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The repressive effect of green tea ingredients on amyloid precursor protein (APP) expression in oral carcinoma cells in vitro and in vivo

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Abstract

In a hamster model of *N*-methyl-*N*-benzylnitrosamine (MBN)-induced oral carcinogenesis, the incidence of buccal pouch (HBP) carcinomas in MBN-treated hamsters (17.8 ± 7.5) was significantly higher than MBN-treated hamsters given tea (10.8 ± 3.9) ($P < 0.05$). Amyloid precursor protein (APP) expression was also significantly increased in MBN-induced HBP carcinomas but was significantly reduced by tea intake ($P < 0.0001$). Furthermore, APP expression and secretion by OECM-1 oral squamous cell carcinoma cells was inhibited by a major polyphenolic ingredient of green tea, (−)-epigallocatechin gallate, in a dose-dependent manner. Thus, APP might promote oral carcinogenesis, whereas green tea ingredients might diminish it by down-regulating APP.

Keywords: APP; Cancer; Carcinogenesis; Mouth; Tea

1. Introduction

Tea is a popular drink in many parts of the world. Tea contains flavanols, glycosides, leucoanthocyanins, and phenolic acid; the flavanols (also known as catechins) (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG) and others are major components of tea [1,2]. Tea and EGCG have been reported to have chemopreventive effects against various cancers [1,3–9]. In vitro studies on oral squamous cell carcinoma (OSCC) have shown that tea may be chemotherapeutic by reducing cell growth [10–12], increasing apoptosis [13,14] and inhibiting angiogenesis [7,12]. In vivo studies have also demonstrated that tea inhibits 7,12-dimethylbenz[a]anthracene (DMBA)-induced HBP carcinogenesis, possibly by inhibiting proliferation and angiogenesis as well as by inducing apoptosis [15,16].

Abbreviations: APP, amyloid precursor protein; EC, (−)-epicatechin; ECG, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin gallate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HBP, hamster buccal paunch; MBN, *N*-methyl-*N*-benzylnitrosamine; OSCC, oral squamous cell carcinoma; PG, propylene glycol; sAPP, secreted form of APP; DMBA, 7,12-dimethylbenz[a]anthracene; EGFR, epidermal growth factor receptor.

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Amyloid precursor protein (APP) overexpression is associated with the neuropathological abnormalities of Alzheimer's disease. Abnormal cleavage of APP is related to the pathogenesis of Alzheimer's disease; however, researchers have recently elucidated additional roles for APP in tissues other than those of neural origin. Pietrzik et al. [17] showed that the secreted form of APP (sAPP) serves as a local mediator of growth in thyroid cells. Hoffmann et al. [18] demonstrated the growth-promoting effects of sAPP on skin keratinocytes. It has also been suggested that APP plays an important role in cellular proliferation and differentiation [19]. Hansel et al. [20] further showed that APP expression is upregulated in pancreatic cancer cells both in vitro and in vivo. APP undergoes high levels of proteolytic processing in cultured pancreatic cancer cells, and sAPP can be detected in the culture medium. Conversely, inhibition of sAPP-mediated signaling reduces pancreatic cancer cell proliferation [20]. In addition, APP expression has been linked to the malignant progression of cancer cells. In human colon carcinoma SW837 cells, APP functions in cellular proliferation and differentiation both in vitro and in vivo [19].

We recently discovered increased APP mRNA expression in OSCC relative to corresponding noncancerous matched oral mucosa and found that APP expression can be used as a biomarker to monitor OSCC prognosis. An antisense oligonucleotide against APP can reduce cellular APP and the growth of the OSCC cell line, OECM-1 [21]. Furthermore, Rezai-Zadeh et al. [22] found that EGCG can reduce cellular APP levels in neuronal cells. In the present study, we further used OECM-1 cells and an HBP carcinogenesis model, in which tumors are induced by *N*-methyl-*N*-benzylnitrosamine (MBN), to address whether green tea or EGCG can regulate APP [3,23].

