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Activation of iCaspase-9 in Neovessels Inhibits Oral Tumor Progression

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Abstract

Tumors of the oral cavity are highly vascularized malignancies. Disruption of neovascular networks was shown to limit the access of nutrients and oxygen to tumor cells and inhibit tumor progression. Here, we evaluated the effect of the activation of an artificial death switch (iCaspase-9) expressed in neovascular endothelial cells on the progression of oral tumors. We used biodegradable scaffolds to co-implant human dermal microvascular endothelial cells stably expressing iCaspase-9 (HDMEC-iCasp9) with oral cancer cells expressing luciferase (OSCC3-luc or UM-SCC-17B-luc) in immunodeficient mice. Alternatively, untransduced HDMEC were co-implanted with oral cancer cells, and a transcriptionally targeted adenovirus (Ad-VEGFR2-iCasp-9) was injected locally to deliver iCaspase-9 to neovascular endothelial cells. *In vivo* bioluminescence demonstrated that tumor progression was inhibited, and immunohisto-chemistry showed that microvessel density was decreased, when iCaspase-9 was activated in tumor-associated microvessels. We conclude that activation of iCaspase-9 in neovascular endothelial cells is sufficient to inhibit the progression of xenografted oral tumors.

Keywords

angiogenesis; neovascularization; apoptosis; suicide gene; bioluminescence

INTRODUCTION

Angiogenesis, the development of new capillaries from existing blood vessels, is required for the progression of solid tumors (Folkman, 1971). Today, several anti-angiogenic drugs designed specifically to disrupt the tumor's microvessel network are being used for the treatment of patients with cancer (Kerbel and Folkman, 2002). The cells recruited to form these networks are non-mutated endothelial cells that have been activated by tumor-cell-derived pro-angiogenic factors. Since these cells have not undergone significant genetic changes, they behave in a fairly predictable manner and are more susceptible to induced cell death than are neoplastic cells (Kerbel, 2000). Tumors of the oral cavity are highly vascularized malignancies, and it is known that oral cancer cells secrete a panel of potent angiogenic factors (Chen *et*

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et al., 1999). Taken together, these results suggest that the microvascular network is an important target for the therapy of oral cancers.

The process of programmed cell death (apoptosis) is executed by a class of cysteine proteases known as caspases (Nunez *et al.*, 1998). Caspase-9 is an initiator of the apoptotic pathway that is activated when recruited by Apaf-1 in the presence of dATP and cytochrome-c (Hu *et al.*, 1998; Li *et al.*, 1998). Caspases that can be artificially activated by chemical inducers of dimerization (CID) were engineered to function as “caspase-based artificial death switches” (MacCorkle *et al.*, 1998; Fan *et al.*, 1999). Caspase-9 was fused to a CID-binding domain, and was named ‘inducible caspase-9’ (iCaspase-9). Exposure of cells to dimerizer drugs (*e.g.*, AP20187) induces activation of iCaspase-9, and triggers the apoptotic signaling pathway (Fan *et al.*, 1999; Shariat *et al.*, 2001; Nör *et al.*, 2002).

Recombinant adenoviruses have been proposed for gene therapy of oral lesions because of their stability *in vivo* and relatively low risk of secondary mutagenesis (Ali *et al.*, 1994; Rudin *et al.*, 2003; St. George, 2003). The feasibility of an artificial death switch as an anti-angiogenic treatment strategy for oral cancer is dependent on whether iCaspase-9 can be specifically expressed in tumor-associated endothelial cells. We have recently reported the development of a novel transcriptionally targeted adenoviral vector that allowed for the expression of iCaspase-9 specifically to neovascular endothelial cells (Song *et al.*, 2005). This vector utilizes the promoter of vascular endothelial growth factor receptor-2 (VEGFR2) to drive expression of iCaspase-9. VEGFR2 is poorly expressed in mature blood vessels and in most other cell types. In contrast, its expression is high in the angiogenic endothelium of most tumors (Patterson *et al.*, 1995; Heidenreich *et al.*, 2000).

Recent advances in imaging technology have allowed for the miniaturization of systems and have greatly enhanced the quality of the data obtainable from small-animal models of neoplasia (Edinger *et al.*, 2002). The use of *in vivo* bioluminescence imaging has emerged as a quantitative assessment tool for evaluating tumor progression (Rehemtulla *et al.*, 2000). This system utilizes the light produced by the catalysis of the cell-permeable substrate luciferin by firefly *Photinus pyralis* luciferase protein (Timmins *et al.*, 2001). Since a luciferase construct can be inserted into the cancer cell’s genome, this technique allows for non-invasive quantitative assessments of tumor progression over time. The intensity of luminescence is directly proportional to the number of metabolically active cancer cells expressing luciferase (Rehemtulla *et al.*, 2000).

