Assessing the Impact of Targeting CEACAM1 in Head and Neck Squamous Cell Carcinoma

Kenric Tam, MM1, David W. Schoppy, MD, PhD1, June Ho Shin, PhD1, Joshua K. Tay, MBBS1,2, Uriel Moreno-Nieves, PhD1, David C. Mundy1, and John B. Sunwoo, MD1

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

Abstract

Objective. In conjunction with advances made in cytotoxic chemotherapy, radiation, and surgery, immunotherapy has emerged as a fourth modality of treatment for head and neck squamous cell carcinoma (HNSCC). Understanding the mechanisms by which HNSCC evades immune-mediated control will aid in the development of new therapies to augment an antitumor immune response. Carcinoembryonic antigen–related cell adhesion molecule 1 (CEACAM1) is a cell surface receptor that is expressed on malignant cells and lymphocytes such as natural killer (NK) cells. We sought to determine whether tumor-derived CEACAM1 inhibits NK cell cytotoxicity and whether blockade of CEACAM1 restores antitumor immunity.

Study Design. In vitro HNSCC cell line study.

Setting. Research laboratory.

Subject and Methods. We utilized a real-time cell analyzer to assess NK cell cytotoxicity against an oral squamous cell carcinoma cell line after modulating CEACAM1 expression by cytokines and shRNA knockdown of CEACAM1 expression.

Results. NK cells and HNSCC cells both demonstrated cytokine-inducible expression of CEACAM1. Coincubation of NK cells and HNSCC cells resulted in the upregulation of CEACAM1 on the tumor cells. When compared with CEACAM1− cells, CEACAM1+ tumor cells exhibited increased cell growth and increased size and number of organoids in 3-dimensional culture. Notably, CEACAM1− HNSCC cells were more resistant to NK cell–mediated killing, but the inhibited expression of CEACAM1 by an shRNA construct restored NK cell cytotoxicity.

Conclusion. Together, these data indicate that CEACAM1 acts as an inducible checkpoint molecule, and they support the idea that targeting CEACAM1 could serve as a novel immunotherapy approach in HNSCC.

Keywords

head and neck squamous cell carcinoma, CEACAM1, NK cells, immunotherapy, immuno-oncology

Received August 27, 2017; revised December 8, 2017; accepted January 11, 2018.

Despite improvements in treatment and diagnosis of head and neck squamous cell carcinoma (HNSCC), patients with advanced-stage disease have a high rate of recurrence, ranging from 25% to 50%, depending on the primary site and association with human papilloma virus.1,2 Over the past 30 years, survival rates have improved minimally, and only 30% to 60% of patients can expect to be cured.3,4 In conjunction with advances made in cytotoxic chemotherapy, radiation, and surgery, immunotherapy has emerged in recent years as a promising fourth modality of treatment for HNSCC. Understanding the fundamental mechanisms by which HNSCC evades the immune system will aid in the development of new therapies and strategies to augment the antitumor immune response.

Carcinoembryonic antigen–related cell adhesion molecule 1 (CEACAM1; CD66a, BGP) is expressed by natural killer (NK) cells and T cells, as well as tumors cells, including HNSCC cells.5,6 CEACAM1 is a negative regulator of NK cells and inhibits cell-mediated cytotoxicity through...
homophilic interactions between NK cells and tumor cells.\textsuperscript{7–9} There are 11 CEACAM1 isoforms—4 of which are expressed at the mRNA level—with differing numbers of extracellular domains (3 or 4) and either a short or long cytoplasmic tail.\textsuperscript{7} The long intracellular domain (CEACAM1-L) contains immunoreceptor tyrosine-based inhibitory motifs, which can transmit inhibitory signals that decrease cell effector functions, such as cytotoxicity or degranulation.\textsuperscript{7} Melanoma cell lines avoid NK cell killing through homotypic CEACAM1 interactions.\textsuperscript{10} Furthermore, patients with CEACAM1\textsuperscript{+} melanoma cells demonstrate poor outcomes associated with metastatic disease.\textsuperscript{11,12}

Like melanoma, HNSCC also expresses CEACAM1, and overexpression of CEACAM1 in HNSCC is associated with a poor prognosis, higher clinical stage, recurrence, and lymph node metastasis.\textsuperscript{5,13} In this study, we investigate the inducible expression of CEACAM1 on HNSCC cells and the ability of CEACAM1 to inhibit NK cell–mediated cytosis. Furthermore, we examine whether blockade of CEACAM1 restores NK cell cytotoxicity and could therefore serve as a novel immunotherapy against HNSCC.