2. Materials and methods

2.1. Animal treatment and tissue samples

The administration of reagents to animals and sample preparation were modified from published protocols [3]. In brief, male Syrian golden hamsters about 6 weeks old were divided into four experimental groups. Both of the buccal pouches of MBN-treated hamsters were painted with a 1% (wt/vol) of MBN (Ash Stevens, Inc., Detroit, MI, USA) dissolved in propylene glycol (PG) every Monday and Friday for 17 weeks. Tea-supplied hamsters received 0.2% (wt/vol) green tea (National Food Industry Research and Development Institute, Taipei, Taiwan) in drinking water that was changed daily. MBN-treated hamsters that also received tea were first treated with MBN as above for 8 weeks to initiate tumorigenesis; they then continued to receive biweekly MBN painting of their buccal pouches and were allowed access to 0.2% green tea in the drinking water for the remaining 9 weeks period. The control group consisted of two hamsters whose buccal pouches were painted with PG lacking MBN for the entire duration. The tea-supplied group was treated likewise, and in addition they received 0.2% tea supplied in the drinking water from week 9 to week 17. Three days after the final treatment, the hamsters were sacrificed and the HBPs were opened by resection. The exophytic tumors were enumerated, and their maximum dimensions were recorded at a 0.5-mm scale. All excised lesions and the remaining pouch tissues were fixed in 10% neutralized formalin at 4 °C for histopathological evaluation. The animal experiments were approved by an animal study evaluation committee.

2.2. Immunohistochemistry

Immunohistochemistry was performed on resected masses and HBP tissues. Following normal processing, sections were incubated with a 1:200 dilution of mouse monoclonal anti-APP (cat no.: 22C11, Chemicon, Temecula, CA, USA) at 25 °C for 2 h in a humid chamber. After rinsing with PBS, standard immunohistochemistry staining was performed according to the manufacturer's protocol using a LSAB2® streptavidin–biotin complex system (Dako, Carpinteria, CA, USA) with amino ethyl carbazol (AEC; Zymed, South San Francisco, CA, USA) as the chromogen. The slides were counterstained with hematoxylin (Zymed) and mounted with Clearmount® (Zymed). For the negative control, equivalent tissue was incubated in PBS lacking the primary antibody. The extent of immunoreactivity was scored based on the percentage of positive cells found in each tissue sample: $<$ 10%, absent (−); 10–25%, weak (+); 25–50%, moderate (++); and $>$50%, strong (+++).

2.3. Cell culture

The OSCC cell line, OECM-1, was grown in RPMI-1640 media (Life Technologies, Gaithersburg, MD, USA) routinely supplemented with 10% FCS, except in cases of specific experimental demands [21]. EGCG was purchased from Sigma (St Louis, MO, USA).

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted using a Tri-reagent® RNA isolation kit (Molecular Research Center, Cincinnati, OH, USA). The RNA was treated with DNase I (Stratagene, La Jolla, CA, USA) to remove contaminating DNA. Five milligram of total RNA was reverse transcribed to cDNA

using an oligo-$dT_{(18)}$ primer and Stratascript reverse transcriptase (Stratagene).

2.5. RT-PCR

cDNA was amplified by PCR in a 25 μl reaction containing 1 U of Prozyme® DNA polymerase (Protech, Taipei, Taiwan), 0.4 mM dNTP, and 0.4 mM primers. The APP primer pair used was: forward, GCAGTGAGAAGAGTACCAAC, and reverse, ACCTCATCACCATCCTCATC [21,24]. Primers used for amplification of the control, GAPDH, were: forward, TGGTATCGTGGAAGGACTCATGAC, and reverse, ATGCCAGTGAGCTTCCCGTTCAGC [25]. The cDNA was denatured for 5 min at 95 °C, followed by 26–30 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for APP or 55 °C for GAPDH for 1 min, and extension at 72 °C for 1 min. The number of cycles for PCR was optimized by pilot studies to assure that amplification was within the logarithmic phase. PCR products were resolved on a 2% agarose gel and visualized by an imaging system (Viber Lourmat, Marne La Valle, France). The intensities of the signals were measured by a densitometer (Amersham, Piscataway, NJ, USA). Relative APP mRNA expression was determined by normalizing to GAPDH expression.

