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

Nico Ullrich, Anja Heinemann, Elena Nilewski, Inka Scheffrahn, Joachim Klode, André Scherag, Dirk Schadendorf, Bernhard B. Singer, and Iris Helfrich

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.

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 CEA-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 Laboratoryware and Accuri C6) 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_001184813.1), CEACAM1-3S(NM_001184816.1),CEACAM1-4L(NM_001712.4), and CEACAM1-4S (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 (IC₅₀) between 0 hour and 70 hours (IC₅₀) was used for determination of statistical differences calculated by the Student t test. Cytotoxicity results are presented as percentages of cytosis 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

Melanoma cells on coverslips were grown to 90% confluence. 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). Cating 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 (\( p \)) 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 identified correlation of CEACAM1-3S with patient clinical stage and overall survival**

Total CEACAM1 expression has been shown to be significantly higher in melanomas compared with benign nevi (39). 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 alone or in combination with other isoforms, while no other CAM isoform was expressed without CEACAM1-3L (Supplementary Table S2). In correspondence to our isoform data, CEACAM1-4L was predominantly expressed (Fig. 1A and Supplementary Table S2). CEACAM1-3S exhibits the lowest expression intensities, whereas absent in stage I/II melanomas, with only one exception that observed CEACAM1 variant expression (Table 1 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 S2).

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-

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 S2).

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**

<table>
<thead>
<tr>
<th>Age at biopsy removal, y</th>
<th>Total (( N = 51 ))</th>
<th>Biopsies positive for specific CEACAM1 splice forms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CEACAM1-3S (( N = 26 ))</td>
<td>CEACAM1-3L (( N = 38 ))</td>
</tr>
<tr>
<td>Mean</td>
<td>62</td>
<td>62</td>
</tr>
<tr>
<td>Range</td>
<td>34–90</td>
<td>34–85</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>28</td>
<td>13</td>
</tr>
<tr>
<td>Female</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td>Clinical stage*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I/II</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Stage III</td>
<td>21</td>
<td>8</td>
</tr>
<tr>
<td>Stage IV</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>Breslow's depth (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT1 (&lt;1.00)</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>pT2 (1.01–2.00)</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>pT3 (2.01–4.00)</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>pT4 (&gt;4.00)</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Unknown</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Localization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin</td>
<td>42</td>
<td>21</td>
</tr>
<tr>
<td>Other</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Ulceration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>18</td>
<td>7</td>
</tr>
<tr>
<td>No</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>Unknown</td>
<td>20</td>
<td>9</td>
</tr>
</tbody>
</table>

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

*According to AJCC criteria 2010.
3L, CEACAM1-4S, and CEACAM1-4L did not affect the clinical outcome (data not shown).

**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.

Figure 1. CEACAM1-3S expression correlates with clinical stage and overall survival in patients with melanoma. A, scatter plot for expression of indicated CEACAM1 splice variant relative to total CEACAM1. Patients are grouped according to their clinical stages, based on the AJCC system (stage I/II, N = 8; stage III, N = 21; stage IV, N = 22). Mann–Whitney Test; *, one-sampled t test to hypothetical value = 0; **, P < 0.05; ***, P < 0.01; ****, P < 0.001; error bars, mean ± SEM. B, Kaplan–Meier curve of patients (N = 51) with (N = 26, solid line) or without (N = 25, dotted line) CEACAM1-3S expression. Short vertical lines represent censored observations. Statistical significance was calculated by log-rank test.
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). In accordance to this finding, expression of CEACAM1-3L ($P = 0.006$) 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).
was consistent with the CEACAM1-3L, CEACAM1-3S, and CEACAM1-4S transfectants analysis. MICA and ULBP2 expression in whole-cell lysates of sion levels in the CEACAM1 transfectants using Western blot

Mel-86a-cell surface (Fig. 6C). Parallel we analyzed ligand expres-

resulted in enhanced recruitment of MICA and ULBP2 to the Ma-

determined by confocal microscopy. Expression of CEACAM1-3S

expression of NKG2D ligands, MICA and ULBP2 expression was

significantly enhanced compared with control cells at an
effector to target ratio of 10:1 (P = 0.031, Fig. 6I) with a corre-

sponding outcome using a 5:1 ratio (data not shown). Blocking
the NKG2D receptor by antibody significantly reduced NK

cell-mediated cytolysis (control, P = 0.0113; 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 CEAC-

CAM1-4L transfectants could dampen the NK cell-mediated kill-
ning, we performed xCelligence-based cytotoxicity assays. Expression
of CEACAM1-3S resulted in significant upregulation of NK

cell-mediated cytolysis compared with control transfectants (Sup-

plementary Fig. S1A, P = 0.0016). The specific lysis of control (P = 0.0257) and CEACAM1-3S transfectants (P = 0.0137) was

reduced by blockage of NKG2D (Supplementary Fig. S1A). The

expression of CEACAM1-4L revealed no significant modulatory
effect although blockage of NKG2D significantly reduced cytolysis
(P = 0.0141, Supplementary Fig. S1A). Nevertheless, data pre-


cented 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 NKG2D ligand
ULBP1 and ULBP3, the DNAM-1 ligands CD112, CD155 and ligands for NKp30 and NKp46 receptors on CEACAM1 transfec-
tants and control cells by flow cytometry (Supplementary Fig. S2).

