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Secretion of VEGF by oral squamous cell carcinoma cells skews endothelial cells to suppress T-cell functions

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Abstract

Patients with oral squamous cell carcinoma (OSCC) have severe defects in anti-tumor immune functions. Endothelial cells are potential regulators of immune cell functions and have therefore been examined to determine their role in tumor-induced immune suppression. The present studies showed that supernatants from endothelial cells exposed to OSCC-conditioned media (Endo^{OSCC-sup}) had elevated levels of the immune suppressive products PGE₂ and VEGF as compared to supernatants from endothelial cells treated with media alone (Endo^{Media}) or with keratinocyte-conditioned media (Endo^{Ker-sup}). Antibody neutralization of OSCC-derived VEGF prevented tumor-conditioned media from inducing endothelial cells to increase production of PGE₂ and VEGF. Furthermore, treatment of T-cells with supernatants from Endo^{OSCC-sup} resulted in diminished T-cell proliferation and decreased IFN- γ production, when compared to T-cells treated with media or supernatants from Endo^{Media} or Endo^{Ker-sup} controls. T-cell levels of granzyme B and perforin were reduced after treatment with supernatant from Endo^{OSCC-sup} compared to control treatments. Addition of VEGF neutralizing antibody to the OSCC-conditioned media prevented endothelial cells from being skewed to downregulate T-cell proliferation and production of IFN- γ , perforin and granzyme B. Taken together, these studies provide support for the use of VEGF targeting therapies as an immunotherapeutic agent to block induction of immune suppressive endothelial cells in patients with OSCC.

Keywords

Oral squamous cell carcinoma (OSCC); vascular endothelial growth factor (VEGF); endothelial cell; T-cell; immune suppression

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Introduction

Despite numerous advances in the treatment of cancer, five year survival rates for patients with oral squamous cell carcinoma (OSCC) have remained relatively unchanged over the last 50 years. Patients diagnosed with OSCC have a 5 year relative survival rate of only about 60%. This low survival rate may be due in part to the highly vascularized and immune suppressive nature of these tumors [1]. OSCC tumor cells secrete numerous pro-angiogenic products including vascular endothelial growth factors (VEGF), transforming growth factor- β (TGF- β), prostaglandin E₂ (PGE₂), interleukin-8 and angiopoietin-1 and -2 [2]. Many of these pro-angiogenic factors also serve a dual role in directly suppressing anti-tumor immune functions. For example, tumor secretion of VEGF promotes endothelial cell migration and proliferation [3]. In addition, VEGF also disrupts dendritic cell maturation and function, and suppresses T-cell development [4,5]. Tumor-secretion of PGE₂ promotes angiogenesis, and is involved in suppression of T-cell proliferation, expression of Th1 cytokines and alters dendritic cell functions [6,7]. During tumor progression, tumor-secretion of TGF- β promotes angiogenesis and serves a dual role as a potent suppressor of T-cell, NK cell, dendritic cell and macrophage functions [8,9]. These and other studies suggest that many of the mechanisms involved in development of the tumor vasculature may also contribute to tumor-induced immune suppression.

Multiple studies have shown that in patients with OSCC, as well other types of solid tumors, defects in immune functions correlate with a poorer prognosis. Patients with OSCC have been found to have reduced numbers of circulating CD3⁺, CD4⁺ and CD8⁺ T-cell subsets as well as diminished responsiveness to anti-CD3 stimulation with alterations in these responses being associated with a reduced survival rate [10-12]. In addition, patients with OSCC have reduced T-cell levels of the cytotoxic mediators granzyme B and perforin as compared to healthy controls and corresponds with diminished T-cell killing of tumor cells [13].

In addition to directly suppressing anti-tumor immune responses through the secretion of immune suppressive products, tumors can indirectly suppress anti-tumor immune responses by recruiting normal host cells to become immune suppressive. Examples of this include immune suppression mediated by tumor-associated macrophages, Treg, myeloid derived suppressor cells and CD34⁺ progenitor cells [14]. For example, in patients with OSCC, increased macrophage infiltrate in the tumor correlates with an increase in tumor aggressiveness. Elevated numbers of immune suppressive Treg cells have been shown in patients with OSCC, though the role of Treg cells in OSCC survival remains to be determined [15]. Recent studies have shown that in patients with hepatocellular carcinoma, an elevated number of tumor-infiltrating Treg cells correlates with a significantly reduced disease free survival [16]. CD34⁺ progenitor cells represent another population of immune suppressive cells identified as being upregulated in patients with OSCC. In patients with OSCC, increased numbers of immune suppressive CD34⁺ cells have been shown to correlate with an increased rate of tumor recurrence and metastasis [17].