**Methods**

**NK Cell Enrichment and Culture Conditions**

Leukocyte-enriched human blood was obtained from the Stanford Blood Center in accordance with an Institutional Review Board–approved protocol. NK cells were isolated with RosetteSep Human NK Cell Enrichment Cocktail (Stemcell Technologies, Vancouver, Canada). NK cell medium consisted of RPMI 1640 (Corning Inc, Corning, New York), 10% fetal bovine serum (FBS; Omega Scientific, Tarzana, California), 1% penicillin-streptomycin (10,000 U/mL; Thermo Fisher Scientific, Waltham, Massachusetts), and 100 IU/mL of IL-2 (Teceleukin; Roche, Basel, Switzerland). For expansion, NK cells were plated with 100-Gy irradiated K562 feeder cells in U-bottom 96-well plates at a ratio of 30,000 NK cells to 60,000 K562 cells per well. Prior to all experiments, expanded cells were sorted for CD3\textsuperscript{+}CD56\textsuperscript{+} populations to &gt;95% purity.

**PCI-13 Culture Conditions and IFN\textgamma Induction**

PCI-13 cells were cultured in filtered DMEM (Corning), 10% FBS (Omega Scientific), and 1% penicillin-streptomycin (Thermo Fisher Scientific). For stimulation of PCI-13 with IFN\textgamma, 50 U/mL of IFN\textgamma (PeproTech, Rocky Hill, New Jersey) was added to the culture medium for 24 hours prior to reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) or flow cytometry analysis.

**Coculture of NK Cells and PCI-13**

Fifty thousand PCI-13 cells and 25,000 CD3\textsuperscript{+}CD56\textsuperscript{+} NK cells were seeded in each well of a 48-well plate and cocultured for 24 hours in RPMI/10% FBS medium. After 24 hours, attached and suspended cells were harvested. Cells were stained with DAPI and antibodies against CEACAM1 (FAB2244P; R&D Systems, Minneapolis, Minnesota) and CD56 (HCD56; Biolegend, San Diego, California) and analyzed with flow cytometry. To detect intracellular IFN\textgamma, 1 \muL/mL of BD GolgiPlug was added at the start of the cocultures. Attached and suspended cells were then harvested, fixed, and permeabilized with BD Cytofix/Cytoperm kit (BD Biosciences, Franklin Lakes, New Jersey) before staining with antibodies against IFN\textgamma (4S.B3, Biolegend).

**NK Cell Cytotoxicity against PCI-13 Assay**

NK cell cytotoxicity assays were performed with a real-time cell analyzer (xCELLigence RTCA SP; ACEA Biosciences, San Diego, California), which quantifies killing and detachment of target tumor cells by measuring changes in impedance across gold nanoparticle–coated plates. NK cells were cultured with feeder cells and IL-2 for 8 days, then sorted for CD56\textsuperscript{+}CEACAM1\textsuperscript{+} and CD56\textsuperscript{+}CEACAM1\textsuperscript{+} NK cell populations. PCI-13 cells (10,000 cells/well) were seeded onto E-Plate VIEW 96 Well Plates (ACEA Biosciences) and expanded for 12 hours, followed by the addition of expanded NK cells (10,000 cells/well). Based on the xCELLigence RTCA SP, results were collected in real time with RTCA software 2.0 (ACEA Biosciences) with cell indices recorded at 15-minute intervals. With xIMT immunotherapy (ACEA Biosciences), percentage-specific cytosis was calculated over time. Controls included PCI-13 alone and expanded NK cells alone.

