Exogenous Expression of Caspase-14 Induces Tumor Suppression in Human Salivary Cancer Cells by Inhibiting Tumor Vascularization

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Abstract. *Background: Current therapeutic approaches to salivary gland cancer are often associated with severe disfigurement and loss of glandular function, which are traumatic to the patients. Exploration of novel treatment approaches, such as gene therapy, is needed. Materials and Methods: The human salivary gland cancer cell line HSG was transiently transfected with full length human caspase-14 cDNA. Photomicroscopy, BrdU assay, cell counting, MTT assay, and TUNEL assay were applied. To determine the tumorigenicity, tumor volume, tumor pathology and vascularization were analyzed in vivo. Results: Cell growth and viability were inhibited significantly by transient caspase-14 expression. Caspase-14 expression resulted in a significant reduction of tumorigenicity. Importantly, a significant decrease in tumor blood vessel formation was observed. Conclusion: Salivary gland cancer cells underwent growth inhibition, cell death, and reduced tumorigenicity in vivo when exogenous caspase-14 was expressed, which could be due, in part, to an inhibitory effect of caspase-14 on tumor vascularization.*

Oral cancer is one of the top ten types of cancer in the United States, with only a 50% five-year survival rate (1). Salivary gland cancer is the most histological diverse type of cancer. It can derive from both major and minor glands, although more than 50% of these tumors are benign (2, 3). Treatment of salivary gland cancer is often associated with disfigurement and loss of glandular function, which can be traumatic to patients (4). Exploration of novel approaches and development of innovative therapies is needed to combat such cancer. Gene therapy for salivary cancer has been evaluated in the past decade, but strategies that specifically target tumor cells remain to be developed (5, 6).

In the normal epidermis, caspase-14 plays an important role in keratinocyte differentiation (7). The stratum corneum of the epidermis consists of denucleated corneocytes cross-linked by proteins, and the intercellular space is occupied by lipids (8). Formation of this waterproof barrier relies on the cornification of the epidermal keratinocytes, which undergo growth arrest, terminal differentiation, and an epidermal-specific cell death, referred to as planned cell death (9). Abnormalities in any of these programmed events may lead to epidermal disorders such as psoriasis and cancer. Studies using a caspase-14 knockout mouse model confirmed that caspase-14 expression is associated with epidermal keratinocyte terminal differentiation, barrier formation and reduced carcinogenesis (10). Consistent with this, keratinocyte differentiation is accelerated when caspase-14 is induced by green tea polyphenols (11, 12).

Many epithelial cancer cell lines, such as human epidermoid cancer A431, human oral carcinoma cell OSC2, and human salivary gland cancer HSG, fail to express caspase-14 (11). We showed previously that stable transfection of human caspase-14 cDNA into A431 human skin cancer cells caused growth inhibition and cell death, associated with reduced tumorigenicity in athymic mice (13). We hypothesized that gene therapy using exogenous caspase-14 expression could be an effective approach to

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inhibit epithelial cancer. However, transient expression would be a preferred gene therapy approach since it would not permanently alter the genome, which has inherent risks. Whether transient expression of caspase-14 is able to achieve such an inhibitory effect is not known. In addition, whether caspase-14 expression has an inhibitory effect on human salivary gland cancer cells has not been determined. The objective of the current study was to use a poorly differentiated human salivary gland cancer cell line (HSG) transiently transfected with an expression plasmid containing a full length cDNA of human caspase-14 to determine the effects of caspase-14 expression on cell growth, cell death, and tumorigenicity.

Materials and Methods

Cell culture. The human salivary gland adenocarcinoma cell line HSG was derived from intercalated ductal epithelium (14). This cell line was maintained in DMEM/Ham's F12 medium, with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml hydrocortisone at 37˚C with 5% CO_2.

Vector construction. The caspase-14 expression vector containing full length cDNA of human caspase-14 in the pCMV6-XL4 plasmid was constructed and provided by OriGene (Rockville, MD USA) by inserting *Homo sapiens* caspase 14 cDNA (729 bp) into the Not I site of the pCMV6-XL4 plasmid. The construct was sequenced to confirm the cDNA sequence.

