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Expression of Caspase-14 Reduces Tumorigenicity of Skin Cancer Cells

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Abstract. *Background: The green tea polyphenol (–)-epigallocatechin-3-gallate (EGCG) possesses anti-carcinogenic properties and was found to induce terminal differentiation in epidermal keratinocytes. Caspase-14, a member of the caspase family associated with epithelial cell differentiation, planned cell death, and barrier formation, is induced by EGCG in normal human epidermal keratinocytes but not in cancer cells. Materials and Methods: A human epidermoid cancer cell line, A431, was co-transfected with a caspase-14-expressing pCMV vector and a GFP/neo-expressing pCMV vector. Cell growth and tumorigenicity of the stable transfectant were determined in comparison to cells transfected with the control GFP/neo-expressing pCMV vector. Results: Expression of exogenous caspase-14 led to growth inhibition and reduced the tumorigenicity of A431 cells. Conclusion: Pending future studies, caspase-14 could be used as a novel approach to skin cancer therapy via gene delivery systems.*

Cancer of the epidermis is the most common cancer type in the United States according to the Centers for Disease Control and Prevention (1). Excluding melanoma, the incidence of basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) of the skin is estimated to exceed one million cases per year (2). At certain stages in tumor development, the balance between proliferation, differentiation, and apoptosis is interrupted, resulting in dysregulated cell proliferation (3, 4).

Caspase-14, a member of the caspase family, is only expressed in epithelial tissues, especially in the differentiating epidermis (5-7) but is also expressed in other tissues with a barrier function (8). The human caspase-14 gene is located on chromosome 19p13.1 and consists of seven exons (9). The native caspase-14 protein, with a molecular weight of 28 kDa, is not catalytically active. Activation requires its proteolysis onto 17 kDa and 11 kDa subunits. These cleaved subunits are found in the stratum corneum of normal human epidermis and are able to specifically cleave tetra-peptide substrates (10). The activity of caspase-14 is associated with epidermal cornification and nuclear destruction in epidermal keratinocytes, and is not involved in the pro-apoptotic caspase cascade (11-13). Indeed, induction of caspase-14 at the transcriptional level was observed during stratum corneum formation (9). Caspase-14 in its various forms is found in the cytoplasm and nucleus of the granular layer, in the nuclear remnants of the transitional layer of the epidermis, and in the stratum corneum (14). Inhibition of differentiation leads to diminished expression of caspase-14 (15). Therefore, caspase-14 is believed to facilitate epidermal differentiation, possibly by activating planned cell death and cornification of the epidermis to form the skin barrier (14). These properties make caspase-14 a potential tool for gene therapy to trigger the differentiation of skin cancer cells.

Green tea polyphenols (GTPs), potent antioxidants with anticancer activities, are found in the leaves of the tea plant (*Camellia sinensis*). The major polyphenols include (–)-epicatechin (EC), (–)- epigallocatechin (EGC), (–)-epicatechin-3-gallate (ECG), and (–)- epigallocatechin-3-gallate (EGCG) (16-19). GTPs are able to selectively induce growth arrest and apoptosis in many types of tumor cells while protecting certain normal epithelial cells from oxidative stress and ultraviolet radiation (20-23). We found that GTPs and EGCG are able to activate a differentiation pathway in normal human epidermal keratinocytes (NHEK), in association with the induction of p57/KIP2, a cyclin-dependent kinase inhibitor involved in growth arrest and differentiation, and without activation of caspase-3, whereas tumor cells undergo caspase-3-mediated apoptosis (24-28).

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We previously reported that EGCG coordinately activates the expression of p57 and caspase-14 in NHEK, both of which facilitate terminal differentiation in these cells, and the expression of p57/KIP2 activates caspase-14 gene expression (29). Recently, we found that expression of p57/KIP2 in OSC2 cells results in a remarkable decrease in tumorigenicity *in vivo* (30). However, whether caspase-14 independently acts as a differentiation inducer to reduce the tumorigenicity of cancer cells has not yet been investigated. In these studies, we investigated the effect of caspase-14 expression on the *in vitro* and *in vivo* growth of epidermoid cancer cells.

Materials and Methods

Reagents. Anti-human actin (I-19) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-caspase-14 antibody was raised against amino acids 24-122, mapping near the N-terminus of human caspase-14 protein (Santa Cruz Biotechnology, H-99). The caspase-14-expression vector containing full-length cDNA of human caspase-14 in pCMV plasmid (pCMV/C14), the pCMV empty vector and caspase-14 siRNA constructs were custom-designed by OriGene (Rockville, MD, USA). The pCMV/C14 vector was sequenced to confirm the cDNA sequence. The pCMV/GFP/neo was provided by Dr. Hubert Stöppler.

