

Molecular Inhibition of Angiogenesis and Metastatic Potential in Human Squamous Cell Carcinomas after Epidermal Growth Factor Receptor Blockade¹

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Abstract

Tumor metastasis represents a complex multistep process that requires migration, invasion, and angiogenesis. In this study, we examined the impact of molecular blockade of the epidermal growth factor receptor on the invasive and metastatic capacity of human squamous cell carcinoma (SCC) of the head and neck using *in vitro* and *in vivo* model systems. Treatment with the anti-epidermal growth factor receptor antibody C225 attenuated the migration of SCC-1 tumor cells through a chemotaxis chamber in a dose-dependent manner. Incubation of SCC cells with 10–100 nM C225 for 4 h resulted in 40–60% inhibition of cell migration. Furthermore, in the presence of C225, the capacity of SCC-1 to invade across a layer of extracellular matrix (Matrigel) was significantly inhibited. Using an *in vivo* orthotopic floor-of-mouth xenograft model, locoregional tumor invasion of SCC-1 into muscle, vessel, bone, and perineural tissues was inhibited in C225-treated mice. This inhibition was additionally characterized by down-regulation in the expression of matrix metalloproteinase-9. These data suggest that inhibition of metastatic potential by C225 may be mediated via decreased migration and invasion of SCC cells. Regarding angiogenesis *in vitro*, we first studied human umbilical vascular endothelial cells, which established a capillary-like network structure (tube formation) in the presence of reconstituted Matrigel. Treatment with C225 reduced cell-to-cell interaction of human umbilical vascular endothelial cells, resulting in disruption of tube formation. The effect of C225 was additionally examined using an *in vivo* tumor xenograft neovascularization model of angiogenesis. Systemic treatment with C225 not only reduced tumor growth and the number of blood capillaries but also hindered the growth of established vessels toward the tumor. Taken together, these results provide evidence that C225 can suppress

tumor-induced neovascularization and metastasis in SCC of the head and neck.

Introduction

SCC⁴ of the H&N represents a worldwide health care problem with ~750,000 new cases diagnosed annually. Conventional therapy involves surgery, radiation, and chemotherapy, either alone or in various combinations. The overall mortality of patients affected by this disease has not improved significantly over recent decades (1). The high mortality rate for patients with advanced SCC of the H&N is in large measure because of uncontrolled locoregional disease, which includes local tissue invasion by the primary tumor as well as regional lymph node metastasis (1, 2). Metastasis represents a multistep process that initially relies on the motility and invasive capacity of the malignant cell. These processes facilitate growth and spread of the malignant cell from its primary site of origin to a secondary site by degrading surrounding ECM and basement membrane. A variety of agents have been shown to stimulate early components of the metastatic process, and one of these includes the EGF.

EGF is shown to increase motility, *in vitro* invasion, and metastatic potential in a number of different tumor cells (3). Overexpression of the EGFR is linked with a growing number of epithelial malignancies, including a possible role in the progression of tumors (4). Studies in breast and H&N cancer show a correlation between overexpression of EGFR and enhancement of *in vitro* cell migration and invasion (5, 6). The ability of cells to invade through Matrigel is found to be greater in cell lines that overexpress EGFR. In addition, highly metastatic human colon cancer cells are shown to express high levels of EGFR mRNA relative to cells with low metastatic potential (7). These findings suggest that EGF and its receptor may play an important role in the progression of some epithelial tumors. Indeed, EGFR has emerged as a highly promising therapeutic target for cancer therapy.

During the past decade, several molecular strategies have been explored to modulate either the EGFR itself or the downstream signal beyond the cell surface receptor (4). The use of anti-EGFR monoclonal antibody C225 represents one of the highly promising approaches to date. Recent studies have identified C225 as a potent antiproliferative agent, capable of inhibiting tumor cell growth kinetics and modulating the response of tumor cells to radiation and chemotherapy (4, 8). In the present study, we have furthered *in vitro* and *in*

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⁴ The abbreviations used are: SCC, squamous cell carcinoma; EGF, epidermal growth factor; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; H&N, head and neck; HUVEC, human umbilical vascular endothelial cell; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor.

vivo investigation to examine the capacity of C225 to attenuate metastatic potential and neovascularization in H&N cancer models. We have studied migration and invasion using *in vitro* systems, and locoregional tissue invasion using an *in vivo* orthotopic floor-of-mouth xenograft model. Antiangiogenic effects of C225 were examined using *in vitro* endothelial capillary tube formation assays and an *in vivo* xenograft angiogenesis model.

Materials and Methods

Chemicals and C225. Cell culture medium was obtained from Life Technologies, Inc. (Gaithersburg, MD). Diff-Quik stain set was purchased from Dade International Inc. (Miami, FL). Human fibronectin was generously provided by Dr. Deane F. Mosher (University of Wisconsin, Madison, WI). Matrigel was obtained from Becton Dickinson (Bedford, MA). MCDB 131 medium without growth supplements was obtained from Life Technologies, Inc. (Grand Island, NY). Primary antibody against MMP-9 was obtained from NeoMarkers (Fremont, CA). Nucleopore membranes and the 48-well chemotaxis chamber were purchased from Nucleopore Corp. (Pleasanton, CA). Transwell inserts for 12-well plates were obtained from Costar (Cambridge, MA). All of the other chemicals were purchased from Sigma (St. Louis, MO). C225, a human-mouse chimeric antibody, was generously provided by ImClone Systems Inc. (New York, NY).