**PCI-13 shRNA CEACAM1 Cell Line**

TRIPZ Human CEACAM1 shRNA (Dharmacon, Lafayette, Colorado), pCMV-dR8.2 (Addgene, Cambridge, Massachusetts), and pCMV-VSV-G (Addgene) were transfected into 293T cells with the CalPhos Mammalian Transfection Kit (Clontech Laboratories). Virus was harvested at 48, 72, and 96 hours posttransfection and pooled after storage at &minus;80°C. The supernatant was filtered through a 0.45-\mu m polyethersulfone filter and centrifuged at 120,000 \times g for 1.5 hours at 4°C. The viral pellet was resuspended in phosphate-buffered saline, and PCI-13 cells were transduced. Seventy-two hours posttransduction, PCI-13 cells were selected with puromycin (1 \mu g/mL) for 48 hours. After doxycycline induction, TURBO-RFP\textsuperscript{+} cells were sorted with a BD FACSaria II (BD Biosciences).

**NK Cell Cytotoxicity against PCI-13 shCC1 Assay**

PCI-13 shCC1 cells (10,000 cells/well) were seeded on E-Plate View 96 Well Plates (ACEA Biosciences) and cultured with IFN\textgamma± doxycycline for 24 hours. Subsequently, expanded NK cells were sorted for CD56\textsuperscript{+}CEACAM1\textsuperscript{+} or CD56\textsuperscript{+}CEACAM1\textsuperscript{−} populations and then cocultured with plated PCI-13 shCC1 at a 1:1 effector:target ratio. Results were collected in real time with the xCELLigence RTCA SP.

**Growth Rate of PCI-13 and PCI-13 shCC1 Cells**

Growth rate was measure with an xCELLigence RTCA SP and expressed as cell index, which is directly proportional to number of cells. X-axis time units were in hours. PCI-13 and PCI-13 shCC1 cells were plated and expanded with
IFNγ (50 U/mL). At 18 hours, doxycycline (1 μg/mL) was added to PCI-13 and PCI-13 shCC1.

Organoid Culture

A 30-mm Millicell insert (Millipore, Billerica, Massachusetts) was placed in the center of a 5-cm culture dish (Corning). A 900-μL mixture of Cultrex Rat Collagen I, Lower Viscosity (R&D Systems), F12 (Thermo Fisher Scientific), and Solution C was added to the Millicell insert and allowed to solidify at 37°C. One hundred thousand PCI-13 or PCI-13 shCC1 cells were resuspended with 900 μL of cold collagen mixture and added to the solidified collagen matrix. Organoids were maintained and expanded with DMEM media containing 10% FBS and IFNγ (50 U/mL) ± doxycycline for 2 weeks.

RNA Extraction and RT-qPCR Analysis

Cells were extracted with Trizol (Thermo Fisher Scientific); RNA was purified with the RNaseasy Mini Kit (Qiagen); and cDNA was synthesized with the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific). RT-qPCR was performed with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). Gene expression was normalized to β-actin and calculated as 2^(-ΔΔCt).

Flow Cytometry Analysis and Cell Sorting

Cells were incubated with Human FeR Blocking Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 minutes at 4°C in complete growth medium, followed by the addition of staining antibodies for 1 hour at 4°C. CD56 (HCD56), CD3 (UCHT), IgG2b (MPC-11), and IgG1 (MOPC-21) antibodies were acquired from Biolegend. Anti-CEACAM1 (FAB2244P) was acquired from R&D Systems. DAPI was used as viability dye.

Statistical Analysis

Experiments were replicated to ensure reproducibility and included biological and technical triplicates. All statistical analysis was performed with a 2-tailed Student’s t test in Excel (Microsoft). Results were considered significant if P < .05.