2.6. Western blotting

Cell lysates and conditioned media were harvested. Conditioned media were concentrated 100-fold with vivaspin tubes (Sartorius, Goettingen, Germany). Proteins (30 μg) were resolved on a 10% SDS-PAGE gel and then transferred onto nitrocellulose membranes (PALL Corp., Ann Arbor, MI, USA). Membranes were blocked with non-fat milk and then incubated with primary antibodies overnight at 4 °C. A mouse monoclonal antibody against APP (cat no.: 22C11, Chemicon) at 1:2000 dilution and a mouse monoclonal antibody against actin (Chemicon) at 1:10,000 dilution were used. The secondary antibody was horseradish peroxidase-conjugated anti-mouse antibody (Amersham). The signals were detected by the western lightening chemiluminescence reagent plus kit (Perkin–Elmer, Wellesley, MA, USA). The intensities of the signals were measured by a densitometer (Amersham). Quantification of the APP signal was achieved by normalization with that of actin or the number of cells cultured.

2.7. Statistical analysis

The Fisher's exact test and unpaired *t*-test were performed. Differences between the values were considered significant when $P<0.05$.

3. Results

3.1. The incidence of HBP carcinoma decreased with green tea intake

Mean water intake for the control and the tea-supplied groups was 17.5 and 16.7 ml/day, respectively. These values were not statistically different. For MBN-treated hamsters and the MBN-treated/tea-supplied hamsters, the mean water intake was 6.5 and 7.6 ml/day, respectively. These values also were not statistically different. Therefore, the tea intake was approximately 0.03 g/day per hamster for the control and tea-supplied groups; and approximately 0.015 g/day per hamster for the MBN-treated groups. No exophytic masses were noted on the smooth buccal pouch surfaces of control or tea-supplied hamsters. The MBN-treated and MBN-treated/tea-supplied hamsters, however, exhibited exophytic tumors on the irregular buccal pouch surfaces. Histopathologically, most of the exophytic masses were well- or moderately differentiated squamous cell carcinomas. Approximately 4% were distinctive pyogenic granulomas and 8% were squamous cell papillomas and thus were excluded from the analysis. Results of the HBP carcinoma induction are summarized in Table 1. The incidence of HBP carcinomas was significantly higher in MBN-treated hamsters than in MBN-treated/tea-supplied animals (17.8 ± 7.5 vs 10.8 ± 3.9; $P<0.05$).

3.2. APP immunoreactivity in buccal pouches and HBP carcinomas

The pouches of control group animals showed scattered APP immunoreactivity in the lower two-thirds section of the pouch epithelium (Fig. 1A). Interestingly, APP immunoreactivity was nearly absent or focally distributed in the pouch epithelium of tea-supplied hamsters (Fig. 1B). APP immunoreactivity was also observed in submucosal mesenchymal cells and skeletal muscle of the pouches from controls

Table 1
Incidence of HBP carcinoma in various groups

Hamster group	No. of hamsters	No. of HBP carcinomas	HBP carcinomas/hamster (mean ± SD)
MBN-treated	6	107	17.8 ± 7.5
MBN-treated/tea-supplied	9	98	10.8 ± 3.9

$P<0.05$, unpaired *t*-test.