Cell Lines and Cell Culture. Human SCC-1 cell line was derived from a patient with the floor-of-mouth cancer and was provided by Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI). SCC cells were cultured routinely in DMEM supplemented with 5% fetal bovine serum, 1 $\mu\text{g/ml}$ hydrocortisone, and 1% penicillin and streptomycin. HUVEC cells were kindly provided by Dr. Deane F. Mosher and cultured in MCDB 131-complete medium purchased from VEC Technologies, Inc. (Rensselaer, NY).

Cell Migration Assays. Experiments assessing cell motility were performed using a 48-well chemotaxis chamber as described (9). SCC-1 cells were grown for 3–4 days and harvested by trypsinization. Cells were then counted and resuspended in DMEM containing 0.1% BSA at a final concentration of 1×10^5 cells/ $50 \mu\text{l}$ and incubated for 1 h at 37°C. The lower compartment of the migration chamber was filled with fibronectin (100 $\mu\text{g/ml}$) dissolved in DMEM + 0.1% BSA (29 $\mu\text{l/well}$), and the cells were added to the upper compartment of the migration chamber (50 $\mu\text{l/well}$). For treatment groups, cells were incubated with 30 nm C225 for 30 min before being placed into the upper wells. The two compartments of the migration chamber were separated by a polycarbonate filter (5- μm pore size). Cells were allowed to migrate for 4 h at 37°C in a humidified atmosphere containing 5% CO_2 . Cells that did not migrate through the filter remained on the upper surface of the filter and were removed mechanically by scraping. Cells that successfully migrated to the lower surface were fixed and stained with Diff-Quik fixative and staining solutions. The filters were densitometrically analyzed (Vista Scan) and quantified by NIH Image.

Cell Invasion Assays. The ability of tumor cells to spread and invade into the basement membrane was determined by

a modification of methods described previously (10) using transwell inserts in a 12-well plate (Fig. 2A). The basement membrane was reconstituted by loading cold ECM (Matrigel; 50% in prechilled DMEM, 150 $\mu\text{l/well}$) on top of the polycarbonate filter of the inserts of the transwell plate. After gelation of Matrigel at 37°C, SCC-1 cells were plated into the inserts (2×10^5 cells/0.8 ml/well), and fibronectin (100 $\mu\text{g/ml}$) was added to the lower wells (1 ml/well). To analyze the effect of C225, C225 (30 nm) was present in either the lower wells or in the cell suspension, Matrigel, and the lower wells. After a 24-h incubation, the noninvasive cells that remained within the inserts were removed by a cotton swab. Cells that traversed through the Matrigel and the polycarbonate filter were attached to the lower surface of the filter, and were fixed and stained using the same method as in the migration assays. The number of invasive cells was additionally quantified by counting 9 random fields/sample under the microscope.

Orthotopic Xenograft Assay of Invasion. The *in vivo* orthotopic floor-of-mouth cancer model was established as described previously with minor modification (11). Female athymic mice (*nu/nu*), 3–4 weeks of age, were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and maintained in a laminar air-flow cabinet under aseptic conditions. The care and treatment of experimental animals were in accordance with institutional guidelines. Human SCC-1 cells ($\sim 1 \times 10^6$ in 0.1 ml) were injected s.c. into the submental space. The cell suspension was slowly injected at a depth superficial to the muscle. After 10 days, treatment was initiated by injecting 0.8 mg of C225 i.p. twice a week for a total of eight injections. Control animals received injections of PBS. Tumor volume was determined by direct measurement with calipers and calculated by the formula; $\pi/6 \times (\text{large diameter}) \times (\text{small diameter})^2$. For histopathologic evaluation of tumor invasion, animal jaws were fixed in 10% buffered formalin, decalcified, routinely processed, and embedded in paraffin. Serial 5- μm sections were cut, and every third section (total: 3 sections) was stained with H&E and microscopically examined by a pathologist (Weixiong Zhong, University of Wisconsin) for the invasion of tumor cells to the locoregional tissue structure.

Immunohistochemical Determination of MMP-9. The expression of proteinase MMP-9 was detected in histological sections of SCC xenografts. Briefly, excised tumor specimens were fixed in 10% neutral buffered formalin. After embedding in paraffin, 5- μm sections were cut, and tissue sections were mounted. Sections were dried, deparaffinized, and rehydrated. After quenching endogenous peroxidase activity and blocking nonspecific binding sites with serum, slides were incubated at 4°C overnight with 1:100 dilution of primary antibody directed against MMP-9, followed by a 30 min incubation of biotinylated goat antimouse secondary antibody. Slides were then incubated with streptavidin peroxidase and visualized using the 3,3'-diaminobenzidine chromogen with a standard immunoperoxidase method (Vectastain ABC kit).