Results

Coincubation of NK Cells and Tumor Cells Upregulates CEACAM1

Because activation CEACAM1 on NK cells and other lymphocytes is known to inhibit cell-mediated cytotoxicity, we hypothesized that chronic immune pressure against tumor cells upregulates CEACAM1 on HNSCC, similar to other checkpoint inhibitory receptors.7-9 To test this hypothesis, we cocultured freshly isolated primary human NK cells with PCI-13, an oral cavity HNSCC cell line, for 24 hours and measured CEACAM1 and IFNγ expression by flow cytometry. CEACAM1 expression on the cocultured PCI-13 cells was compared with PCI-13 cultured alone in growth medium. Similarly, CEACAM1 expression on the cocultured NK cells was compared with NK cells cultured alone. After coculture with NK cells, 52.1% of PCI-13 cells expressed CEACAM1, as opposed to 19.2% of control (Figure 1; P < .01). Coculture also resulted in a significant increase of CEACAM1 expression on CD56+ NK cells, from 5.1% to 16.4%, and IFNγ expression by the NK cells, from 1.6% to 8.5% (Figure 1; P < .01).

Given the upregulation of CEACAM1 expression on the NK cells and the tumor cells, we sought to determine whether this concurrent expression of CEACAM1 could inhibit NK cell–mediated cytotoxicity. Because freshly isolated human NK cells do not express CEACAM1 but IL-2-activated NK cells upregulate expression of this molecule, we expanded human primary NK cells in K562 feeder culture with IL-2 for 8 days. Cell viability, as determined by DAPI staining, was 89.8% (data not shown). When percentage-specific cytosis of CEACAM1+ and CEACAM1− CD56+ NK cell populations were compared against PCI-13 HNSCC cells, we found that CEACAM1+ NK cells demonstrated a significantly greater percentage-specific cytosis (76.5%) than CEACAM1+ NK cells (56.2%; Figure 2; P < .01).

Cytokine Induction of CEACAM1 on PCI-13 and NK Cells

Because the number of IFNγ+ NK cells increased after coculture with tumor cells and because one of the principal effector functions of activated NK cells is the production of IFNγ, we hypothesized that upregulation of CEACAM1 on PCI-13 after coculture with NK cells may be induced by this cytokine. To assess this, PCI-13 cells were cultured in growth medium containing recombinant human IFNγ (50U/mL) for 24 hours. Using flow cytometry, we observed that incubation of the tumor cells with IFNγ increased the percentage of CEACAM1+ expression from 20% to 72% (Figure 3A and 3B; P < .01). Furthermore, RT-qPCR analysis confirmed that IFNγ induces CEACAM1 expression at the transcription level and that the HNSCC tumor cells predominately express the short isoform of CEACAM1 (CEACAM1-S), which lacks the cytoplasmic domain (Figure 3C). Note, expression of the short isoform is 1 order of magnitude greater than the long isoform (CEACAM1-L).

As mentioned, primary NK cells isolated from peripheral human blood do not express CEACAM1 at baseline. However, stimulation of primary NK cells with IL-2 (100 IU/mL) for 24 hours resulted in a significant increase in CEACAM1 expression to 10.3% (Figure 4A and 4B; P < .01). Furthermore, the expansion of primary NK cells in K562 feeder culture and IL-2 for 8 days resulted in a dramatic increase of CEACAM1+ NK cells (65.3%, P < .01). In contrast to PCI-13 cells, RT-qPCR demonstrated that NK cells predominately express the long isoform of CEACAM1 (Figure 4C; P < .01), which contains immunoreceptor tyrosine-based inhibitory motifs.

CEACAM1+ PCI-13 Tumor Cells Exhibit Increased Growth and Resistance to NK cell cytotoxicity

To determine whether blockade of CEACAM1 restores NK cell killing of tumor cells, we transfected a plasmid
Figure 1. Carcinoembryonic antigen–related cell adhesion molecule 1 (CEACAM1) is upregulated on PCI-13 and natural killer (NK) cells after coculture. (A) PCI-13 and NK cells were cocultured and analyzed by flow cytometry. (B) Percentage CEACAM1 − PCI-13 and NK cells. n = 3. Error bars indicate SEM. *P < .05. **P < .01.