While CD34⁺ progenitor cells can be immune suppressive, the presence of tumor-derived products skews CD34⁺ cells to become endothelial cells that incorporate into the tumor vasculature [18]. In addition to being components of the vasculature, endothelial cells have also been shown to be regulators of immune cell functions [19]. Since immune suppressive CD34⁺ progenitor cells can be skewed to become endothelial cells, we hypothesized that OSCC-derived factors could induce endothelial cells to inhibit T-cell functions. Accordingly, we examined the role of OSCC-derived VEGF in inducing endothelial cells to suppress T-cell functions. These studies show that OSCC-secretion of VEGF induces the formation immune suppressive endothelial cells capable of disrupting T-cell functions and demonstrates a novel role of endothelial cells in tumor-induced immune suppression.

Materials & Methods

Tissue culture

Human microvessel-derived endothelial cells (HMEC-1) were maintained on fibronectin-coated plates (BD Biosciences, San Jose, CA) in 50:50 DMEM/Ham's F12 with 5% heat-inactivated fetal bovine serum (FBS), 200 U/ml penicillin G, 200 µg/ml streptomycin sulfate, 500 µg/ml amphotericin B, 10 µM hydrocortisone and 5×10^{-5} M 2-mercaptoethanol (Sigma-Aldrich, St. Louis, Missouri).

Human primary tumor lines were established from tissue samples of patients undergoing surgical excision of OSCC tumors. Fresh OSCC tumor specimens were rinsed in antibiotic and antimycotic solutions containing 100 µg/ml penicillin, 100 µg/ml streptomycin and 200 µg/ml neomycin. Tissues were finely minced and then seeded onto gelatin-coated tissue culture plates (Sigma-Aldrich, St. Louis, Missouri). Primary, OSCC cells were maintained in culture in phenol free RPMI-1640 medium containing 15% heat-inactivated FBS, 50 µg/ml penicillin, 50 µg/ml streptomycin, 100 µg/ml neomycin and 5×10^{-5} M 2-mercaptoethanol. Adherent cells were then passed and maintained on gelatin-coated tissue culture plates. For the studies presented in this paper, lines were established from 5 different patients and were used for no more than 20 passages. Tumor lines were established from OSCC tissue collected from the oral cavity, larynx, supraglottis and tonsil, ranging in staging from T1 through T4.

Controls for tumor cells were primary human keratinocyte cultures (Invitrogen, Carlsbad, CA). Cells were maintained in Defined Keratinocyte-SFM, as per the manufacturer's instructions.

T-cell isolation

T-cells were isolated from peripheral blood collected from five healthy donors and maintained separately. Blood was layered over Ficoll-Hypaque and centrifuged for 15 minutes at 2000 RPM. The peripheral blood mononuclear leukocytes (PBML) were then removed and plated in 24-well plates and allowed to adhere for 2 hours at 37°C to remove macrophages/monocytes. Non-adherent T-cells were collected, washed and plated in 96 well, round-bottom plates at a density of 2.5×10^5 cells per well on immobilized anti-CD3 (R&D Systems, Minneapolis, MN). T-cells were maintained in RPMI culture medium with 10% heat-inactivated FBS, 200 U/ml penicillin G, 200 µg/ml streptomycin sulfate, 500 µg/ml amphotericin B, 5×10^{-5} M 2-mercaptoethanol (Sigma-Aldrich, St. Louis, Missouri) and 10 units/ml recombinant human IL-2 (R&D Systems, Minneapolis, MN).