In Vitro Angiogenesis Assay (HUVEC Capillary-like Network Formation). For reconstitution of a basement membrane, Matrigel was diluted 2-fold with cold DMEM and added to the 24-well tissue culture plate (250 $\mu\text{l/well}$) at 4°C.

The 24-well plate was brought to a 37°C cell culture incubator and incubated for 1 h to allow the Matrigel to solidify. HUVEC cells were trypsinized, counted, resuspended in MCDB-131, and added on top of the reconstructed basement membrane (1000 cells/ml/well) in the absence or presence of C225 (30 nM). Cells were incubated for the indicated time intervals to allow capillary-like structure formation. For ease of handling and optimal visualization of endotubes, medium was removed at the end of incubation culture, and agarose (250 μ l/well, 0.5% in PBS, ~50°C) was gently added drop-by-drop to the cells. After solidification of agarose (2–5 min at room temperature), the immobilized endotubes were fixed with formalin (200 μ l/well, 30 min) and washed twice with 250 μ l of PBS. The endotubes were then stained with Diff-Quik solution for 20 min, followed by extended washing with water (6 \times 1 ml/well over at least 24 h). The number of endotubes was additionally quantified by counting 9 random fields/sample under the microscope.

Tumor Xenograft Angiogenesis Assay (Matrigel Plug).

The Matrigel plug neovascularization assay was developed by Drs. Nasim Akhtar and Robert Auerbach (University of Wisconsin). Athymic nude mice were injected s.c. along the abdominal midline with 0.5 ml of Matrigel. After 24 h, SCC-1 cell (5×10^5 in 5 μ l) suspension was soaked with a polyvinyl sponge (1 \times 1 \times 0.5 mm). The sponge was then introduced into a surgically created micropocket in the center of Matrigel plug formed within the mouse abdominal wall. After 5 days, mice began receiving 0.8 mg of C225 (i.p. injection) or PBS (control) twice a week. On day 21, mice were injected with 0.2 ml of a 50 mg/ml FITC-Dextran (molecular weight, M_r ~2,000,000) solution via the tail vein for the purpose of visualizing the vessels within the Matrigel plug. After 20 min, mice were sacrificed, and the Matrigel plugs were removed and fixed in 10% formalin solution. To visualize the general layout of the Matrigel plug and the presence of perfused blood vessels, phase-contrast microscopy and fluorescence microscopy were used, respectively. The intensity of fluorescence was additionally quantified by Adobe Photoshop software (Adobe Systems, Mountain View, CA).

Statistical Analysis. In all of the experiments, the differences among treatment groups were examined by Student's *t* test. The incidence of tumor locoregional invasion was analyzed by the χ^2 test.

Results

Fibronectin-mediated Migration of SCC Cells *in Vitro*. To assess the effect of C225 on the metastatic potential of SCC cells, we first examined the impact of C225 on fibronectin-mediated chemotaxis of SCC cells *in vitro*. The effect of C225 on SCC-1 cell migration was investigated using the chemotaxis chamber over a period of 4 h with various concentrations of C225. As shown in Fig. 1, the migration of SCC-1 cells was significantly decreased ($P = 0.0087$) from a value of 78.0 ± 10.0 densitometric units in untreated cells to 47.7 ± 4.6 in cells treated with 10 nM C225, and to 32.8 ± 7.9 in cells treated with 100 nM C225. This represents a motility decrease of 40–60% in cells treated with C225. The inhibitory effects of C225 were not attributable to cytotoxicity at 10 and 100 nM, as neither concentration produced notable ef-

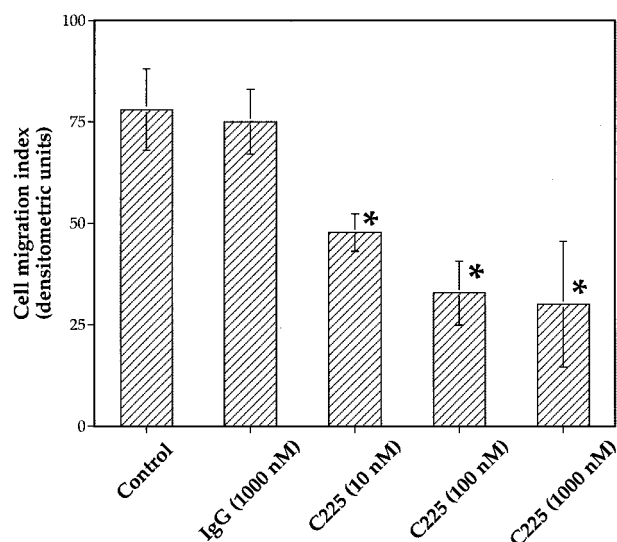


Fig. 1. Effect of C225 on SCC cell migration. Cell migration was determined using a 48-well microchemotaxis chamber as described in "Materials and Methods." SCC-1 cell migration in response to fibronectin was measured in the presence of various concentrations (0–1000 nM) of C225. Each bar represents the mean \pm SD of triplicate determinations. * denotes values that are significantly different from control (dose = 0), $P < 0.01$. Similar results were obtained in replicate experiments.

fects on SCC-1 proliferation or viability (data not shown). Specificity of the C225 inhibition of SCC cell migration was confirmed by the use of nonspecific human IgG, which was found to exert no influence on cell migration (Fig. 1).