Here we used *in vivo* bioluminescence imaging to evaluate the effect of iCaspase-9 activation in neovascular endothelial cells on tumor progression. We tested the hypothesis that disruption of the neovascular network with an artificial death switch targeted to the endothelial cells is sufficient to inhibit progression of oral tumors xenografted in immunodeficient mice.

MATERIALS & METHODS

Generation of Oral Cancer Cell Lines Stably Expressing Luciferase

The pFB-neo-luc was generated by digestion of pGL2-Basic (Promega, Madison, WI, USA) with Kpn I and PflMI to release the luciferase cassette, which was inserted into the Sal I site of pFB-neo (Stratagene, La Jolla, CA, USA) by blunt ligation. Ecotropic packaging cells (PE501, gift from A.D. Miller) were transfected with either the pFB-neo-luc or with the negative control pFB-neo plasmid, with Lipofectin (Invitrogen Corp., Carlsbad, CA, USA), according to the manufacturer’s instructions. Twenty-four hrs after transfection, retrovirus-containing supernatants were collected, centrifuged, and used to infect the amphotropic packaging cells PA317 (gift from A.D. Miller) in the presence of 4 µg/mL polybrene (Sigma-Aldrich Corp., St. Louis, MO, USA), as described (Nör *et al.*, 1999, 2002). Cells were selected

for 7 days with Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 400 µg/mL G418 (Fisher Scientific, Hanover Park, IL, USA). Retrovirus-containing culture medium was collected for 24 hrs and used to infect OSCC-3 (Oral Squamous Cell Carcinoma-3, gift from Mark Lingen), or UM-SSC-17B (University of Michigan-Squamous Cell Carcinoma-17B, gift from Tom Carey), in the presence of 4 µg/mL polybrene (Sigma). Tumor cells were selected for at least 14 days with 1 mg/mL G418 (Fisher). To confirm the expression of luciferase, we subjected OSCC-3-luc and UM-SSC-17B-luc cells to lysis and measured luciferase activity with the substrate LAR II (Promega) in a luminometer (Lmax; Molecular Devices, Sunnyvale, CA, USA).

Severe Combined Immunodeficient (SCID) Mouse Model of Human Tumor Angiogenesis

To investigate the effect of iCaspase-9 dimerization on tumor microvessel density and tumor progression, we co-implanted endothelial cells and luciferase-expressing tumor cells using the severe combined immunodeficient mouse model of human angiogenesis (Nör *et al.*, 2001a,b). Human dermal microvessel endothelial cells (HDMEC; Cambrex Corp., East Rutherford, NJ, USA) stably expressing iCaspase-9 (HDMEC-iCasp-9) (Nör *et al.*, 2002), or empty vector controls (HDMEC-LXSN), were cultured in endothelial growth medium 2-microvascular (EGM2-MV; Cambrex) supplemented with 250 µg/mL G418 (Fisher). Each poly-L-lactic acid (PLLA; Medisorb, Germantown, NY, USA) biodegradable scaffold was seeded with 1×10^5 OSCC-3-luc and either 9×10^5 HDMEC-LXSN or 9×10^5 HDMEC-iCasp-9, as described (Nör *et al.*, 2001a,b, 2002). Scaffolds were implanted into the dorsal subcutaneous of each severe combined immunodeficient mouse (CB-17 SCID; Taconic, Germantown, NY, USA). Starting 18 days after implantation, each mouse received a daily intraperitoneal injection of 2 mg/kg AP20187 (ARIAD Pharmaceuticals, Cambridge, MA, USA) in a solution of 10% PEG 400 and 1.7% Tween 20 for 3 consecutive days. Alternatively, scaffolds containing untransduced endothelial cells (HDMEC) and UM-SSC-17B-luc were implanted into severe combined immunodeficient mice (CB-17 SCID), as described above. Twenty-one days after implantation, mice received local injections of 10^{10} Ad-hVEGFR2-iCaspase-9 particles/scaffold or 10^{10} Ad-iCaspase-9 particles/scaffold (no promoter control), as described (Song *et al.*, 2005). Starting one day after viral delivery, mice received 2 consecutive daily injections of 2 mg/kg AP20187 (ARIAD) or phosphate-buffered saline (PBS; Invitrogen). Following the animals' death, tumors were retrieved, measured with calipers, weighed, and fixed in 10% buffered formalin at 4°C overnight. At least 4 mice were evaluated *per* experimental group. The treatment and care of animals were performed in accordance with the University of Michigan standards.