Experimental set-up and T-cell treatments

Twenty-four hours prior to endothelial cell treatment, OSCC and keratinocyte cultures of equal plating density were washed and resuspended in RPMI with 15% fetal bovine serum (FBS). Supernatants from primary OSCC cultures and keratinocyte cultures were used at a 40% concentration to treat subconfluent cultures of endothelial cells. Endothelial cells treated with media alone served as an additional control. As indicated in specific experiments, 0.025 µg/ml of neutralizing antibodies to VEGF-A₁₆₅ or goat IgG isotype control antibody (R&D Systems, Minneapolis, MN) were added to OSCC-conditioned media immediately preceding treatment of endothelial cells. After a 24 hour incubation with conditioned media or controls, endothelial cells were washed and fresh media was added for an additional 24 hours of incubation. Supernatants from endothelial cells that were exposed to media (Endo^{Media}), keratinocyte-conditioned media (Endo^{Ker-sup}) and OSCC-conditioned media (Endo^{OSCC-sup}) were collected and assayed for the presence of immune modulatory products and were used to treat freshly isolated T-cells.

For experimental treatments, T-cells were incubated for 24 hours with either medium alone or 40% supernatant from Endo^{Media}, Endo^{Ker-sup} or Endo^{OSCC-sup}. After the treatment period, T-cells were washed and new medium was added for an additional 24 hours of incubation. Supernatants from T-cells were collected for cytokine quantification, T-cell proliferation. Other T-cell functional analyses were also performed as described in individual experiments.

Immune function assays

Endothelial cell and T-cell secretion of immune regulatory factors was assessed by ELISA. These include measurement of PGE₂, VEGF, IL-6 (R & D Systems, Minneapolis, MN USA) and IFN- γ (BD Biosciences, San Jose, CA). All ELISA's were performed according to the manufacturers' instructions.

T-cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) analysis (Promega, Madison, WI). MTS is a tetrazolium compound that is reduced to formazan by metabolically active cells and was detected by measuring the absorbance at 492 nm. T-cell proliferation was assessed 24-hours after the removal of endothelial cell supernatants.

T-cell intracellular cytokine levels and cytotoxic granule production were measured by flow cytometric analysis of immunostained cells. T-cells were treated with monensin (GolgiStop) for two hours prior to antibody staining. Anti-CD16/CD32 monoclonal antibodies against the Fc γ II/III receptors and mouse serum were used to block nonspecific binding. Cell surface antigen staining was performed using anti-CD4 and anti-CD8 monoclonal antibodies. After staining, cells were washed twice, fixed and permeabilized with Cytotfix/Cytoperm. Cells were then stained with anti-IFN- γ , anti-granzyme B or anti-perforin antibodies. Marker channels were set using isotype control antibodies. Flow cytometric analysis was performed on a BD FACSCanto flow cytometer using FACS Diva flow cytometry analysis software. All flow cytometry reagents were obtained from BD Biosciences (San Jose, CA).

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 4.03 software. ANOVA analysis with post-hoc student *t* test was used to calculate statistical significances between experimental groups. Data shown are mean values $\pm$ SD or SEM of multiple experiments, as indicated in individual experiments. Histograms are representative results of multiple experiments.

Results

OSCC-secreted factors skew endothelial cells to disrupt T-cell proliferation and IFN- γ production in response to anti-CD3 stimulation

In our previous studies, we determined that products secreted by murine Lewis lung carcinoma cells were capable of skewing endothelial cells to suppress splenic T-cell functions. To expand on these findings, we tested whether OSCC-secreted products were capable of skewing endothelial cells to disrupt human T-cell responses to anti-CD3 stimulation. After exposure to media alone or media conditioned by endothelial cells (Endo^{Media}, Endo^{Ker-sup} or Endo^{OSCC-sup}), T-cells were washed and fresh media was added. T-cells were then allowed to incubate for an additional 24 hours. MTS analysis was then utilized to determine if OSCC-secreted products were capable of skewing endothelial cells to disrupt T-cell proliferation (Figure 1). These studies showed that T-cells treated with Endo^{Media} and Endo^{Ker-sup} had significantly higher levels of proliferation than those treated with media alone ($p = 0.0091$ and $p = 0.006$, respectively). However, treatment of T-cells with Endo^{OSCC-sup} reduced T-cell proliferation compared to control treatments with supernatants from Endo^{Media} ($p = 0.002$) or Endo^{Ker-sup} ($p = 0.0017$).