Invasion of SCC Cells through ECM Gel. The traverse of SCC-1 cells through a layer of ECM gel (Matrigel) was assayed using a 12-well transwell plate. Fig. 2A illustrates the layout of the well. C225 was either absent (control), present in lower well only (lower well), or present in lower wells, Matrigel, and the cell suspension (all). Results are shown in Fig. 2, B and C. In the control setting, SCC cells traversed across the Matrigel and subsequently occupied almost the full surface area of the filter. After C225 exposure within the medium of the lower well, the number of invading cells was substantially reduced. When C225 was present in the lower wells, Matrigel, and cell suspension (all), invasion of SCC cells across the Matrigel was almost completely inhibited. These results demonstrate that C225 can inhibit the *in vitro* invasion potential of SCC cells.

Locoregional Invasion in Orthotopic H&N Cancer Model. We also examined the capacity of C225 to inhibit *in vivo* invasion using a murine orthotopic floor-of-mouth cancer model. SCC-1 cells were inoculated into mice in the s.c. tissues underlying the floor of the mouth. After inoculation and establishment of a tumor, SCC-1 tumor invasion progressed in an orderly fashion involving the muscular, vascular, bone, and neural structures of the floor of the mouth and tongue region (Fig. 3A). Fifty days after inoculation, 6 of the 8 control mice showed tumors that involved local muscle tissue, 5 of 8 showed invasion into vessels and bone, and 3 of 8 tumors demonstrated perineural infiltration (Table 1). Conversely, only 3 of 8 C225-treated mice showed tumor