In vivo Bioluminescence Imaging

Mice were imaged on a cryogenically cooled imaging system (Xenogen, Alameda, CA, USA) coupled to a data acquisition computer. Mice were first anesthetized in an acrylic chamber with a 1.5% Isoflurane/air mixture, and injected intraperitoneally with 40 mg/mL luciferin potassium salt (Xenogen) in PBS at a dose of 320 mg/kg body weight. Digital gray images were captured and overlaid with pseudocolor images, which represent photon counts emitted from active luciferase within viable tumor cells. Luminescence emitted from each animal was integrated for one-minute intervals, from 5–20 min after the injection of Luciferin. Image processing and photon count quantification were conducted by means of Living Image software (Xenogen).

Immunolocalization of Tumor Blood Vessels

We used immunohistochemical staining with a polyclonal anti-human Factor VIII antibody (Lab Vision Corp., Fremont, CA, USA) to visualize the microvascular networks, as described

(Nör *et al.*, 2001b). The number of microvessels was counted in 10 random fields *per* implant in a light microscope (at 200x).

Statistical Analysis

Statistical significance was determined at $p \leq 0.05$, by *t* tests or one-way ANOVA, followed by the Student-Newman-Keuls test with SigmaStat 2.0 software (SPSS, Chicago, IL, USA).

RESULTS

We have recently demonstrated that activation of iCaspase-9 results in apoptosis of endothelial cells, and a decrease in tissue microvessel density *in vivo* (Nör *et al.*, 2002; Song *et al.*, 2005). To evaluate whether iCaspase-9 activation and subsequent apoptosis of endothelial cells affect tumor microvessel density, we co-implanted OSCC-3 with HDMEC-iCasp-9 in poly-L-lactic acid scaffolds in the severe combined immunodeficient mouse model of human angiogenesis (Nör *et al.*, 2001b). We allowed the tumors to grow for 18 days. Then, we activated the apoptotic cascade by delivering the dimerizer drug AP20187. We observed a 2.8-fold decrease ($p < 0.05$) in tumor microvessel density when the tumor microvessel network was lined with HDMEC-iCasp-9 cells, as compared with tumors vascularized with the empty vector control HDMEC-LXSN (Fig. 1).

To evaluate the impact that activation of iCaspase-9 in endothelial cells has on oral cancer progression, we performed *in vivo* bioluminescence imaging. We first established and characterized two lines of oral squamous cell carcinomas stably expressing luciferase. We observed that luciferase expression was higher ($p < 0.05$) in two clones (M1 and M2), than in control cells *in vitro* (Fig. 2A). We selected the UM-SSC-17B-luc M2 clone to evaluate luciferase expression further, using serial dilutions. Bioluminescence was detectable even at relatively small cell numbers *in vitro*, and the intensity was cell-number-dependent (Fig. 2B). The results observed for the OSCC3-luc clones followed similar trends (data not shown).

To improve our understanding about the use of *in vivo* bioluminescence to evaluate the progression of a xenografted oral tumor, we co-implanted endothelial cells and OSCC3-luc in severe combined immunodeficient mice. We imaged the mice 1–20 min post-injection of luciferin and observed that the saturation point for bioluminescence was reached within 12–14 min of injection (Figs. 2C, 2D). We imaged the mice over time to evaluate the stability of luciferase expression, and concluded that these cells express luciferase for at least 35 days (Fig. 2E).

Once the model has been characterized, we decided to use it to evaluate the effect of iCaspase-9 activation in neovascular endothelial cells on overall tumor progression. Sixteen mice were implanted with scaffolds containing UM-SSC-17B-luc cells and primary human dermal microvascular endothelial cells. The mice were imaged 3 days after implantation so that baseline bioluminescence values could be measured (Fig. 3A). Following 20 days of tumor growth, each mouse received a local intra-tumor injection of the engineered adenovirus Ad-hVEGFR2-iCasp9 (targeted to microvascular endothelial cells, with the endothelial cell-specific promoter VEGFR2) or the negative control Ad-iCasp9 (without promoter) as described (Song *et al.*, 2005), and was imaged for determination of pre-treatment bioluminescence values (Fig. 3A). Starting on day 21, mice received daily injections of AP20187 or phosphate-buffered saline (PBS) for 3 days. Luciferase expression was measured just prior to AP20187 injection and on the day of death (Fig. 3A). A decrease was noted in the growth rate (*i.e.*, the slope of the curve between day 21 and day 25) of the tumor that received Ad-hVEGFR2-iCasp-9 followed by the administration of the dimerizer drug (Fig. 3E), as compared with control tumors (Figs. 3B–3D). The observed inhibition of tumor progression in mice injected with Ad-VEGFR2-iCasp9 and treated with AP20187 (Figs. 4A, 4B) was correlated with lower

microvessel density (Fig. 4D). In contrast, control mice showed an increase in tumor progression (Figs. 4A, 4B) that was associated with higher microvessel density (Fig. 4C).