Transfection. Transfection was performed using Lipofectamine 2000 according to the manufacturer's instructions (13). The HSG cells were transfected with the pCMV plasmid containing human caspase-14 cDNA. Control cells were transfected with empty pCMV vector. Expression of caspase-14 was confirmed by Western blotting and transfection efficiency determined by immunocytochemistry.

Western blot analysis. Cell lysate preparation and SDS-PAGE were performed using a previously described method (12).

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The MTT assay method is described elsewhere (15). Briefly, 10^4 cells were seeded in each well of a 96-well plate. At different time points, medium was replaced with 100 μl of 2% MTT and the plate was incubated at 37˚C for 30 minutes. A volume of 100 μl of 0.2 M Tris, pH 7.7, 4% formalin was then added to each well. After incubation at room temperature for 5 minutes, liquids were removed and the wells were allowed to dry. Each well was rinsed with 200 μl water followed by addition of 100 μl dimethylsulfoxide (containing 6.35% 0.1 N NaOH) to each well. The optical absorption of each well at 562 nm was measured using a spectrophotometer.

Cell growth assay. Cell growth assays were performed on HSG cells at 24, 48 and 72 h post transfection. Cells were initially plated in triplicate in each well (10^5/well) of a 24-well cell culture plate at time 0. Cell quantification was achieved by trypsinization of the cultured cells and cell counting using a hemacytometer.

BrdU assay. The BrdU incorporation assay to measure DNA synthesis was analyzed by using a BrdU Cell Proliferation Assay Kit (Oncogene Research Products, Boston, MA, USA). The method is described elsewhere (16).

Immunocytochemistry. Cells were grown on 8-well chamber slides (Nagle Nunc International, Naperville, IL, USA). Specific antibodies were used to stain the cells for the presence of caspase-14 protein, or other targeted proteins, by a method described elsewhere (12).

Animals and xenograft experiments. All animal protocols in this study were approved by the Institutional Animal Care and Use Committee. Female athymic (nu/nu) mice at the age of 4-6 weeks were purchased from the National Cancer Institute (Bethesda, MD, USA). Xenografts from HSG cells were injected into the abdominal area subcutaneously at 1 million cells in 100 μl PBS. Each animal was xenografted with HSG pCMV cells on one side and HSG pCMV Caspase-14 cells on the contralateral side. Animals that exhibited tumor growth by day 3 of xenograft were monitored. Measurements of tumor size started immediately following the appearance of tumors. The volume of the tumor was calculated using the following equation: Volume=width^2 x length/2 (mm^3).

Immunohistochemistry. The tumor tissues were fixed and paraffin embedded. Five micron-thick serial sections were cut and stained with hematoxylin and eosin (H&E) for histopathological evaluation.

Immunofluorescence. New blood vessel formation was analyzed in the salivary gland tumor with and without caspase-14 transfection by using paraffin-embedded sections blocked for 30 minutes with 3% normal goat serum. The sections were then incubated overnight at 4˚C in 15 μg/ml of the vascular cell-specific marker biotinylated *Griffonia simplicifolia* isolectin B4 (GSI; Vector Laboratories, Burlingame, CA, USA). This was followed by incubation with 7 μg/ml Avidin-conjugated Texas Red (Vector Laboratories). LSM 510 confocal microscopy (Carl Zeiss, Thornwod, NY, USA) was used to localize the expression of isolectin B4 in the salivary gland sections. The density of the microvasculature within each image was determined by using computer-assisted morphometric software (Image-1/Metamorph Imaging System; Universal Imaging Corporation).

Apoptosis analysis (TUNEL assay). TUNEL assays using the ApopTag Plus Peroxidase *in situ* apoptosis detection kit (Chemicon International, CA, USA) were performed according to the manufacturer's directions, and as described elsewhere (17). Briefly, after fixation of the cells or after deparaffinization of tumor sections, endogenous peroxidase activity was quenched with 3% hydrogen peroxide in PBS for 5 min at room temperature. The wells or slides were incubated with terminal deoxynucleotidyl-transferase (TdT) enzyme in a moist chamber for 60 min at 37˚C. Incubation with antidigoxigenin-conjugate for 30 min at room temperature was conducted, followed by color development with diaminobenzidine substrate.

Statistical analysis. All data are reported as means±SD or SEM. Two-tailed Student's *t*-tests were used to analyze statistical significance. Differences were considered statistically significant at $p<0.05$.