Next examined were the effects of supernatant from Endo^{OSCC-sup} on T-cells ability to produce the inflammatory mediator IFN- γ in response to anti-CD3. Flow cytometric analysis of intracellular IFN- γ expression demonstrated that compared to treatment with media alone, the percent of total T-cells immunostaining positive for IFN- γ was increased by treatment with media conditioned by Endo^{Media} ($p = 0.0012$) or Endo^{Ker-sup} ($p = 0.0004$) (Figure 2A-E). In contrast, treatment of endothelial cells with OSCC-conditioned media disrupted their ability to stimulate T-cell IFN- γ production to levels induced by Endo^{Media} or Endo^{Ker-sup} treatments ($p < 0.0001$ for both treatment groups). Further analysis showed that CD8⁺ T-cells, and not CD4⁺ T-cells (Figure 2F), were responsible for the observed changes in IFN- γ production. CD8⁺ IFN- γ production was found to be increased upon treatment with media conditioned by Endo^{Media} and Endo^{Ker-sup} ($p = 0.0004$ for both treatments). However, supernatants of Endo^{OSCC-sup} reduced CD8⁺ T-cell IFN- γ production compared to treatment with supernatant from Endo^{Media} or Endo^{Ker-sup} ($p = 0.004$ and $p = 0.0016$ respectively). These results demonstrate that OSCC-derived products are capable of disrupting endothelial cell stimulation of CD8⁺ T-cell responses to anti-CD3.

Neutralization of OSCC-derived VEGF blocks their modulation of endothelial cell secretion of immune regulatory products

After observing the ability of supernatants from Endo^{OSCC-sup} to suppress T-cell proliferation and IFN- γ production, studies were conducted to identify the OSCC-secreted factor responsible for inducing suppressive endothelial cells. We hypothesized that VEGF was the OSCC-derived factor responsible for inducing the formation of suppressive endothelial cells due to its ability to modulate a spectrum of endothelial cell functions. Consistent with the results of our previous studies and studies of others, OSCC primary cell lines secreted elevated levels of VEGF-A as compared to levels secreted by keratinocyte controls (Table 1) [20].

First examined was the ability of OSCC-derived VEGF to modulate endothelial cell secretion of VEGF, IL-6 and PGE₂. OSCC-conditioned media induced endothelial cells to produce elevated levels of PGE₂, compared to levels produced by endothelial cells that were treated with media alone ($p = 0.01$) or with keratinocyte-conditioned media ($p = 0.001$) (Figure 3A). Addition of VEGF neutralizing antibody to OSCC-conditioned medium prevented endothelial cells from being induced to increase secretion of PGE₂ ($p < 0.0001$ compared to Endo^{OSCC-sup} + Isotype control). There were no significant differences in the levels of PGE₂ secreted by endothelial cells that were treated with media alone, keratinocyte-conditioned media or OSCC-conditioned media whose VEGF was neutralized with antibodies.

Endothelial cell secretion of VEGF followed similar trends as secretion of PGE₂ (Figure 3B). Compared to supernatant from Endo^{Media} and Endo^{Ker-sup} controls, Endo^{OSCC-sup} that were treated with a mixture of isotype control antibodies plus OSCC-derived conditioned media produced increased levels of VEGF ($p = 0.0003$ and $p = 0.0016$ respectively). Neutralization of OSCC-secreted VEGF prevented endothelial cells from being induced to increase secretion of VEGF compared to levels in Endo^{OSCC-sup} conditioned media admixed with isotype control ($p < 0.001$). Endo^{OSCC-sup} that were treated with a mixture of isotype control antibody and OSCC-conditioned media also produced increased levels of IL-6 compared to levels produced by Endo^{Media} and Endo^{Ker-sup} controls ($p = 0.0048$ and $p = 0.0003$ respectively) (Figure 3C). However, neutralization of OSCC-derived VEGF failed to reduce endothelial cell secretion of IL-6 to control levels. These results demonstrate that OSCC-derived VEGF can induce endothelial cells to increase production of VEGF and PGE₂. Furthermore, OSCC-induction of endothelial cells to increase production of IL-6 is not regulated by OSCC-derived VEGF.

