specific variations. In line with this idea, Markel and colleagues presented data that melanoma patient-derived NK cells show an irregular phenotype and lower levels of NKp46, NKp30, and CD16 while the expression NKG2D was not altered (31). Furthermore, they showed that NK cells from healthy donors behaved differently than patient-derived NK cells. Thus, utilizing melanoma patient-derived NK cells expressing increased amounts of CEACAM1 on their cell surface, could result in stronger interaction with CEACAM1-4L on melanoma cells (Fig. 3A) and consequently show higher susceptibility to CEACAM1-mediated inhibition of NKG2D-triggered lysis.

Interestingly, treatment with marimastat reverted the soluble level of MICA in medium conditioned by CEACAM1-4L–expressing melanoma cells almost back to the level of control cells. These data implicate a possible involvement of MMPs in the regulation of NKG2D ligand shedding by CEACAM1-4L in melanoma cells. Data from Liu and colleagues strengthen our hypothesis. They showed that MMP mediates MICA shedding independent of disintegrin; other MMPs and ADAMs (49). Our data implicate the possibility that CEACAM1-4L–expressing melanoma cells reduce the tumor–immune response by MMP-mediated shedding of NKG2DL but further studies are clearly needed to unravel the involvement of all the players in this process. Taken together, our results provide for the first time evidence that the expression of CEACAM1-3S, CEACAM1-3L, CEACAM1-4S, and CEACAM1-4L differentially affect disease progression in malignant melanoma. Importantly, we clearly demonstrated that CEACAM1 variants
differentially modulate attenuation of immune surveillance by regulating ligand expression for the NKG2D receptor on tumor cells in malignant melanoma. These findings are of major interest, since CEACAM1 is discussed for use in clinical applications of immunotherapy. Caused by the fact that CEACAM1-3S potentially inhibits melanoma cell migration and invasion, and drives these cells through NK cell-mediated cytolysis, whereas CEACAM1-4L is acting in the opposite direction by supporting tumor cell growth in human lung adenocarcinoma cells. PLoS ONE 2010;5:e8747.


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References
CEACAM1 Splice Variants in Melanoma

CEACAM1-3S Drives Melanoma Cells into NK Cell-Mediated Cytolysis and Enhances Patient Survival

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