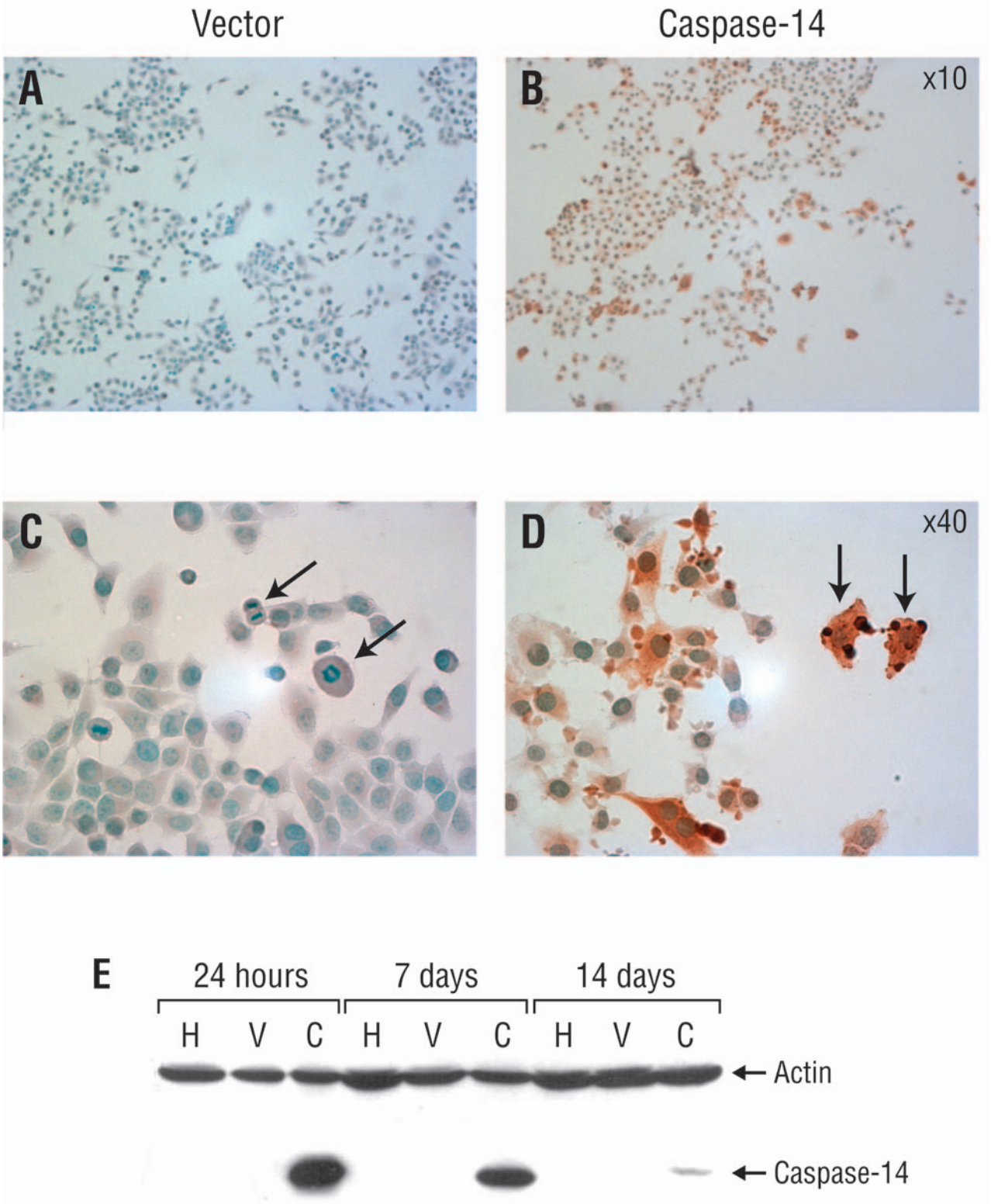

Figure 1. *Caspase-14 immunostaining of transiently transfected HSG cells shows efficient transfection. A, Vector-transfected only, and B, caspase-14-transfected HSG cells (×10); C, vector-transfected only, and D, caspase-14-transfected HSG cells (×40). Dark brown staining represents caspase-14 protein. Nuclei are stained blue. Arrows in C point to mitotic cells, arrows in D to cells with heavy staining of caspase-14. Caspase-14 expression persisted through two weeks as shown by E, Western blot result of caspase-14 protein levels in HSG cells transfected with either control vector or caspase 14-pCMV6-XL4 at 24 h, day 7 and day 14 post transfection. H: HSG, V: control vector, C: caspase-14 vector.*