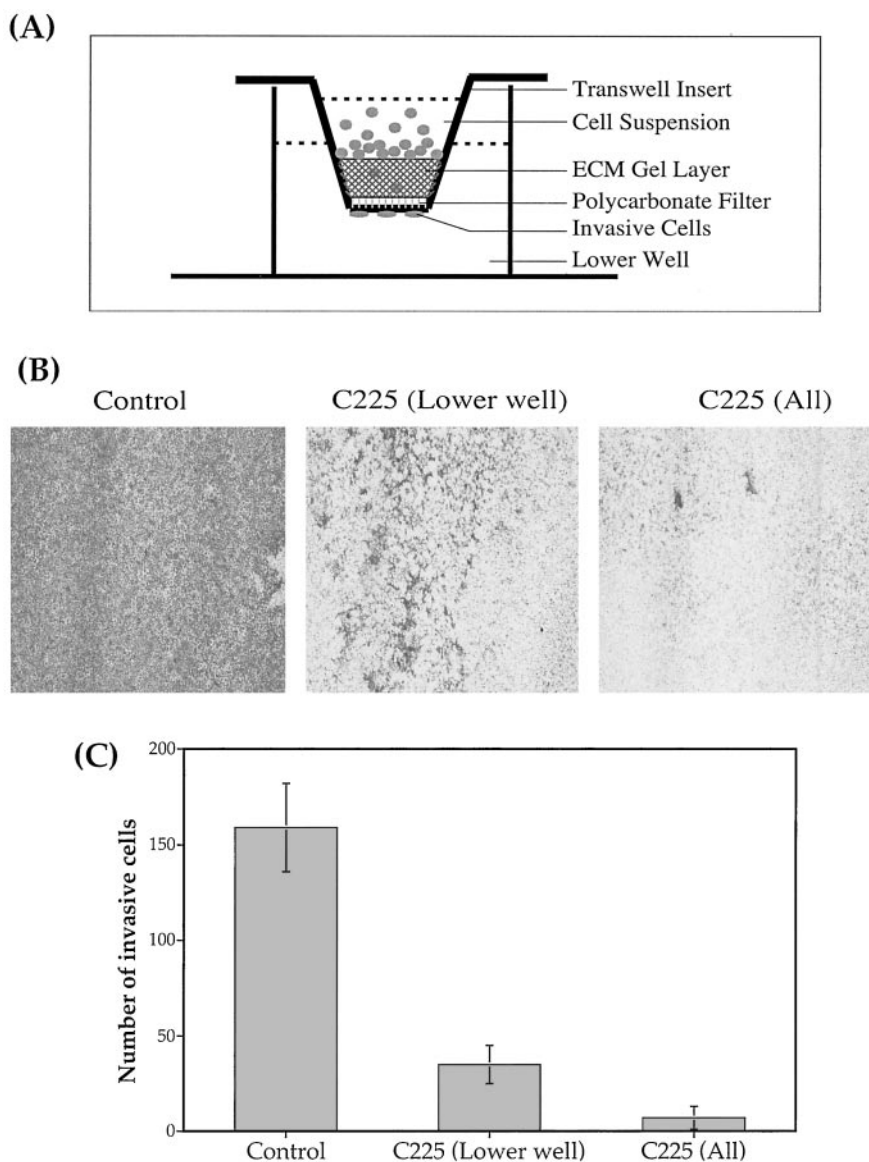


Fig. 2. Effect of C225 on *in vitro* invasion of SCC cells. The invasion of SCC-1 cells through a layer of ECM gel was assayed using a 12-well transwell plate as described in "Materials and Methods." C225 (30 nM) was either absent (*control*), present only in the lower well (*Lower well*), or present in the lower well, ECM gel, and cell suspension (*All*). A, schematic representation of transwell plate and insert. B, photographs ($\times 200$) of control and C225-treated samples. C, average number of invasive cells per microscopic field ($\times 400$) from two separate experiments; bars, \pm SD.

invasion into muscle. Among these 3 mice, 1 had muscle invasion accompanied by both vascular and bone invasion. No perineural invasion was observed in the C225-treated mice. In addition, the expression of MMP-9 was reduced in C225-treated mice compared with controls (Fig. 3B). These results suggest that treatment with C225 inhibited the local invasion of SCC-1 tumors arising in the H&N. This effect may be in part mediated by inhibition in the expression of the cell invasion-associated proteinase, MMP-9.

Capillary-like Network Formation in HUVEC Cells. To investigate the effect of C225 on angiogenesis, we first examined the formation of capillary-like network (tube formation) in an *in vitro* model of angiogenesis using HUVEC cells. HUVEC cells were shown to attach to the reconstituted ECM matrix (Matrigel) when plated, and were capable of forming tube-like structures. It is believed that these processes in ECM are representative of the latter stages in

angiogenesis, during which vessels complete their differentiation and become stable (12, 13). In the present study, HUVEC cells were observed to spread out and generate lateral processes 2 h after plating onto Matrigel. By 6 h, the alignment of cells and the formation of intercellular tubular connections become evident (Fig. 4A). At 18 h, the inhibitory effect of C225 was clearly noted. Whereas the endotubes formed in control cells continued to differentiate and mature, cells treated with C225 gradually lost their cell-to-cell connections. This destabilizing effect became more obvious at 30 h, when the capillary-like network of HUVEC cells displayed additional fragmentation and dissociation, resulting in the loss of most intercellular contacts. The number of endotubes was substantially reduced in the C225 treated group (Fig. 4B). These observations suggest that C225 can reduce the ability of HUVEC cells to form stable capillary-like network structures *in vitro*. Specificity

(A) H&E

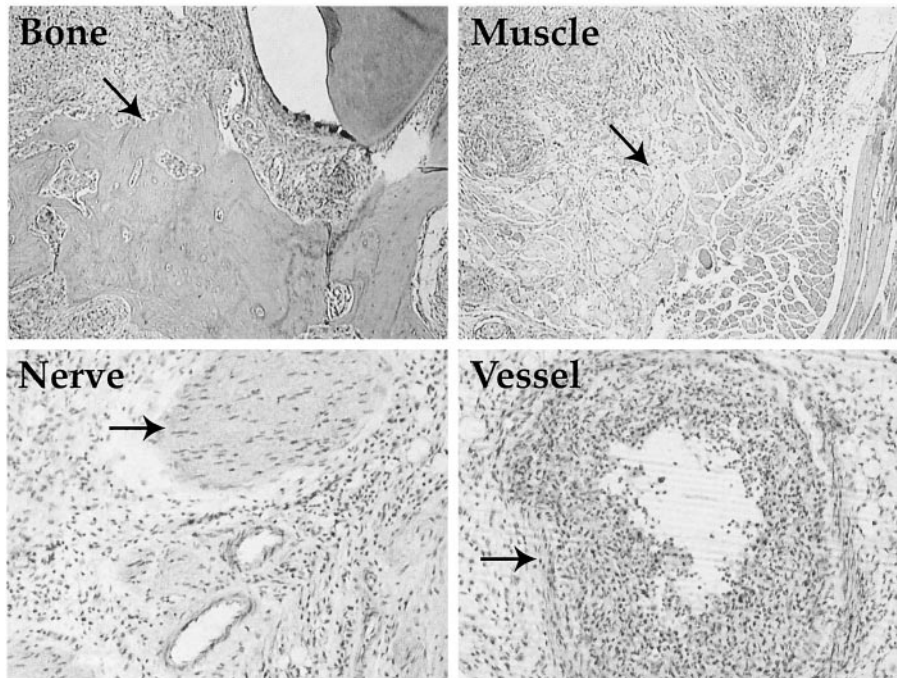


Fig. 3. A, histological sectioning of tumors growing in the floor of the mouth region in the orthotopic mouse model system. SCC-1 cells demonstrate capacity for locoregional invasion into surrounding tissues including bone, muscle, nerve, and vessel as indicated by arrows. Photographs are taken from representative tissue sections stained with H&E. B, effect of C225 on the expression of MMP-9. Immunohistochemical staining of MMP-9 was determined using human SCC-1 tumor sections taken from control or C225-treated mice. Positive (brown) staining indicates the expression of MMP-9. Photographs show representative tissue sections from the control and the treatment group, respectively (a total of three sections were stained per group).