Figure 2. CEACAM1 + natural killer (NK) cells exhibit decreased cytolysis against PCI-13. (A) Percent specific cytolysis of CD56 + CEACAM1 + and CD56 + CEACAM1 − NK cells against PCI-13, measured by a real-time cell analyzer. (B) Percent specific cytolysis at 5 hours. Effector:target = 1:1. n = 3. Error bars indicate SEM. **P < .01. CEACAM1, carcinoembryonic antigen–related cell adhesion molecule 1.
containing a doxycycline-inducible Tet-ON CEACAM1 shRNA into PCI-13 cells. While exposure of this inducible CEACAM1-shRNA cell line (PCI-13 shCC1) to IFN-γ for 24 hours resulted in an increase of CEACAM1 expression, the addition of doxycycline significantly inhibited all CEACAM1 isoforms (Figure 5).

Using the xCELLigence RTCA SP, we compared the growth curves of PCI-13 shCC1 with and without doxycycline. PCI-13 shCC1 and PCI-13 parental cell lines were plated with medium containing IFN-γ and allowed to expand for 18 hours, at which point doxycycline was added (Figure 3).
6A). PCI-13 shCC1 + doxycycline + IFNγ (dashed, orange) had reduced growth compared with PCI-13 shCC1 + IFNγ (without doxycycline; solid, blue); that is, CEACAM1 PCI-13 expanded more slowly than CEACAM1 PCI-13. The addition of doxycycline did not affect the control, indicating that there was no direct toxicity by the doxycycline.

Figure 4. IL-2 induces carcinoembryonic antigen–related cell adhesion molecule 1 (CEACAM1) on natural killer (NK) cells. (A) CEACAM1 expression of NK cells in varying culture conditions. Horizontal line indicates CEACAM1 expression. (B) Percentage CEACAM1 NK cells measured by flow cytometry. (C) Reverse transcription quantitative real-time polymerase chain reaction of CEACAM1 isoforms of NK cells. n = 3. Error bars indicate SEM. *P < .05. **P < .01.
Similarly, when grown in a 3-dimensional collagen culture with and without doxycycline, CEACAM1\(^+\) cells formed a greater number and size of organoids as compared with CEACAM1\(^-\) cells (Figure 6B).

CEACAM1 expression on tumor cells inhibited CEACAM1\(^+\)-activated NK cells (Figure 2). To investigate this further, we utilized the inducible CEACAM1 shRNA PCI-13 cells and assessed cytolysis by NK cells (Figure 7). Because CEACAM1 contributed to greater proliferation over a long time course (>30 hours), we assessed killing at 5 hours using the ACEA Biosciences xCELLigence RTCA SP. To further diminish the role of proliferative differences in our cytotoxicity assay, percentage-specific cytolysis was calculated with CEACAM1\(^+\) and CEACAM1\(^-\) PCI-13 alone as controls; furthermore, CEACAM1\(^+\) PCI-13 control cells had a lower cell index (ie, fewer cells) than that of CEACAM1\(^+\) PCI-13. We consistently observed significantly greater killing of CEACAM1\(^+\) PCI-13 cells (cultured with doxycycline) than CEACAM1\(^+\) PCI-13 cells (no doxycycline). Interestingly, the difference in cytolysis was independent of whether CEACAM1\(^+\) or CEACAM1\(^-\) NK cells were used in the killing assay.

**Discussion**

In this study, we examined a mechanism by which HNSCC evades and inhibits the antitumor immune response. HNSCC tumor cells undergoing chronic immune pressure and cytokine stimulation from NK cells upregulate CEACAM1, which in turn inhibits NK cell killing and cytotoxicity (Figure 8). We show that inhibition of tumor cell CEACAM1 with an inducible shRNA construct is sufficient to restore NK cell killing and effector functions. These data suggest that blockade of CEACAM1 with a targeted antibody or small molecule could serve as a novel immunotherapy for HNSCC.

Tumor-derived CEACAM1 inhibits T-cell- and NK cell–mediated cytotoxicity through transmolecular interactions with lymphocyte CEACAM1 or Tim-3. Transhomophilic binding of target cell–derived CEACAM1 and CEACAM1 expressed on NK cells inhibits NK cell cytotoxicity through reduction of NKG2D.\(^8,10\) Melanoma expressing CEACAM1-3S inhibits NK cell killing by binding to NK-derived CEACAM1-3L, and nearly all melanoma tumor-infiltrating...
lymphocytes express CEACAM1. An antibody targeted against CEACAM1 was sufficient to restore NK cell killing against melanoma tumors. Tumor-derived CEACAM1 also interacts with Tim-3 on T cells through their respective N-terminal domains, resulting in T-cell inhibition and exhaustion.