Cell line. A431, a human epidermoid cancer cell line, was purchased from ATCC and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma, St. Louis, MO, USA) according to established protocols.

Stable expression of caspase-14 in A431 cells and siRNA knockdown of caspase-14. A431 cells were co-transfected with pCMV/C14 and pCMV/GFP/neo using Lipofectamine 2000 according to the manufacturer's instructions, followed by subclone selection using G418 at 500 ng/ml. One subclone, A431/GFP/C14, was able to grow and populate. An A431 subclone expressing GFP/neo, A431/GFP, was also selected. These subclones, along with the parental A431 cells, were analyzed for caspase-14 expression by Western blotting with the anti-caspase-14 antibody H-99 and siRNA knockdown method.

Cell growth assay. Cell growth assays were performed on exponentially growing A431-derived cells either expressing GFP (A431/GFP) or expressing both GFP and caspase-14 (A431/GFP/C14) in complete DMEM medium. Cells at 10^5 were initially plated in each well of a 6-well cell culture plate in duplicate at time 0. Cell quantification was achieved by counting using a hemacytometer and trypan blue exclusion at 24 and 48 h.

Western blot. Cells were washed in ice-cold PBS and lysed for 10 min in RIPA buffer containing 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10 μg/ml leupeptin, 3 μg/ml aprotinin and 100 mM phenylmethylsulfonyl fluoride (PMSF). Samples of lysates containing 40 μg protein were loaded in each lane and electrophoretically separated on a 12% SDS polyacrylamide gel. Following electrophoresis, proteins were transferred to a nitrocellulose (NC) membrane (Santa Cruz Biotechnology). The membrane was blocked for 1 h with 5% (w/v) non-fat dry milk powder in TBST (0.1% Tween-20 in TBS) and then incubated for 1 h with anti-human rabbit polyclonal antibodies against caspase-14 or actin (Santa Cruz Biotechnology). The membrane was washed three times with TBST and incubated with peroxidase-conjugated, affinity-purified anti-rabbit IgG (Santa Cruz Biotechnology) for 1 h. Following extensive washing, the immunoreactive proteins were visualized by enhanced chemiluminescent staining using ECL Western blotting detection reagents (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA).

Animals and xenograft experiments. Female athymic (nu/nu) mice at the age of 4-6 weeks were purchased from the National Cancer Institute (Bethesda, MD, USA). Ten nude female mice were assigned randomly to two groups with five animals per group. Xenografts from A431-derived cells (A431/GFP and A431/GFP/C14) were injected into the abdominal area subcutaneously at a concentration of 1.5 million cells in 100 μl PBS. In one group, A431/GFP/C14 was introduced into the left abdominal area while A41/GFP was introduced into the right abdominal area. In the second group, the cells were injected into the opposite sides. The animals were monitored daily and the first signs of tumor growth recorded. 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). The difference in tumor size between the caspase-14-transfected cells and the GFP-transfected cells was analyzed with a two-tailed Student's *t*-test.

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

Results

Analysis of A431-derived subclones. To determine caspase-14 protein levels, A431 and A431-derived subclones were examined by Western blotting. A431/GFP/C14 cells expressed high levels of caspase-14, while the A431/GFP subclone and the parental A431 cells failed to express detectable caspase-14 (Figure 1A, three right hand lanes). To determine whether caspase-14 protein was down-regulated by the caspase-14 siRNA constructs designed by OriGene, five different caspase-14 siRNA expressing vectors were used, along with an empty vector (pCMV), to transfect A431/GFP/C14 cells. siRNA constructs #1 and #2 showed significant down-regulation of protein levels (Figure 1A, six left lanes).

The morphology of A431/GFP/C14 cells was significantly different when compared with that of A431/GFP cells (Figure 1B). A431/GFP/C14 cells demonstrated a large cell size and a flattened shape, characteristics of more highly differentiated cells.