We found enhanced expression for CD155 in Ma-Mel-86a-CEA-
CAM1-3S (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 CEACAM1-3S (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 NKG2D 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-NKG2D decreased the lysis of CEACAM1-3S transfectant (6.6%), and to lesser extend the vector control (1.7%). This confirmed our prior observation (Fig. 6H). 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 NKG2D and DNAM-1 resulted in

To verify whether CEACAM1-3S indeed affected cell surface expression of NKG2D ligands, MICA and ULBP2 expression was
determined by confocal microscopy. Expression of CEACAM1-3S
resulted in enhanced recruitment of MICA and ULBP2 to the Ma-

Mel-86a-cell surface (Fig. 6C). Parallel we analyzed ligand expres-

sion 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). Further-

more, we confirmed that CEACAM1-3S–transfected cells upregu-

lated 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 exclu-

sively 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 medi-

um 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 cytotoxicity assay. As shown in Fig.

6I 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
effectortarget ratio of 10:1 (P = 0.031, Fig. 6I) with a corre-

sponding outcome using a 5:1 ratio (data not shown). Blocking

Furthermore, we validated the expression of the NKG2D ligand
ULBP1 and ULBP3, the DNAM-1 ligands CD112, CD155 and ligands for NKp30 and NKp46 receptors on CEACAM1 transfec-
tants and control cells by flow cytometry (Supplementary Fig. S2).

We found enhanced expression for CD155 in Ma-Mel-86a-CEA-
CAM1-3S (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 CEACAM1-3S (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 NKG2D 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-NKG2D decreased the lysis of CEACAM1-3S transfectant (6.6%), and to lesser extend the vector control (1.7%). This confirmed our prior observation (Fig. 6H). 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 NKG2D 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 = 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 populations including cells of the immune system. Most recent studies discussed CEACAM1 as a novel promising target for immunotherapy of malignant melanoma patients (3, 45).

Here, we report CEACAM1-3S, CEACAM1-3L, CEACAM1-4S, and CEACAM1-4L expression in melanoma cell lines and biopsies. 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 primary tumors. Interestingly, CEACAM1-3L was predominately and CEACAM1-3S was solely expressed in progressed melanoma.
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 biological functions of the four CEACAM1 isoforms on 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 CEACAM1-3 variants (66%) 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–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. Sadekova 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 NK2GD in colon cancer cells (22). Contrarily, 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 NK2GD ligands MICA, ULBP2, and DNAM-1 ligand CD155 on the melanoma cell surface, while 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 NK2GD 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 NK2GD 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 NK2GD-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 NK2GD ligand shedding by CEACAM1-4L in melanoma cells. Data from Liu and colleagues strengthen our hypothesis. They could show 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 NK2DL1 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

(Continued)
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 progression, it is indispensable to define the impact of different CEACAM1 variants to secure therapeutic efficiency. Hence, determination of all four CEACAM1 isoforms opens up new possibilities for diagnosis and prognosis of melanoma patients.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: N. Ullrich, A. Heinemann, I. Scheffrahn, D. Schadendorf, B.B. Singer, I. Helfrich


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Ullrich, A. Heinemann, I. Scheffrahn, I. Klode, B.B. Singer, I. Helfrich

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Ullrich, A. Heinemann, I. Scheffrahn, A. Scherag, B.B. Singer, I. Helfrich

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Ullrich, I. Scheffrahn, I. Klode, B.B. Singer, I. Helfrich

Writing, review, and/or revision of the manuscript: N. Ullrich, A. Heinemann, I. Scheffrahn, J. Klode, A. Scherag, D. Schadendorf, B.B. Singer, I. Helfrich

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Ullrich, I. Scheffrahn, J. Klode, B.B. Singer, I. Helfrich

Study supervision: N. Ullrich, B.B. Singer, I. Helfrich

**Acknowledgments**

The authors thank Christiane Breuer, Birgit Maranaka-Hüwel, Bärbel Gobs-Holvek, Mohamed Benchellal, and Nadine Hochard for excellent technical assistance; Antje Sacker for providing Skin Cancer Biobank information of melanoma samples and established melanoma cell lines; Dr. Peter Horn (Institute for Transfusion Medicine) for providing PBMCs; and Dr. Ingo Stoffels for providing fresh melanoma biopsies. Microscopic imaging and analyses were done in the Imaging Center Essen (IMCES) in consultation with Dr. Anthony Squire and Prof. Matthias Gunzer and professional manuscript editing made by Dr. Kathy Astrahtanteff.

**Grant Support**

This work was supported by Hiege Stiftung gegen Hautkrebs (I. Helfrich) and by the Federal Ministry of Education and Research (BMBF), Germany, FKZ: 01EO1002 (A. Scherag).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 11, 2014; revised January 30, 2015; accepted February 17, 2015; published OnlineFirst March 5, 2015.

**References**


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

Nico Ullrich, Anja Heinemann, Elena Nilewski, et al.

Cancer Res  Published OnlineFirst March 5, 2015.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-14-1752

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2015/03/07/0008-5472.CAN-14-1752.DC1 http://cancerres.aacrjournals.org/content/suppl/2015/04/17/0008-5472.CAN-14-1752.DC2

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.