We demonstrate that NK cell immune pressure upregulates CEACAM1 on HNSCC tumor cells as well as primary human NK cells (Figure 1). In addition, coincubation of NK cells and PCI-13 tumor cells results in an increased number of IFNγ-producing NK cells (Figure 1). This suggests that tumor cells, when placed under immune pressure, upregulate CEACAM1 to inhibit NK cell killing. The mechanism by which tumor cells upregulate CEACAM1-S is through IFNγ, a cytokine secreted by NK cells (Figure 3). IL-2 induces predominantly CEACAM1-L isoforms on primary human NK cells (Figure 4). The CEACAM1-L isoforms have an intracellular immunoreceptor tyrosine-based inhibitory motif, suggesting that activation of this pathway inhibits immune function. To determine whether CEACAM1 inhibits NK cell effector functions, we compared percentage-specific cytolysis of CEACAM1+ and CEACAM1− NK cells against PCI-13 tumor cells and found that CEACAM1+ NK cells had 20% less killing (P < .01; Figure 2).

In the absence of a commercially available monoclonal antibody that targets the N-terminus active site of CEACAM1 receptor, we created a PCI-13 cell line with an inducible CEACAM1 shRNA construct. Interestingly,
CEACAM1⁺ PCI-13 cells exhibited an increased rate of growth when compared with CEACAM1⁻ PCI-13 cells and formed a greater quantity and size of organoids in 3-dimensional culture (Figure 6). The mechanism by which CEACAM1 confers a tumor cell–intrinsic proliferation advantage is not known; current efforts are underway to understand this. In addition to the growth advantage, CEACAM1⁺ PCI-13 cells were more resistant to killing as compared with CEACAM1⁻ PCI-13 cells, regardless of NK cell CEACAM1 expression. Interestingly, this suggests that tumor-derived CEACAM1 may be inhibiting NK cell cytotoxicity through an additional signaling pathway (Figure 7). Although the mechanism by which this inhibition occurs is not well understood, the cytoplasmic portion of CEACAM1 possesses 2 immunoreceptor tyrosine-based inhibitory motif domains that probably mediated this effect.

In summary, we identified CEACAM1 as one of the mechanisms by which HNSCC evades NK cell killing, and we demonstrated that inhibition of CEACAM1 restores NK cell killing. These data suggest that CEACAM1 acts as a checkpoint molecule and that a blocking antibody against CEACAM1 could release its immunosuppressive effects and serve as a novel immunotherapeutic approach similar to blockade of the PD-1:PD-L1 axis.

Author Contributions
Kenric Tam, conception and design of the project and studies, acquisition of data, interpretation of data, writing of the manuscript, final approval, agreement to be accountable; David W. Schoppy, conception and design of the project and studies, acquisition of data, interpretation of data, review and revision of the manuscript, final approval, agreement to be accountable; June Ho Shin, acquisition of data, interpretation of data, review and revision of the manuscript, final approval, agreement to be accountable; Joshua K. Tay, acquisition of data, interpretation of data, review and revision of the manuscript, final approval, agreement to be accountable; June Ho Shin, acquisition of data, interpretation of data, review and revision of the manuscript, final approval, agreement to be accountable; John B. Sunwoo, conception and design of the project and studies, interpretation of data, writing of the manuscript, final approval, agreement to be accountable.

Disclosures
Competing interests: None.
Sponsorships: Kenric Tam, Stanford University Medical Scholars Fellowship; Joshua K. Tay, Ministry of Health Healthcare Research Scholarship, National Medical Research Council, Singapore (grant NMRC/ Scholarship/0001/2014); John B. Sunwoo, National Institutes of Health (R01CA158516).
Funding source: National Institutes of Health funding supported the research activity and studies described in the article.

References