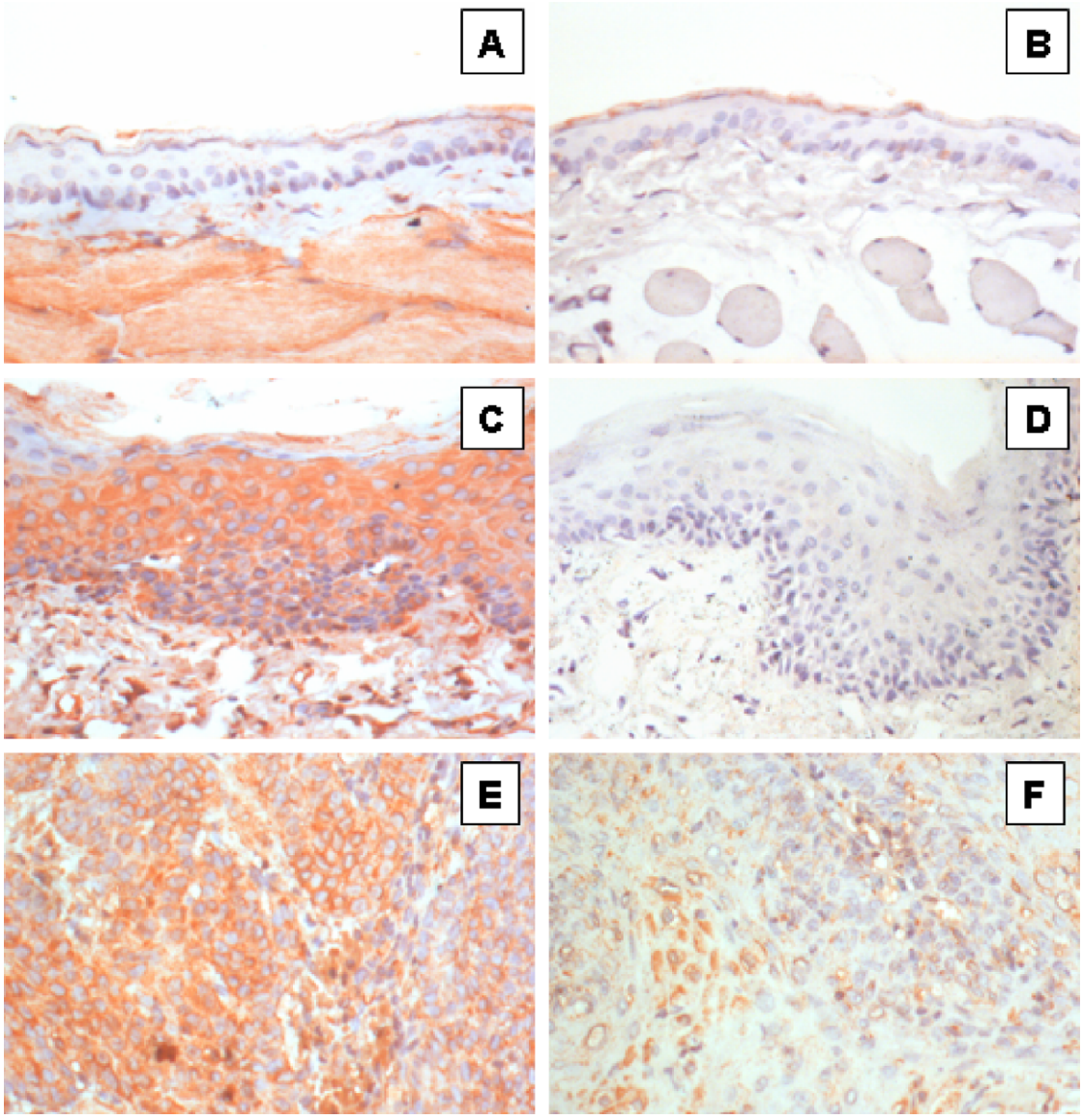

Fig. 1. APP immunoreactivity in hamster buccal pouches and HBP carcinomas. (A) A control pouch. Scattered cytosolic APP immunoreactivity can be seen in stratum spinosum cells and basal cells. Note the intense APP immunoreactivity in muscle cells and submucosal mesenchymal cells. (B) A pouch from a tea-supplied hamster. APP immunoreactivity is nearly absent in the whole epithelium layer, stromal cells and muscle cells. Only some faint immunoreactivity is seen in the keratinized surface layer. (C) Pouch epithelium from an MBN-treated hamster. Strong APP immunoreactivity can be seen in the full thickness of both the hyperplastic and hyperkeratotic pouch epithelium as well as in submucosal mesenchymal cells. (D) A pouch from an MBN-treated/tea-supplied hamster. APP immunoreactivity is not present in either the hyperplastic or hyperkeratotic epithelium, and neither is APP immunoreactivity found in any submucosal mesenchymal cells. (E) Well-differentiated MBN-treated and (F) MBN-treated/tea-supplied HBP carcinomas exhibit strong and weak APP immunoreactivity, respectively. The stromal cells and extracellular matrix in (C) and (F) exhibit strong APP immunoreactivity (200×). The oral epithelium in (A) was scored as APP (+) according to the criteria defined in Section 2; (B) and (D) were scored as APP (−); (C) and (E) were scored as APP (+++); (F) was scored as APP (+).