OSCC-derived VEGF skews endothelial cell modulation of T-cell cytokines

The above studies showing OSCC-secreted VEGF alters endothelial cell VEGF and PGE₂ production led to studies examining if OSCC-secretion of VEGF was the factor responsible for inducing endothelial cells to disrupt T-cell IFN- γ production (Figure 4). Endothelial cells that had been treated with a mixture of isotype control antibodies plus OSCC conditioned media suppressed intracellular expression of IFN- γ in CD8⁺ T-cells compared to levels of intracellular T-cell expression of IFN- γ after treatment with supernatant from Endo^{Media} ($p = 0.0003$) or Endo^{Ker-sup} ($p = 0.0006$). Addition of neutralizing antibody to OSCC-derived VEGF blocked induction of endothelial cells that are suppressive to T-cell IFN- γ expression ($p = 0.0008$ compared to Endo^{OSCC-sup} containing isotype).

Next, T-cell secretion of IFN- γ was assessed by ELISA (Figure 5). Consistent with the results of the flow cytometric analyses of T-cell IFN- γ expression, T-cells treated with supernatant from Endo^{Media} or Endo^{Ker-sup} had increased IFN- γ secretion compared to IFN- γ secretion by T-cells treated with media alone. Figure 5 shows that T-cells treated with supernatant from endothelial cells that had been exposed to a mixture of OSCC-conditioned media plus isotype control antibodies had reduced levels of IFN- γ secretion compared to IFN- γ secretion by T-cells treated with control supernatants from Endo^{Media} and Endo^{Ker-sup} ($p < 0.0001$ compared to either control treatment). Addition of VEGF neutralizing antibody to OSCC-conditioned medium blocked OSCC cells ability to induce endothelial cells that disrupt T-cell IFN- γ secretion ($p < 0.0001$ compared to Endo^{OSCC-sup} with isotype control antibodies). These results demonstrate that OSCC secretion of VEGF diminishes endothelial cells capacity to stimulate T-cell IFN- γ production.

OSCC-derived VEGF induces endothelial cells to diminish T-cell proliferation

Similar to the data shown in Figure 1, MTS analyses were used to determine if OSCC-secreted VEGF could skew endothelial cells to disrupt T-cell proliferation (Figure 6). Consistent with previous results, T-cells treated with supernatants from Endo^{OSCC-sup} were less able to mount a proliferative response to anti-CD3 compared to T-cells treated with the control supernatant from Endo^{Media} ($p = 0.003$) or Endo^{Ker-sup} ($p = 0.0006$). Neutralization of OSCC-derived VEGF prevented the induction of endothelial cells that suppress T-cell proliferation to anti-CD3 stimulation ($p = 0.0019$). These data demonstrate that OSCC secretion of VEGF skews endothelial cells to disrupt T-cell proliferation.

OSCC-derived VEGF skews endothelial cells to reduce T-cell production of cytotoxic mediators

CD8⁺ T-cell production of granzyme B and perforin were examined as additional indicators of T-cell function and cytotoxicity (Figure 7A and 7B). Unlike IFN- γ production, treatment of T-cells with media conditioned by Endo^{Media} and Endo^{Ker-sup} did not alter T-cell levels of granzyme B or perforin. However, supernatants from endothelial cells that were treated with a mixture of isotype control antibodies plus OSCC-conditioned media significantly reduced T-cell granzyme B and perforin production compared to levels in T-cells that were treated with Endo^{Media} and Endo^{Ker-sup} ($p = 0.01$ compared to either treatment). Neutralization of OSCC-derived VEGF prevented the induction of endothelial cells that down regulate T-cell granzyme B and perforin production ($p = 0.04$ and $p = 0.01$, respectively), compared to when endothelial cells were treated with isotype antibodies plus OSCC-conditioned media. While the intensities of T-cell granzyme B and perforin staining were reduced upon treatment with media conditioned by Endo^{OSCC-sup}, the percentage of T-cells staining positive for granzyme B or perforin was not altered significantly. These results demonstrate that OSCC-derived VEGF induces endothelial cells to diminish T-cell production of the cytotoxic mediators granzyme B and perforin.