(B) MMP-9

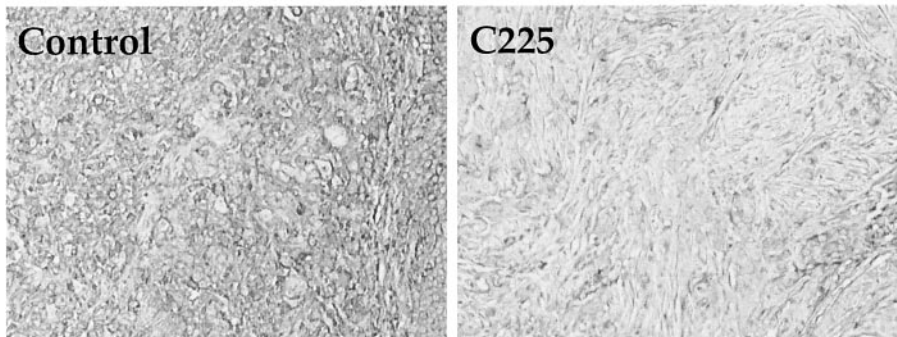


Table 1 Locoregional invasion of SCC-1 tumors in the orthotopic floor-of-mouth model

Therapy ^a	Median tumor volume (mm ³)	Median body weight (g)	Muscle invasion	Vascular invasion	Bone invasion	Perineural invasion
Control	220	25	6/8 ^b	5/8	5/8	3/8
C225	15	23	3/8 ^c	1/8 ^d	1/8 ^d	0/8 ^d

^a Therapy began on day 10 when median tumor size was 19 mm³. PBS (control) or C225 (0.8 mg) was injected i.p. biweekly for 4 weeks. Tumor weight and invasion incidence were evaluated at necropsy (day 50).

^b Number of mice with invasion per number of injected mice.

^c $P = 0.13$.

^d $P < 0.05$.

of the C225-inhibition of tube formation was additionally confirmed using nonspecific human IgG, which was found to exert no influence on tube formation.

Tumor-induced Vascularization of Matrigel Plugs. To additionally investigate the influence of C225 on tumor-induced angiogenesis *in vivo*, we adapted a tumor xenograft angiogenesis assay using Matrigel matrix. In this assay, Ma-

trigel was injected s.c. into the abdominal wall of mice thereby forming semisolid plugs. Later (24 h), SCC-1 cells were introduced into a polyvinyl sponge that was then inserted into a surgically created pocket within the Matrigel plug. In each experimental group, at least three grafts were analyzed in two independent experiments. Plugs with Matrigel alone were pale in color and visibly clear, with no iden-