(Fig. 1A). However, no APP immunoreactivity was observed in mesenchymal cells or muscle cells in pouches of tea-supplied animals (Fig. 1B). Histopathologically, pouches from MBN-treated and MBN-treated/tea-supplied hamsters exhibited epithelial hyperplasia and hyperkeratosis. APP immunoreactivity was noted in the full thickness of MBN-treated pouch epithelium (Fig. 1C) but was absent in MBN-treated/tea-supplied pouch epithelium (Fig. 1D). Similarly, strong APP immunoreactivity was observed in

Table 2
APP immunoreactivity in HBP carcinomas with invasive growth

	MBN-treated hamsters		MBN-treated/tea-supplied hamsters	
	<2 mm	≥2 mm	<2 mm	≥2 mm
−, Absent, <10%	0	3	26	6
+, Weak, 10–25%	3	0	3	4
++, Moderate, 25–50%	6	1	3	6
+++, Strong, >50%	39	14	2	2

$P<0.0001$, MBN-treated hamsters vs MBN-treated/tea-supplied hamsters; $P<0.05$, <2 mm vs ≥2mm in MBN-treated/tea-supplied hamsters. Fisher exact test.

mesenchymal cells in pouches of MBN-treated hamsters, whereas it was completely absent in like tissue from MBN-treated/tea-supplied animals (Fig. 1C and D). Sixty-six (62%) MBN-treated and 52 (53%) MBN-treated/tea-supplied HBP carcinomas with remarkable invasive growth were examined by immunohistochemistry. Ninety-one percent (60/66) of MBN-treated invasive HBP carcinomas exhibited strong (Fig. 1E) or moderate APP immunoreactivity (Table 2). However, 75% (39/52) of MBN-treated/tea-supplied invasive HBP carcinomas showed weak or no APP immunoreactivity (Fig. 1F). The extent of APP immunoreactivity was statistically different between these two groups ($P<0.0001$, Table 2).

In MBN-treated/tea-supplied hamsters, only 15% (5/34) of HBP carcinomas with a size <2 mm exhibited strong or moderate APP immunoreactivity, whereas 44% (8/18) of HBP carcinomas with a size ≥2 mm exhibited such APP immunoreactivity. This was a statistically significant difference ($P<0.05$, Table 2) that might suggest an association between APP immunoreactivity and the size of HBP carcinomas in this subset.

3.3. APP expression in OECM-1 cells is down-regulated by EGCG

Our preliminary studies demonstrated that EGCG inhibits OECM-1 cell growth in a time- and dose-dependent manner (not shown). OECM-1 cells were treated with 0.5–20 μM EGCG for 18 and 24 h, and APP mRNA expression was measured. RT-PCR analysis demonstrated that APP mRNA expression was down-regulated by EGCG at dosages >2.5 μM for both time points (Fig. 2A and B). Western blotting analysis of APP in cell lysates and sAPP in conditioned media from OECM-1 cells following 5 and 10 μM EGCG treatment for 24 h further confirmed that APP