DISCUSSION

A key component of the pathobiology of cancer is aberrant angiogenesis (Folkman, 1995; Polverini, 1995). Tumor cells depend on the establishment of a neovascular network to fulfill their oxygen and nutrient demands. This dependency has become a target for anti-cancer therapies, and recently, the first anti-angiogenic drug (Avastin) has received approval for the treatment of colorectal cancer. Notably, the neovascular network is more accessible to viral vectors for gene therapy than the solid tumor cells themselves. We have developed a transcriptionally targeted adenoviral vector that is capable of delivering an inducible caspase specifically to neovascular endothelial cells (Song *et al.*, 2005). Here, we showed that delivery of iCaspase-9 with this vector, followed by its activation with a dimerizer drug, is sufficient to retard tumor progression *in vivo*.

We have used the severe combined immunodeficient (SCID) mouse model of human angiogenesis to evaluate the therapeutic efficacy of several anti-angiogenic strategies (Nör *et al.*, 2001; Dienst *et al.*, 2005; Song *et al.*, 2005). This model was also used to investigate the effect of iCaspase-9 activation on the microvascular networks *in vivo* (Nör *et al.*, 2002). Following administration of the dimerized drug, we have previously observed a decrease in implant microvessel density that was correlated with induction of endothelial cell apoptosis (Nör *et al.*, 2002; Song *et al.*, 2005). Here, we co-implanted endothelial cells stably expressing iCaspase-9 with oral cancer cells. These experiments demonstrated for the first time that the activation of iCaspase-9 in endothelial cells is sufficient to mediate a decrease in tumor neovascularization.

These results encouraged us to evaluate the effect of disrupting vascular networks in xenografted oral tumors over time. Our previous studies required the death of the animal at a series of time points, followed by histological examination. Here, we used *in vivo* bioluminescence imaging that allowed us to monitor tumor growth and evaluate the impact of treatment in real time. We found that *in vivo* bioluminescence improved the quality of the data obtained, and decreased the number of animals required to obtain information about tumor progression.

During the process of optimization of this assay for the cell lines used here, we learned the following:

1. Tissue saturation of luciferin is reached within 12–15 min, which is consistent with previous reports (Rehemtulla *et al.*, 2000).
2. Luciferase expression is maintained *in vivo* for at least 35 days.
3. A single implant should be placed in each mouse. In previous experiments, where imaging was not conducted, the mice typically carried 2 implants (Nör *et al.*, 2001a). When bioluminescence imaging is being conducted, only one scaffold should be implanted, to prevent ‘bleed-over’ luminescence from one tumor to the other.
4. OSCC-3 tumors grow far too rapidly to evaluate the anti-angiogenic treatment efficacy using long-term bioluminescence imaging. Following 35 days of implantation of OSCC3 cells, the tumor burden required termination of the experiment. Therefore, most experiments presented here were conducted with UM-SSC-17B-luc. These tumors grow at a more manageable rate, and the tumor burden was within acceptable levels. These studies showed that *in vivo* bioluminescence is a useful tool for evaluating the efficacy of novel therapeutic strategies for oral cancer.

The activation of iCaspase-9 delivered with an adenoviral vector transcriptionally targeted to neovascular endothelial cells mediated a decrease in tumor microvessel density that was correlated with inhibition of tumor progression. The tumors were not eliminated within the short duration of this experiment (*i.e.*, 4 days). However, we believe that the inhibition in tumor progression rate observed here warrants further investigation of this novel anti-angiogenic gene therapy for oral cancer.

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Figure 1.

Activation of iCaspase-9 in neovascular endothelial cells is sufficient to decrease tumor microvessel density. (A) Graph depicting the number of microvessels in tumors populated with OSCC-3 (oral squamous cell carcinoma cells) and stably transduced HDMEC-iCasp-9 or control cells (HDMEC-LXSN) retrieved from mice treated with 3 consecutive daily intraperitoneal injections of 2 mg/kg AP20187. Each mouse received one scaffold seeded with HDMEC-iCasp-9 + OSCC-3, and one control scaffold seeded with HDMEC-LXSN + OSCC-3. We evaluated 5 tumors from 5 independent mice *per* condition, and the data presented in the graph represent mean values ($\pm$ SD) of 10 microscopic fields *per* tumor. Statistical significance (asterisk) was determined at $p \leq 0.05$, with the microvessel density for the HDMEC-LXSN group used as control. (B,C) Photomicrographs of representative histological sections depicting immunohistochemistry with Factor VIII antibody to identify blood vessels (arrows). Tumor microvessels are relatively small and can be identified only by immunohistochemistry, presumably due to the rapid growth of xenografted OSCC-3 tumors (scale bar, 50 μ m for B–C).