Discussion

Our previous work examined the ability of murine Lewis lung carcinoma cells to induce endothelial cells to suppress T-cell, NK cell and macrophage functions [21]. The purpose of the present studies was to confirm and extend the results of our prior studies in a human tumor model, to further define the effects of suppressor endothelial cells on T-cell functions and to identify the tumor-secreted factor responsible for inducing endothelial cells to suppress T-cell functions. We hypothesized that OSCC-derived VEGF could be a candidate suppressor-inducing factor based on its abundant secretion by OSCC tumor cells and the potent effect it has on endothelial cell functions. In the normal oral mucosa, VEGF is produced in very low levels, but increases in early oral dysplasia and continues to increase throughout tumor progression [22].

Consistent with the results of our previous murine studies [21], the present studies identified that control human endothelial cells secrete products that heighten CD8⁺ T-cell IFN- γ production in response to anti-CD3 stimulation, while OSCC-exposed endothelial cells are less able to stimulate T-cell IFN- γ production. Additionally, OSCC-derived VEGF was identified to be the mediator that diminishes the capacity of endothelial cells to stimulate T-cell IFN- γ production (summarized in Figure 8). The effects of suppressor endothelial cells on T-cell IFN- γ production may contribute to the observed decreases in serum IFN- γ levels in patients with OSCC [23]. These studies also demonstrate that OSCC secretion of VEGF induces endothelial cells to down regulate CD8⁺ T-cell levels of the cytotoxic mediators, granzyme B and perforin. These results are consistent with recent studies demonstrating that PBMCs from patients with OSCC have down regulated expression of granzyme B and perforin compared to expression in healthy age-matched controls [13]. Down regulation of these mediators could provide a survival advantage to tumors by diminishing the T-cells' ability to destroy tumor cells.

The present studies also identified that OSCC secretion of VEGF induces endothelial cells to increase production of the potent immune suppressant, PGE₂. PGE₂ has been shown to disrupt T-cell and B-cell activation, suppress T-cell proliferation, skew T-cells towards Th2 immune responses, suppress T-cell and NK cell cytotoxic functions and diminish macrophage secretion of the immune stimulatory cytokine IL-12 [24]. PGE₂ also induces the formation of myeloid-derived suppressor cells [25]. Therefore, use of VEGF targeting therapies may reduce endothelial cell PGE₂ production and improve numerous aspects of anti-tumor immunity.

In addition to blocking the induction of immune suppressive endothelial cells, VEGF targeting therapies may have a role in blocking other mechanisms of tumor-induced immune suppression, including chemoattraction of immune suppressive CD34⁺ progenitor cell and disruptions in dendritic cell and T-cell maturation [26-28]. To date, two clinical trials have examined the use of VEGF targeting therapies to improve immune function. Fricke *et al.* demonstrated that treatment with VEGF-Trap, a fusion protein that binds all forms of VEGF-A, did not alter the total number of dendritic cells, myeloid-derived suppressor cells or regulatory T-cells. Conversely, the study showed that the proportion of mature dendritic cells in the peripheral blood significantly increased. Unfortunately, no improvements in T-cell function were observed in patients receiving VEGF Trap treatment. The applicability of this study to patients with OSCC may be limited. The study had a small sample size (n=15) and did not include patients with OSCC [29]. The other VEGF targeting trial examined the effects of the anti-VEGF antibody bevacizumab on immature myeloid cells, dendritic cells and T-cell functions in patients with colorectal cancer. This study by Osada *et al.* showed that bevacizumab administration restored defects in dendritic cell maturation and functions [30]. As with the Fricke study, the Osada study was limited to a small patient population (n=16) and only examined patients with colon cancer. Overall, the results of early studies examining VEGF targeting therapies as an immunotherapy show promise. >Future clinical trials may include

examining if VEGF targeting therapies block the induction of suppressive endothelial cells and if disrupting suppressor endothelial cells improves the functions of tumor-infiltrating lymphocytes in patients with OSCC as well other solid tumors.