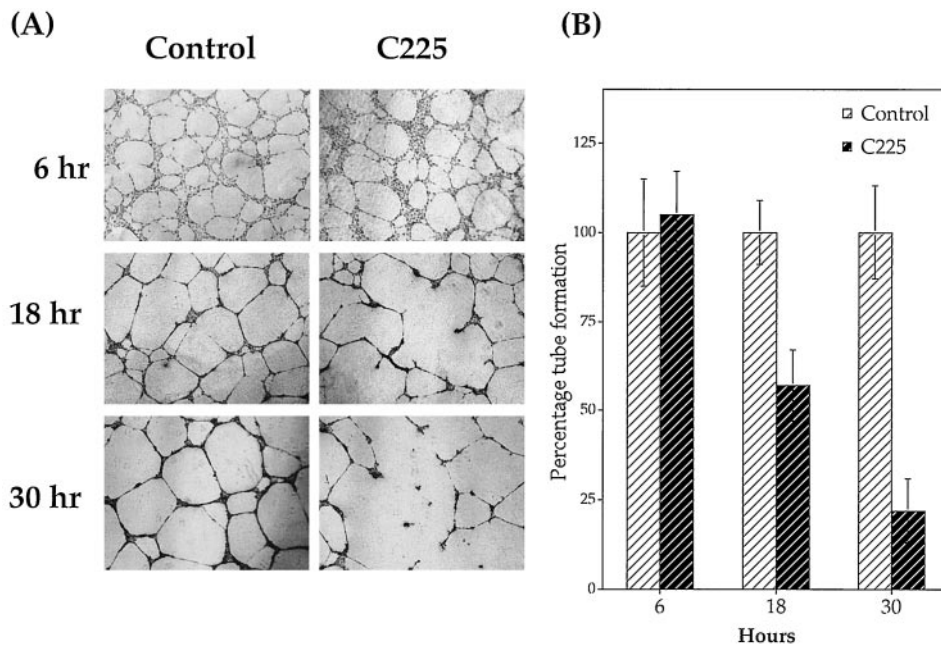
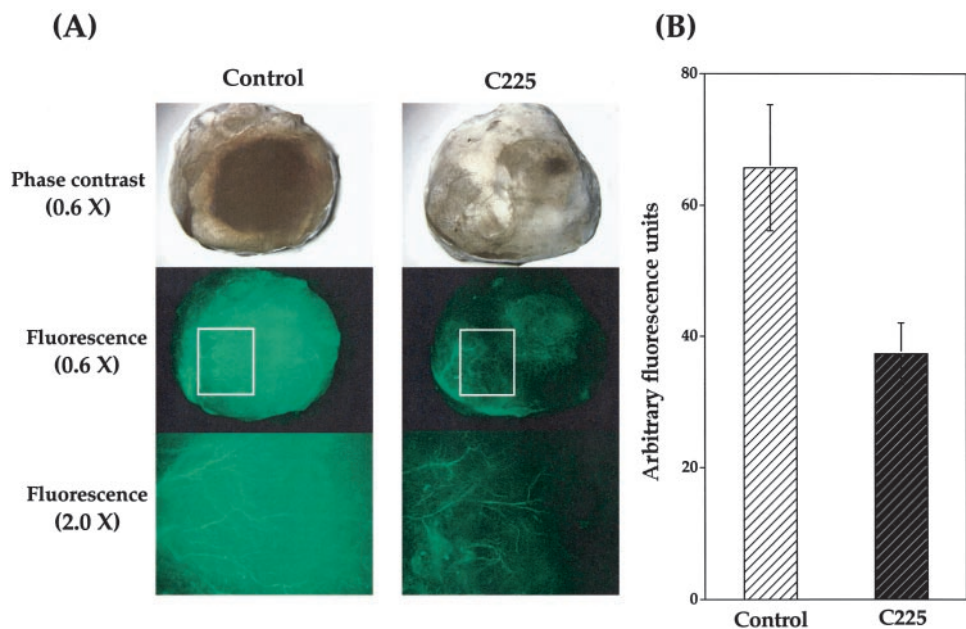


Fig. 4. Effect of C225 on capillary-like network (tube) formation in HUVEC cells. Tissue culture plates were coated with 250 μ l Matrigel for 1 h, and HUVEC cells (1000 cells/ml/well) were added with or without C225 (30 nM). At 6, 18, and 30 h, plates were fixed and stained. *A*, photomicrographs of tube formation in the absence or presence of C225. *B*, average number of tubes per microscopic field from three separate experiments. Data were normalized to the control group for each time interval; bars, \pm SE.

Fig. 5. Effect of C225 on tumor xenograft angiogenesis. SCC-1 cells were implanted in athymic mice as described in "Materials and Methods." Biweekly administration of C225 (0.8 mg in 0.4 ml) or PBS was initiated on day 5. On day 21, mice were injected (i.v.) with FITC-dextran before removal of Matrigel plugs and examination using phase-contrast and fluorescence microscopy. *A*, blood vessel distribution visualized (green) under fluorescence microscopy. *Bottom panels* represent higher resolution photographs ($\times 2$) taken from the designated box within the *middle panels*. *B*, the intensity of fluorescence was additionally quantified as described in "Materials and Methods." The results were obtained from 6 mice/group in two independent experiments; bars, \pm SD.



tifiable blood vessels migrating into the Matrigel. After implantation with tumor cells, blood capillaries were observed to grow from the edge of the Matrigel plug toward the implanted tumor as visualized by fluorescent microscopy (Fig. 5A). Those plugs containing SCC-1 cells showed extensive vascularization and growth of vessels toward the tumor core within 21 days. In contrast, plugs in those mice treated with C225 showed markedly reduced vascularization (Fig. 5B). In addition, dramatic inhibition in the growth of SCC-1 cells in C225-treated mice was observed, as visualized by phase-contrast microscopy (Fig. 5A). These results suggest that

C225 can inhibit angiogenesis, which may in part derive from its inhibitory effect on tumor growth.

Discussion

SCCs of the H&N are characterized by a marked propensity for local invasion and dissemination to regional lymph nodes. The majority of H&N SCC tumors show frank overexpression of EGFR compared with normal squamous epithelium. Studies have shown that high levels of EGFR expression correlate with aggressive behavior, increased metastasis, and de-

creased survival in SCC of the H&N (6, 14). Recently, we examined C225 as a potent antiproliferative agent for SCC of the H&N, capable of inhibiting tumor cell growth kinetics and modulating the response of SCC cells and xenografts to radiation (15, 16). In this study, we extend these previous observations to demonstrate that C225 can significantly inhibit initial events in the metastatic processes, *i.e.*, migration and local invasion of tumor cells. The *in vitro* results indicate that C225 can attenuate SCC-1 cell motility in a dose-dependent manner and inhibit the invasion of SCC-1 cells through reconstituted ECM matrix. Similar results have been confirmed in other human SCC cell lines including SCC-6.⁵ Using an orthotopic murine floor-of-mouth model, we additionally demonstrate that systemic administration of C225 decreases the frequency and extent of tumor invasion into locoregional tissue structures.