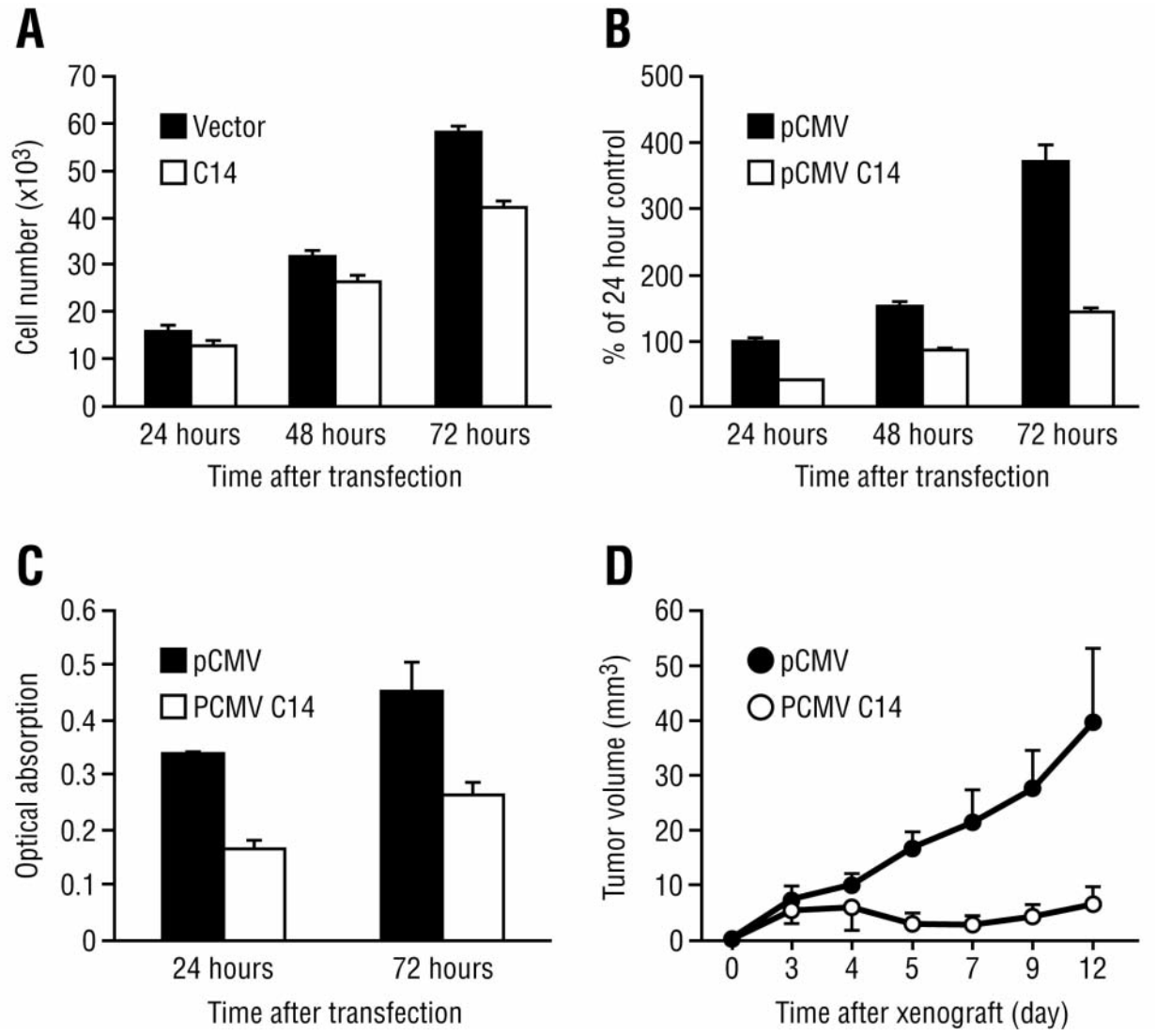

Figure 2. *Growth inhibition and reduced tumorigenicity resulted from transient exogenous caspase 14 expression. A, Cell growth measured by cell counting of HSG cells either transfected with empty vector or caspase 14-pCMV6-XL4 at time points indicated. B, MTT assay of HSG cells transfected with either empty vector or caspase 14-pCMV6-XL4 at time points indicated. C, BrdU incorporation assay performed after transfection of either empty vector or pCMV caspase-14 into HSG cells. D. Tumor volumes in mm3 measured in athymic mice at the time points indicated. ANOVA two-tailed-t-test was performed on the above data, $p<0.05$ in each case.*

Results

Transfection and confirmation of caspase 14 expression by pCMV plasmid in HSG cells. The plasmid was transfected into HSG cells at approximately 46% efficiency, as determined by immunocytochemistry (Figure 1A-D). Transfection efficiency and persistence of expression was examined by Western blot analysis at three time points: 24 h, 1 week and 2 weeks after transfection (Figure 1E). Caspase-14 protein was expressed efficiently at 24 h. As expected for a transient transfection, levels declined with time after transfection. However, significant expression was still observed after 7 days. By day 14 post transfection, caspase 14-expression had declined to low levels but was still detectable (Figure 1E).

Morphological changes in transfected cells. The caspase 14-transfected cells exhibited significant morphological changes in comparison with the cells transfected with empty vector only (Figure 1C and 1D). Cytological evaluation showed that the caspase-14-positive tumor cells assumed a larger, more irregular and flatter morphology (Figure 1D) consistent with