Expression of caspase-14 inhibits A431 cell growth. A431/GFP/C14 cells exhibited significantly delayed cell growth compared to A431/GFP cells (Figure 2). After 24 h, the cell number of A431/GFP/C14 increased from 1x105

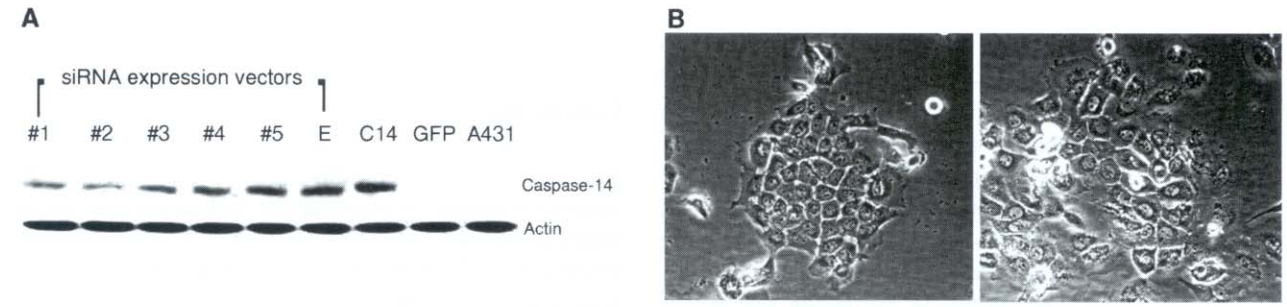

Figure 1. *A) Western blot of cell lysates from A431, A431/GFP (GFP), A431/GFP/C14 (C14), and A431/C14 cells transfected with siRNA constructs (#1-#5), and an empty vector (E). Vectors #1 and #2 showed a significant down-regulation. B) Morphological differences were observed between cultured A431/GFP (left) and A431/GFP/C14 (right) cells.*

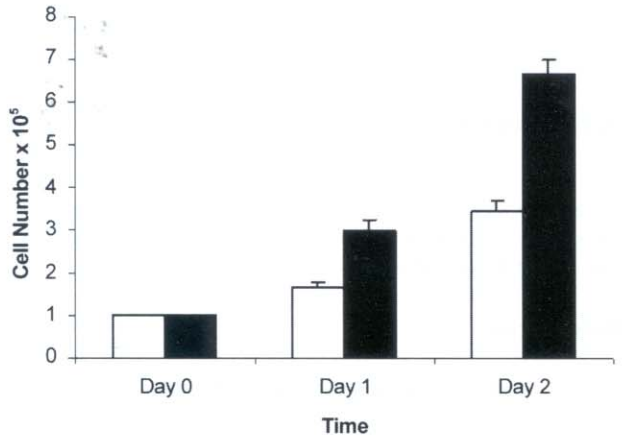

Figure 2. *The increase in cell numbers over time of A431/GFP/C14 (open bars) and A431/GFP cells (solid bars). Cells were seeded at 10^5 per well in duplicate at day 0. At 24 and 48 h, two wells of cells from each cell type were counted and are presented as the average cell number $x10^5$. A431/GFP/C14 cells exhibited significantly reduced growth at these time points compared to A431/GFP cells.*

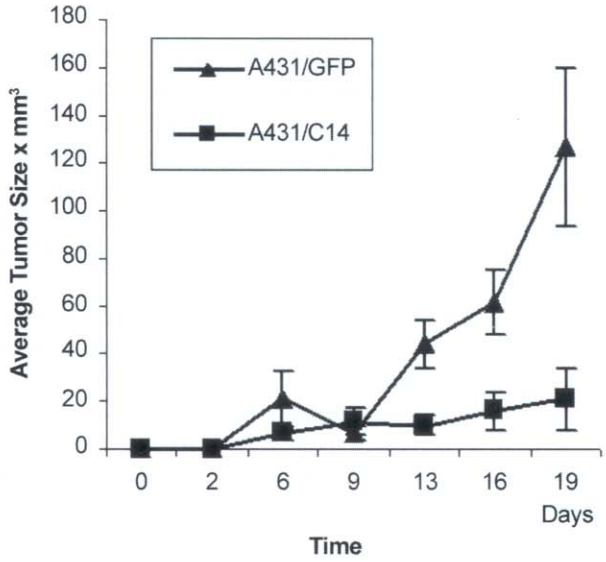

Figure 3. *Xenografts of transfected A431 cells in athymic mice form tumors. A431-derived cells expressing either exogenous caspase-14 (A431/GFP/C14) or GFP only (A431/GFP) were injected into athymic mice. Each developed tumor was measured every 3-4 days over a 3-week period. The mean size of the tumors formed by A431/GFP/C14 cells was significantly smaller than those formed by A431/GFP cells (two tailed Student's t test, $p<0.02$, $n=10$).*

cells to $1.655x10^5$ cells, while the cell number of A431/GFP attained $3x10^5$ cells during the same time period. At 48 h, the number of A431/GFP/C14 cells increased to $3.45x10^5$, while A431/GFP cells reached $6.655x10^5$. Thus, the cell number of A431/GFP/C14 cells is less than 60% of that of A431/GFP cells (55% and 51% at 24 h and 48 h, respectively).