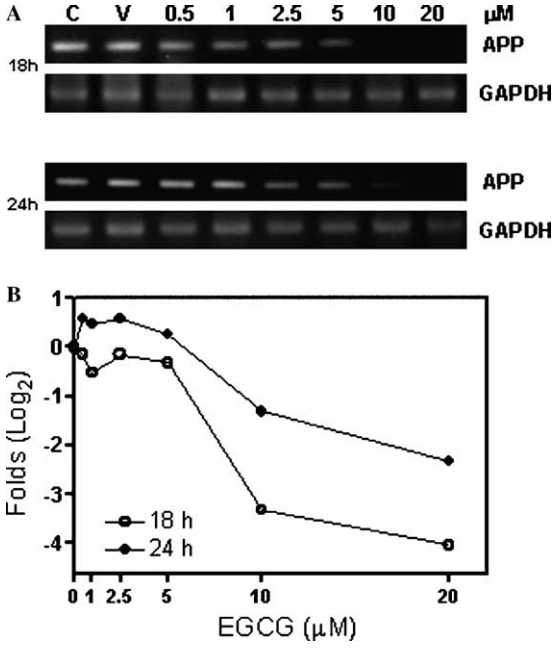

Fig. 2. The effect of EGCG on APP mRNA expression in OECM-1 cells. (A) APP mRNA expression is inhibited by EGCG when the treatment dosage increases. (B) Quantitation shows a dose-dependent decrease of APP mRNA expression at EGCG treatment dosages >2.5 μM at 18 and 24 h. The 18-h treatment seemed to have better inhibitory effects than the 24-h treatment.

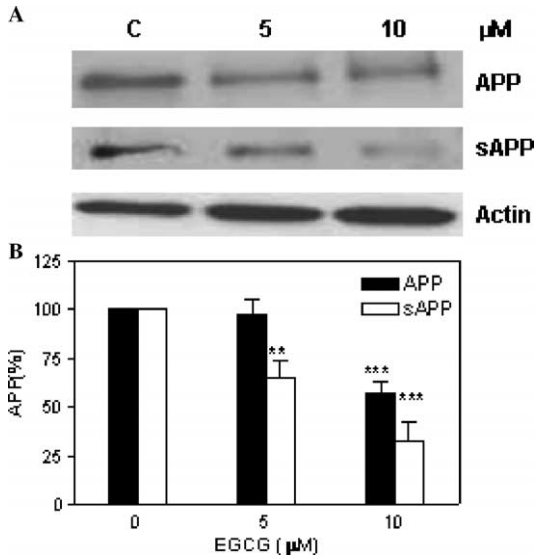

Fig. 3. Regulation of APP and sAPP by EGCG in OECM-1 cells. (A) Western blot of APP in cell lysates and sAPP in conditioned media. A decrease in APP and sAPP protein levels following 5 or 10 μM EGCG treatments is noted. (B) Quantitation shows a statistically significant decrease in APP with 10 μM EGCG treatment, and a decease in sAPP with both 5 and 10 μM EGCG treatment.

and sAPP were significantly down-regulated by EGCG. APP levels decreased by ~43% with 10 μM EGCG treatment, and sAPP levels decreased by ~35 and 67% with 5 and 10 μM EGCG treatment, respectively (Fig. 3A and B).

4. Discussion

Tea has been demonstrated to reduce cell growth, increase apoptosis and inhibit angiogenesis in OSCC [4,7,10–12,14,15,26]. It is effective in preventing animal carcinogenesis in different experimental systems [15,27,28]. In this study, we found that green tea in drinking water significantly reduced the incidence of MBN-induced carcinomas at the post-initiation stage in hamster buccal pouches. With a similar regimen, Li et al. [15] demonstrated the inhibitory effect of tea in drinking water against DMBA-induced carcinogenesis at the post-initiation stage in hamster buccal pouches. Our data further substantiates tea's effects in blocking the progression of chemically induced oral carcinogenesis. EGCG induces higher cytotoxicity in OSCC cells than in normal oral keratinocytes [29]. Together, these findings suggest that tea could be a useful chemopreventive agent against OSCC with less toxicity on normal cells. Shabany et al. [23] developed a system using whole-mount staining of hamster buccal pouches and γGT as a marker for rapid detection of chemopreventive effects against MBN-induced HBP carcinogenesis. Such an assay might be applied to validate tea-mediated regimens for chemoprevention of oral carcinogenesis in the future.