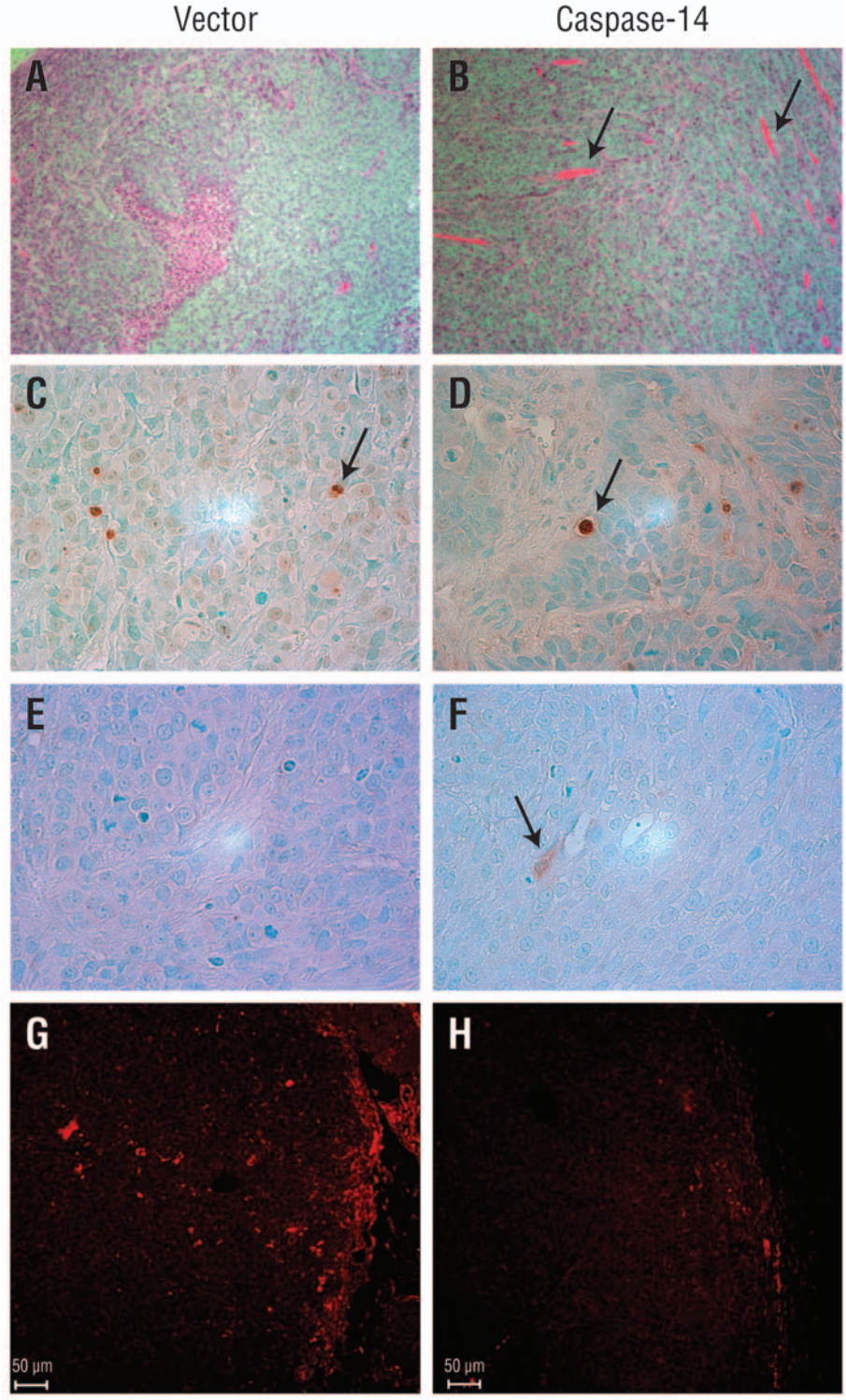

Figure 3. *Representative images of tumor samples formed by either empty vector-transfected or caspase 14-pCMV6-XL4-transfected HSG cells show reduced tumorigenicity with caspase-14 expression. A and B, H &E histological sections of tumors formed in athymic mice. Although all xenografts exhibited features of poorly differentiated adenocarcinoma, the caspase-14-positive xenografts showed significantly more zones of extensive tumor necrosis (arrows) than did the controls. C and D, Tumor sections stained using the ApopTag Plus Peroxidase in situ apoptosis detection kit. TUNEL-positive cells are stained with brown nuclei (arrows). In both cases, there are relatively few apoptotic cells. E and F, Caspase-14 immunostaining of tumor sections (collected three weeks post xenograft) formed by empty vector-transfected or caspase 14-pCMV6-XL4-transfected HSG cells. Arrow points to a caspase-14-positive cell. G and H, Representative immunofluorescence staining of blood vessels in tumor sections formed by control (G) and caspase-14 expressing cells (H).*