Expression of exogenous caspase-14 reduced the tumorigenicity of A431 cells in athymic mice. Upon subcutaneous introduction of A431 cells into athymic mice, tumors appeared in most mice approximately two days after the xenografts were injected. There was no significant difference between the sizes of the tumors formed from A431/GFP/C14 or A431/GFP xenografts until day 13 after their introduction. At this time, the average size of A431/GFP tumors was 43.8 mm^3, while the average size of A431/GFP/C14 tumors was 9.9 mm^3 (two tailed *t*-test, $p<0.01$, n=10, Figure 3). By day 16 after the xenografts were introduced, the average size of the A431/GFP tumors was 61.2 mm^3, while the average size of the A431/GFP/C14 tumors was 16.1 mm^3 (two tailed *t*-test, $p<0.02$, n=10, Figure 3). On day 19, the average size of the A431/GFP tumors reached 123.2 mm^3, while the average size of the A431/GFP/C14 tumors was only 20.9 mm^3 (two tailed *t*-test, $p<0.01$, n=10, Figure 3).

Discussion

Altered expression of caspase-14 was found in many types of human cancer (31) and in skin disorders such as psoriasis (29), which suggests that normal expression and activity of caspase-14 is necessary to maintain homeostasis of the epidermis. The hypothesis tested in the current study is that restoration of caspase-14 expression reduces the tumorigenicity of cancer cells derived from the epidermis. We recently found that caspase-14 expression was absent from A431 cells and from a salivary gland cancer cell line HSG, and was expressed at very low levels in an oral squamous carcinoma cell line OSC2 (data not shown). Expression of exogenous caspase-14 in HSG and OSC2 cells resulted in an unidentified type of cell death that made subclone selection impossible (data not shown). This death might be due to the high levels of caspase-14 expressed in these cells under the control of the CMV promoter. In the subclones of A431 cells transfected with pCMV containing caspase-14 cDNA, only one subclone, A431/C14 was able to proliferate, possibly due to relatively low levels of caspase-14 protein expressed in this particular subclone. Morphological changes were observed in this subclone when compared with the control subclone A431/GFP (Figure 1B) and the parental A431 cells. These changes, enlarged cell size and flattened cell shape, indicated that the caspase-14-expressing A431 cells underwent intrinsic modification toward a more differentiated phenotype, possibly catalyzed by the activity of caspase-14. As expected, this subclone exhibited a significantly reduced growth rate (51% to 55% of control) compared to the control subclone A431/GFP (Figure 2). In fact, repopulation of this subclone was very slow initially, but the rate increased gradually with each passage. This increase may be due to a gradual decrease in caspase-14 expression, possibly caused by an intrinsic mechanism that suppresses caspase-14 (as measured with Western blotting, data not shown). Unlike p57/KIP2, for which expression significantly prevented tumor formation in a highly metastatic oral carcinoma cell line OSC2 (32), caspase-14 expression failed to prevent tumor formation *in vivo*. However, exogenous caspase-14 significantly reduced the tumorigenicity of A431 cells in athymic mice (Figure 3). At day 19 after xenografts were introduced, the average tumor size of control (A431/GFP) was almost 6-fold greater than that of the caspase-14 expressing cells (A431/C14, Figure 3). Since caspase-14 expression and activation are rather late events in epidermal differentiation compared to p57/KIP2 expression (11-14, 29), the downstream effects resulting from caspase-14 expression in A431 cells may not be sufficient to prevent tumor formation, but instead cause reduced cell growth/tumorigenicity. However, the mechanism involved in this inhibitory effect is not clear. Several possibilities resulting from the expression of caspase-14 in A431 cells exist: i) growth arrest is induced, ii) nuclear destruction, iii) cell death arises, iv) angiogenesis is inhibited, and v) a combination of any of the above.

Conclusion

The current study demonstrated, for the first time, that exogenous caspase-14 expression in skin cancer cells induced significant growth inhibition and reduced tumorigenicity in athymic mice. Thus, in addition to its functions in epidermal keratinocyte differentiation and barrier formation, reduced caspase-14 expression and activity may also be useful as a marker for epithelial cancer. More importantly, since the expression and activation of caspase-14 are normal events in the epidermis, and skin cancer cells lose the ability to express caspase-14, the gene for caspase-14 could potentially be used with topical gene delivery methods as a novel approach to modulate skin cancer growth by inducing differentiation, although further studies are necessary.

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