APP has been reported to be a positive regulator of carcinogenesis [17,18,30,31]. Our previous data indicated that APP mRNA and protein are overexpressed in OSCC tissues [21]. In the present study, we found strong APP immunoreactivity in MBN-treated pouches and HBP carcinomas in vivo. We also observed a significant decrease of APP immunoreactivity in tea-supplied hamster buccal pouches overall as well as in HBP carcinomas; these two lines of evidence support the notion that tea can drastically attenuate APP expression in oral keratinocytes. In addition, we have demonstrated that EGCG can decrease the level of cellular APP and secreted APP in OECM-1 cells. In the same cells, we have determined that APP is essential for OSCC growth [21]. Thus, our study demonstrates for the first time, with tissue and molecular evidence, that major tea constituents can reduce APP expression in oral keratinocytes, both in vivo and in vitro. APP may be indirectly involved in growth of oral keratinocytes; however, with regard to tumor suppression, further work is required to elucidate the combined impact of, and interactions between, the down-regulation of APP expression and EGCG-regulated signaling cascades [5].

Interestingly, preinvasive lesions, including hyperkeratosis, epithelial hyperplasia and dysplasia, appeared in pouches from both MBN-treated and MBN-treated/tea-supplied hamsters. The pouches of

the MBN-treated group also had high APP immunoreactivity and a higher incidence of invasive HBP carcinomas. In contrast, APP immunoreactivity was nearly absent and the incidence of HBP carcinomas was rather low in pouches from MBN-treated/tea-supplied animals. These data might suggest that APP expression may be involved in the conversion from preinvasive to invasive carcinomas. These findings also suggest that a fraction of MBN-induced HBP carcinomas may bypass a requirement for APP during pathogenesis.

Herzog et al. [32] have demonstrated that full-length APP appears to facilitate keratinocyte adhesion due to its ability to interact with the extracellular matrix. Other reports have suggested that APP is a cell surface receptor [33–35], a matrix-binding protein [36–39], or a kinesin adapter protein [40,41] that might play an important role in linking epithelial cells with stromal components. APP can be modified to sAPPα by α-secretase and secreted to the extracellular matrix [18,42,43]. sAPPα seems to act as a growth factor related to keratinocyte proliferation and migration [18,41,44–46]. Tripodi et al. [47] identified stromal myofibroblasts as the source of APP in microcystic adenoma and suggested an interaction between epithelial cells and mesenchymal cells through APP. In our study, buccal pouches from MBN-treated hamsters and HBP carcinomas showed tremendous APP immunoreactivity in mesenchymal cells in addition to epithelial cells. These findings suggest that MBN potentially upregulates APP in oral epithelial cells and mucosal mesenchymal cells. Ingestion of tea abolished APP expression in both epithelial and mesenchymal cellular fractions, leading us to speculate that the inhibitory effects of tea ingredients on APP expression in both cell types might contribute to the suppression of tumorigenesis associated with APP in HBP.

We have shown that EGCG reduces both RNA and protein levels of APP. It has been reported that EGCG suppresses cell growth via inhibition of the epidermal growth factor receptor (EGFR) signaling pathway [48,49]. In OECM-1 cells, activation of EGFR can elicit the activity of ERK kinase [50]. Increases in APP expression and sAPP are mediated via the ERK signaling pathway [51]. Moreover, Slack et al. [52] demonstrated that activation of EGFR can stimulate α-secretase activity and the release of sAPP in A431 keratinocytes. In concert with these findings, Ju et al. [5] demonstrated that the repression of colon cancer formation in rodents by EGCG is possibly mediated through the attenuation of carcinogenic events, including ERK and AKT activation. Rezai-Zadeh et al. [22] have shown recently that EGCG modulates the increase in α-secretase activity to further reduce APP accumulation in neuronal cells and the genesis of cerebral amyloid in experimental animals. Therefore, further study is required to determine whether EGCG is involved in post-transcriptional regulation or processing of APP that could underlie the changes in APP expression related to oral tumorigenesis.

Although the epidemiological association between tea consumption and the risk of cancer yet be definitively established [53], our study provides evidences for the chemopreventive and repressive effects of tea ingredients on the neoplastic process. Furthermore, our work gives additional insight into the inhibitory potential of tea ingredients on APP expression.

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