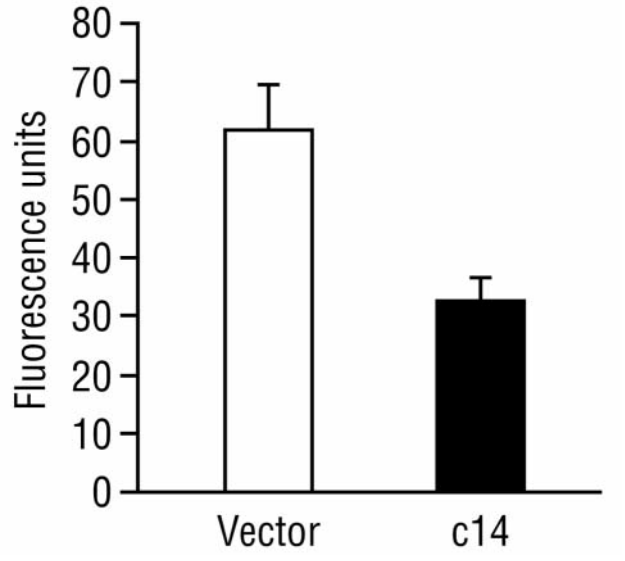

Figure 4. *Caspase-14 expression reduced vascularization. Statistical analysis showed significantly reduced (>50%) lectin staining in tumors formed by caspase-14-expressing HSG cells ($p<0.05$).*

effete and dying cells, in comparison to their caspase-14-negative counterpart. Some (3.6%) caspase-14-positive cells were larger in size with a flattened shape and showed heavy staining in the cytoplasm and nucleus (Figure 1D, arrows). Sacs with heavy caspase-14 staining appeared near the cell surface. These cells appeared to be undergoing cell death. Prominent and numerous punched-out, oval and round, cytoplasmic pinocytic vesicles exhibiting intense caspase-14 staining also are present among this group (Figure 1D, arrows). The appearance of these cells is morphologically consistent with dying cells, suggesting that these are under stress. Other caspase-14-positive cells (6.7% with heavy nuclear staining, and 36% with light staining) also exhibited differences in morphology.