Figure 2.

Characterization of luciferase expressing squamous cell carcinoma cells by *in vivo* bioluminescence. **(A)** Luciferase activity of two clones of UM-SSC-17B-luc and controls, *i.e.*, UM-SSC-17B-neo (transduced with empty vector), or substrate only without cells (–). Cells were seeded in six-well plates (50,000 cells/well, triplicate wells/condition) and cultured for 48 hrs. Statistical significance (asterisk) was determined at $p \leq 0.05$, with the luciferase activity obtained for the UM-SSC-17B-neo cells used as control. **(B)** Luciferase activity of 0–100,000 UM-SSC-17B-luc (M2) cells cultured for 48 hrs in six-well plates (triplicate wells/condition), demonstrating that the intensity of bioluminescence *in vitro* is directly correlated to the number of cells. Statistical significance was determined at $p \leq 0.05$, with the baseline luciferase activity obtained for substrate only (no cells) used as control. **(C,D)** Determination of saturation times for *in vivo* bioluminescence imaging of 1 representative mouse ($N = 1$) that received 1 scaffold containing HDMEC-iCasp-9 and OSCC3-luc (lower, righthand side), and 1 scaffold containing HDMEC-LXSN and OSCC3-luc (upper, lefthand side). This mouse was treated with 2 mg/kg AP20187 for 3 consecutive days. Images were acquired 1–16 min post-injection of luciferin. The graph presented in panel (C) depicts a time-course for bioluminescence intensity after injection of luciferin in the mouse depicted in panel (D). **(E)** *In vivo* bioluminescence imaging of 1 representative mouse ($N = 1$) bearing a xenografted tumor (HDMEC-LXSN and OSCC-3-luc) from day 14 through day 35 post-implantation, for evaluation of luciferase expression over time.

Figure 3. In vivo

bioluminescence analysis of the effect of iCaspase-9 activation on tumor progression. (A) Representative images of mice injected with either Ad-iCaspase-9 (no promoter) or Ad-VEGFR2-iCaspase-9 (with endothelial cell-specific promoter), and treated with either the dimerizer drug AP20187 (2 mg/kg) or the negative control phosphate-buffered saline (PBS) solution. *In vivo* bioluminescence imaging was performed 3 days after tumor implantation (baseline), 20 days post-implantation (day of adenovirus injection), 21 days post-implantation (just prior to treatment with AP20187), and 25 days post-implantation (just prior to death). (B–E) Each graph depicts, presented in the left side of this Fig. (panel A), the quantification of bioluminescence over time for the corresponding mouse (N = 1).

Figure 4.

Tumor progression is inhibited by intratumoral injection of the adenovirus Ad-VEGFR2-iCasp9 and administration of AP20187. **(A)** Graph depicting bioluminescence values ($\pm$ SD) of mice bearing tumors (UM-SCC-17B-luc cells) injected with either Ad-VEGFR2-iCasp-9 or Ad-iCasp-9 (no promoter control), and treated with either 2 mg/kg AP20187 or negative control phosphate-buffered saline (PBS). Statistical significance (asterisk) was determined at $p \leq 0.05$, with the bioluminescence values obtained for the Ad-iCasp-9 + PBS group in each individual time point used as controls. **(B)** Graph demonstrating the percent change in bioluminescence from day 21 (beginning of treatment with AP20187) to day 25 (death). Statistical significance was determined at $p \leq 0.05$, with the bioluminescence values obtained for the Ad-VEGFR2-iCasp-9 + PBS group as control. We evaluated 5 tumors *per* condition from 5 independent mice, and the data presented in the graphs (panels A and B) represent mean values ($\pm$ SD) of 10 microscopic fields *per* tumor. **(C,D)** Photomicrographs of representative histological sections after immunochemistry with Factor VIII antibody to identify tumor blood vessels (arrows). Tumors (UM-SCC-17B-luc) were retrieved from mice that received Ad-VEGFR2-iCaspase-9, and were treated with either PBS (C) or 2 mg/kg AP20187 (D) (scale bar, 50 μ m for C–D).