Tumor invasion is a complex process that requires active interplay between the invading tumor cell and the ECM, as well as other stromal elements (17). MMPs, a family of proteinases, represent key enzymes involved in these processes. MMPs assist tumor cell invasion by degrading either cell surface-associated molecules or the subjacent matrix itself. Among the proteinase family, MMP-9 has been shown to exhibit high expression and strong correlation with the malignant phenotype in SCC of the H&N (18). In a recent study, strong association between EGFR status, MMP-9 expression, and invasive capacity was observed across a variety of H&N SCC cells (6). It has been suggested that EGFR signaling pathway may play an important role in the invasive behavior of tumor via selective up-regulation of MMP-9 (19, 20). In the present study, we demonstrate that the expression of MMP-9, as indicated by immunohistochemical staining, is decreased in tumor specimens obtained from C225-treated mice compared with that of controls. These results suggest another potentially important therapeutic effect of EGFR blockade, namely, the capacity to block induction of MMP-9, which plays a major role in the process of invasion and metastasis in SCC of the H&N.

The inhibitory effect of C225 on the metastatic potential of SCC cells is accompanied by a reduction in tumor-induced angiogenesis, as assessed by the Matrigel plug neovascularization model in nude mice. Our results demonstrate that C225 significantly reduces vessel formation in the Matrigel plug and inhibits the propensity of vessels to grow toward the central core of SCC-1 tumor implantation (Fig. 5). This inhibition of angiogenesis is associated with a significant delay in tumor growth. The inhibitory effect on vessel formation may represent a direct effect of C225 on endothelial cells or on the tumor cells.

A direct effect of C225 on endothelial cells is suggested by our present results indicating that C225 can decrease cell-to-cell interaction and disrupt endotube formation (Fig. 4). Endothelial cells within several neoplasms have been shown to express EGFR (21). Binding of transforming growth factor α to EGFR on endothelial cells has been shown to stimulate their proliferation (22). In addition, blockade of the EGFR

leads to apoptosis of endothelial cells and reduction in neovascularity (23). These results suggest that C225-induced disruption of tube formation in endothelial cells may be attributable, in part, to a direct effect of C225 on endothelial cells. This hypothesis is additionally supported by previous studies showing that C225 can modulate the expression of angiogenic factors such as VEGF, basic fibroblast growth factor, or interleukin 8 from several tumor cells (15, 24, 25). Because VEGF and interleukin 8 can act as survival factors for immature endothelial cells (26) and protect endothelial cells from apoptosis (27), decreased production of VEGF induced by C225 may enhance endothelial cell apoptosis, contributing to the observed reduction in neovascularity.

An alternative explanation for the observed C225-induced disruption of tube formation in endothelial cells is that C225 may influence the interaction between endothelial cells and the basement membrane. Formation of new vessels involves the migration and proliferation of endothelial cells. Once endothelial cells have assembled to form a new vessel, the cells secrete a basement membrane that helps to stabilize and maintain the vessel wall. Therefore, tube formation involves cellular matrix receptors and the production of matrix proteins. Alterations in the production of these components would be expected to influence tube formation. Two major components of the matrix, laminin and collagen IV, have been shown to possess endothelial cell-binding sites which regulate vessel stability (28, 29). VE-cadherin, which is localized at sites of intercellular contact, has been shown to mediate endothelial cell capillary tube formation in fibrin and collagen gel (30). Additional studies are under way to investigate whether the observed antiangiogenic effects of C225 are mediated by mechanisms involving these matrix components.

SCC of the H&N is an aggressive malignancy, and the prognosis is highly dependent on local and regional metastatic spread of this disease. Those patients that are refractory to surgery, radiation, and available chemotherapeutic agents are faced with a high mortality rate, often with uncontrolled locoregional disease. Therefore, demonstration that molecular blockade of EGFR signaling pathways can inhibit the invasive, metastatic, and angiogenic potential of SCC tumors may prove clinically important. Indeed, C225 is but one of several highly promising EGFR inhibitory agents currently moving through the preclinical and clinical trials process. We have recently performed additional preclinical studies with the EGFR-selective tyrosine kinase inhibitor ZD1839 (Iressa), which confirms the capacity of this agent to inhibit HUVEC tube formation and tumor angiogenesis (Matrigel plug assay) in a similar fashion to that observed with C225 (31). Findings from the present study provide evidence that blockade of EGFR signaling may represent a valuable treatment strategy for epithelial tumors that rely on EGFR systems for growth (*i.e.*, SCC of the H&N). Furthermore, the use of C225 in combination with conventional modalities of therapy (radiation and chemotherapy) may improve the efficacy of these treatments. In this regard, clinical trials examining the role of C225 (Phase II-III) are currently in progress for H&N cancer patients, either combined with radiation or with cisplatin chemotherapy.

⁵ Unpublished observation.

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