Cell growth and DNA synthesis. Expression of caspase-14 in HSG cells induced a significant inhibition of growth during the 72 h period following transfection, as measured by cell counting (Figure 2A). At the 24 h time point, the caspase-14-transfected cell number was 82% of the number of vector-transfected cells ($p=0.048$). Forty-eight hours after transfection, the number of caspase-14-transfected cells was approximately 84.1% of the number of vector-transfected cells ($p=0.0074$), and at the 72 h time point, 71.9% of the vector-transfected cells ($p<0.0001$). Consistent with this pattern of reduced cell number, MTT assays demonstrated that caspase-14-transfected cells showed lower growth and/or reduced viability (Figure 2B). At 24 h post transfection, caspase-14-transfected cells gave an MTT value only 43% ($p<0.0001$) of the empty vector-transfected control cell value. At 72 h post transfection, MTT values in the caspase-14-transfected cells were 38% ($p<0.0001$) those of that in the control cells (Figure 2B). Similarly, BrdU incorporation in caspase-14-transfected cells at 24 h post transfection was approximately 50% of that in empty vector-transfected cells (Figure 2C). At 72 h post transfection, the BrdU incorporation rate in caspase-14-transfected cells was still only 58% of that in control cells ($p<0.05$) (Figure 2C). Thus, during the 72 h period following caspase-14 transfection, cell growth and proliferation were inhibited significantly, and this effect was not simply due to transfection *per se*.

Cell death in transfected HSG cells. At 24 h post transfection, overall cell density appeared to be less in the caspase-14-transfected cells (Figure 1D) in comparison to the control cells (Figure 1C), consistent with cell growth measurements described above. TUNEL assay results from cells collected at 24 h, 7 days and 14 days post transfection did not show extensive apoptotic activity in the cells (data not shown). Cells in the control appeared healthy, and a significant proportion (6.5%) were mitotic (Figure 1C, arrows). Apoptotic cells were rare (not shown). In contrast, the caspase-14-transfected cells appeared stressed, and few mitotic cells were visible (Figure 1D), consistent with the reduced BrdU incorporation and lower cell numbers described above.

Tumorigenesis of HSG cells transfected with either pCMV-caspase-14 or the empty vector. Caspase-14-transfected HSG cells induced significantly smaller tumors in athymic mice in comparison with cell containing empty vector-transfected cells (Figure 2D). Small tumors appeared in each animal 3 days after xenografting. A difference in tumor size was evidenced after day 4 post xenografting. At day 12 post xenografting, caspase-14-expressing HSG cells formed tumors averaging at 6.4 mm^3, while the control cells formed tumors averaging at 39.8 mm^3 per tumor (Figure 2D).

Tumor cell and tissue pathology analysis. The pathological analysis was performed by a certified pathologist (Dr. Kalu Ogbureke) to determine any morphological, architectural, or cytological differences between caspase-14-positive tumor cell transplants and caspase-14-negative tumor cell transplants. H&E histological tumor sections (Figure 3A, B) revealed that although all transplants exhibited features of poorly differentiated adenocarcinoma, the caspase-14-expressing HSG cell xenografts showed significantly more zones of extensive tumor necrosis (arrows) (Figure 3C) than did the control HSG cell xenografts (Figure 3A). Taken together with the results from *in vitro* culture, these results indicate that the presence of caspase-14 in the tumor cells likely facilitated tumor death by necrosis. TUNEL staining

showed only a small number of cells in the tumor undergoing apoptosis in both the caspase 14-transfected and control vector-transfected cells (Figure 3C, D, arrows), consistent with TUNEL results from the *in vitro* experiments described above. Similar to Western blot analysis of cultured cells, at week 3 post xenograft, the expression of caspase-14 was substantially diminished in the tumor samples (Figure 3F, arrow).

Angiogenesis of tumors formed from HSG cells transfected with either pCMV-caspase-14 or the empty vector. Figure 3G and H show the results from lectin immunofluorescence staining of blood vessels in tumor sections from vector-transfected HSG cells and caspase-14-expressing HSG cells respectively. Blood vessel formation in the tumors formed by caspase-14-expressing HSG cells was significantly less (52.8% , $p<0.05$) than that in the tumors formed by the vector-transfected HSG cells (Figure 4).