Many questions were raised by the results of the present study that remain to be answered. Studies are currently ongoing to identify the product(s) secreted by suppressor endothelial cells that disrupt T-cell functions. Additional studies are needed to examine if immune suppressive endothelial cells are being induced within that tumor mass of patients with OSCC. Examination of patients undergoing VEGF targeting therapies is also need to determine if such therapies can block tumor induction of immune suppressive endothelial cells. Blocking the induction of immune suppressive endothelial cells may also aid in improving the efficacy of existing immunotherapies. In patients with OSCC, several immunotherapeutic approaches have been attempted including adoptive T-cell transfer, dendritic cell-based vaccines and cytokine based therapies. Many of these studies have demonstrated limited efficacy in treating OSCC and has been attributed in part to the immune suppressive effects of OSCC tumors [31,32]. Disruption of suppressive endothelial cells may improve the function of immune infiltrating T-cells as they must pass alongside tumor-associated endothelial cells to infiltrate into the tumor. Whether or not this could lead to systemic increases in immune competency is less clear. In addition to their role in disrupting tumor vascular, VEGF targeting therapies may play an important role as an immunotherapeutic agent.

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Abbreviations

Endo^{Ker-sup}, endothelial cells treated with keratinocyte-conditioned media
 Endo^{Media}, endothelial cells treated with media
 Endo^{OSCC-sup}, endothelial cells treated with OSCC-conditioned media
 FBS, fetal bovine serum
 IFN- γ , interferon- γ
 IL, interleukin
 NK cell, natural killer cell
 OSCC, oral squamous cell carcinoma
 PBML, peripheral blood mononuclear leukocytes
 PGE₂, prostaglandin E₂
 TGF- β , transforming growth factor- β
 Treg, T regulatory cell
 VEGF, vascular endothelial cell growth factor-A

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Figure 1.

Effects of tumor-exposed endothelial cell supernatant on T-cell proliferation. Healthy donor T-cell proliferation was assessed by MTS analysis in response to anti-CD3 stimulation and exposure to various endothelial cells supernatants. (*) indicates that $p \leq 0.002$ compared to Endo^{Media} or Endo^{Ker-sup} treatments. There is no significant difference between media and Endo^{OSCC-sup} treatments. Data presented is average optical density (OD) $\pm$ SD with $n = 6$ and is representative of 3 experiments with similar results.

Figure 2.

T-cell IFN- γ production in response to anti-CD3 stimulation and endothelial cell supernatant treatment. (A-D) Representative histograms of immunostaining for total T-cell IFN- γ expression. Dark gray peak represents cells staining positive for IFN- γ , with the percent staining positive indicated by the number above the peak. (E) Mean percent of total T-cell, staining positive for IFN- γ $\pm$ SEM with $n \geq 4$ per treatment group. (*) indicates that $p < 0.0001$ compared to Endo^{Media} or Endo^{Ker-sup} treatments. There is no significant difference between media alone and Endo^{OSCC-sup} treatments. (F) Mean fluorescence intensity of CD8⁺ T-cells and CD4⁺ T-cells staining for IFN- γ $\pm$ SEM with $n \geq 3$ per treatment group. (**) indicates that $p \leq 0.002$ compared to Endo^{Media} or Endo^{Ker-sup} treatments. There is no significant difference between media alone and Endo^{OSCC-sup} treatments. (NS) indicates no significant difference in CD4⁺ T-cell IFN- γ production among any of the treatment groups.

Figure 3.

Neutralization of OSCC-secreted VEGF blocks the capacity of OSCC-conditioned media to stimulate endothelial cell PGE₂ and VEGF secretion, but not IL-6 secretion. VEGF neutralizing antibody was added to OSCC-conditioned media prior to being used to treat endothelial cells. 24-hours after the addition of OSCC-conditioned media, endothelial cells were washed and new media was added. After this time, endothelial cell supernatants were collected and assayed by ELISA for presence of immune modulatory products. (*) indicates that $p \leq 0.01$ compared to Endo^{Media} or Endo^{Ker-sup} treatments. (**) indicates that $p \leq 0.0001$ compared to Endo^{OSCC-sup} plus isotype. (#) indicates that $p \leq 0.005$ compared to Endo^{Media} or Endo^{Ker-sup} treatments. (NS) Indicates that there is no significant difference between addition of isotype (Endo^{OSCC-sup} + Isotype) versus anti-VEGF antibodies (Endo^{OSCC-sup} + α VEGF) to the OSCC-conditioned media prior to exposure to endothelial cells. Data presented is mean + SEM with $n \geq 4$ per treatment group.