Discussion

Caspase-14 is important in epidermal differentiation, possibly activating planned cell death and cornification of the epidermis to form the skin barrier. In the epidermis, induction of caspase 14 at the transcriptional level was noted during stratum corneum formation (18). Upon inhibition of cell differentiation, caspase-14 expression is diminished (19). Consistent with this function, in pathological conditions such as psoriasis (in which cornification is interrupted), the normal expression pattern of caspase-14 is lacking (7). We previously reported that proteolytic cleavage of caspase-14 from a 30 kDa procaspase-14 to an 18 kDa caspase-14 is associated with an improved epidermis in a mouse model for human psoriatic lesions (11). Although caspase-14 mutation is not a likely cause for transformation of normal cells, many types of cancer, such as epidermoid, colon, gastric, breast, lung and liver, are associated with altered expression of caspase-14 (13, 20). A caspase-14 knockout mouse model exhibits a susceptibility be increased epidermal cancer (10). Clinically, most epithelial carcinomas are associated with reduced expression of caspase-14, and lower expression of caspase-14 is associated with shorter survival and poor prognosis (21). These observations indicate that caspase-14 expression is critical for normal epithelial function and that caspase-14 is down-regulated in proliferative cells, including tumor cells. Consistent with this, we previously showed that stable transfection of a skin cancer cell line with caspase-14 cDNA caused reduced tumorigenicity (13). Here, we show a similar effect following transient transfection of exogenous human procaspase-14 into a human salivary gland cancer cell line.

In the current study, only the procaspase-14 form (28 kDa) of the protein was detected by Western blot analysis of the transfected HSG cells (Figure 1E). Despite this apparent lack of processing, HSG cells exhibited reduced growth as measured by cell number, MTT assay, and BrdU incorporation (Figure 2A-C). We did not observe an increase in apoptosis in caspase-14-transfected cells that could account for the reduced cell numbers. A small number of cells (3.6%) did show morphological changes, consistent with an unspecified necrosis-like cell death (Figure 1D). Therefore, the reduced tumorigenicity of HSG cells transiently transfected with caspase-14 (Figure 2D) was most likely due in part to inhibition of cell growth and division, although a contribution by cell death remains possible.

Interestingly, although the transfection efficiency was 46% under the conditions used (*i.e.* 54% of cells were not transfected), and caspase-14 expression declined significantly several days after transfection, by day 12 post xenograft, the tumor volume of caspase-14-positive cells was only 16% that of control cells (Figure 2D). This suggested additional inhibition of caspase-14-positive HSG cell growth *in vivo*. Consistent with this, we observed that blood vessel formation in the tumors formed by caspase-14-positive HSG cells was less than half that of the control cells (Figure 4). This would decrease tumor growth and lead to necrosis, as was observed (Figure 1D and 3B). This observation raises the interesting possibility that caspase 14 expression in the skin inhibits surface vascularization.

To our best knowledge, this study represents the first report describing the use of a differentiation-associated gene, caspase-14, to inhibit human salivary gland cancer cell growth both *in vitro* and *in vivo*. Rapidly growing tumor cells are under a strong signal for proliferation. We hypothesize that caspase-14 expression acts as a strong differentiation signal that can override proliferative signals and slow cell growth. A conflict between these signals might also direct some cells to undergo a specific cell death other than apoptosis. This would be consistent with the poor growth and eventual elimination of stably-transfected tumor cells *in vivo* [(3), and unpublished data].

In conclusion, HSG cells underwent considerable growth inhibition and some cell death by a non-apoptotic mechanism when exogenous caspase-14 was transiently expressed in these poorly-differentiated epithelial tumor cells. Transient caspase-14 expression in HSG cells also reduced tumorigenicity *in vivo*. Importantly, we have shown that it is not necessary to transfect a large majority of cells to achieve marked inhibition. These results suggest that it may be practical to develop a gene therapy approach to treat epithelial cancer, for example, by using an adenovirus delivery system to efficiently express caspase-14 in tumor cells. This work is currently underway in our laboratories. We believe that this approach, reported for the first time, could provide an effective strategy to treat epithelial cancer and precancerous lesions in the skin, oral cavity and salivary glands.

Acknowledgements

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