Figure 4.

Neutralization of OSCC-secreted VEGF blocks endothelial cells from being skewed to suppress CD8⁺ T-cell IFN-γ production. Mean fluorescent intensity of immunostaining of T-cells that are double positive for IFN-γ⁺ and CD8⁺ ± SEM (n ≥ 3 per treatment group). (*) indicates that $p \leq 0.0003$ compared to Endo^{Media} or Endo^{Ker-sup} treatments. (**) indicates that $p = 0.0008$ compared to results of when isotype control antibodies were added to OSCC-conditioned media. There is no significant difference in T-cell IFN-γ production between Endo^{Media} or Endo^{Ker-sup} control treatments and results of when VEGF antibody was added to OSCC supernatants (Endo^{OSCC-sup} + αVEGF).

Figure 5.

Endothelial cell supernatant's modulation of T-cell activation to produce IFN- γ in response to anti-CD3 stimulation. T-cell secretion of IFN- γ was measured by ELISA. Mean levels $\pm$ SEM with $n \geq 4$ of IFN- γ production following exposure to various endothelial cell conditioned medium treatments. (*) indicates that $p < 0.0001$ compared to Endo^{Media} or Endo^{Ker-sup} treatments. (**) indicates that $p < 0.0001$ compared to results of when isotype control antibodies were added to OSCC-conditioned media (Endo^{OSCC-sup} + Isotype treatment).

Figure 6.

Neutralization of OSCC-secreted VEGF blocks induction of endothelial cells that suppress T-cell proliferation in response to anti-CD3 stimulation. The effects of various endothelial cell supernatants on proliferation of healthy donor T-cells in response to anti-CD3 stimulation were assessed by MTS analysis. Data presented are average optical densities (OD) $\pm$ SD with $n = 7$ and is representative of four separate experiments. (*) indicates that $p \leq 0.003$ compared to Endo^{Media} or Endo^{Ker-sup} treatments. (**) indicates that $p = 0.002$ compared to results of when isotype antibodies were added to OSCC-conditioned media (Endo^{OSCC-sup} + Isotype).

Figure 7.

Neutralization of OSCC-secreted VEGF prevents induction of endothelial cells that reduce T-cell levels of the cytotoxic mediators granzyme B and perforin. T-cell production of cytotoxic mediators was examined by flow cytometric analysis of intracellular immunostaining. Mean levels $\pm$ SEM of perforin and granzyme B following CD8⁺ T-cell exposure to endothelial cell conditioned media are in (A) and (B) respectively. (*) indicates that $p = 0.01$ compared to Endo^{Media} or Endo^{Ker-sup} treatments. (**) indicates that $p = 0.04$ and (#) indicates that $p = 0.01$ compared to results of when isotype control antibodies were added to OSCC-conditioned media (Endo^{OSCC-sup} + Isotype). Results are means of analyses from three separate experiments to measure granzyme B and four different experiments to measure perforin.

Summary of Results

Figure 8.

Summary of results. OSCC-secretion of VEGF induces endothelial cells to increase production of VEGF and PGE₂. Furthermore, OSCC-derived VEGF skews endothelial cells to produce factors that diminish endothelial cells' ability to heighten T-cell proliferative responses to anti-CD3 and to downregulate T-cell IFN-γ, granzyme B and perforin production. It is hypothesized that elevated secretion of PGE₂ and VEGF by suppressor endothelial cells contributes to their disruption of T-cell functions. Additionally, other OSCC-secreted factors may contribute to induction of immune suppressive endothelial cells by inducing endothelial cells to increase production of IL-6, though this does not appear to be involved in altering T-cell functions.

Table 1

VEGF secretion by primary OSCC cells Analysis of VEGF-A secretion by primary OSCC cultures. Results expressed are the mean $\pm$ SEM from supernatants collected at various times throughout passage with three to five collections per cell line.

Cell line	VEGF-A (pg/ml) + SEM
Media	0 $\pm$ 0
Primary Keratinocyte	74 $\pm$ 4
OSCC 1	999 $\pm$ 41
OSCC 2	785 $\pm$ 101
OSCC 3	513 $\pm$ 21
OSCC 4	823 $\pm$ 133
OSCC 5	900 $\pm$ 30