

Protective Effects of EGCG on Salivary Gland Cells Treated with γ -Radiation or Cis-platinum(II)Diammine Dichloride

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Abstract. Dysfunction of salivary glands is often associated with aging and cancer therapy. Green tea polyphenols were previously found to protect normal epithelial cells from reactive oxygen species, and to induce apoptosis in tumor cells. The current study investigated whether (-) epigallocatechin-3-gallate (EGCG), the major green tea polyphenol, protects normal salivary gland cells from the effects of γ -irradiation and the chemotherapy drug cis-platinum(II)diammine dichloride (CDDP). Human immortalized salivary acinar and ductal cells, and oral squamous cell carcinoma cells were irradiated with γ -rays or treated with CDDP, with or without pretreatment with EGCG, followed by MTT and BrdU incorporation assays. The results demonstrated that EGCG protected the normal salivary gland cells from chemical or irradiation-induced damage. However, protection of oral cancer cells by EGCG was also observed if EGCG was at physiologically achievable salivary concentrations but not at higher concentrations, suggesting that the combination of green tea consumption with cancer therapy requires further evaluation.

In mammals, the salivary glands produce saliva, a fluid secretion that provides protection and lubrication of oral surfaces, and initiates digestion of food. Within the glands, the bulk of the fluid is produced by the terminal secretory acinar cells, and a series of ducts collect and modify the secretion. Xerostomia (dryness of the mouth) occurs in

approximately 30% of the population over age 65 years (1). Xerostomia can result from several causes, such as senility, iatrogenic factors (medications and irradiation), the autoimmune disorder Sjögren syndrome or infection (2, 3). Severe xerostomia can occur in patients treated for head and neck cancers, especially oral cancers due to treatment-induced loss of the acinar cell population, which may not be fully replaced. The mechanism for this loss is not fully known. Patients with severely dysfunctional salivary glands often suffer from impaired speech, infection, and loss of taste perception and appetite (4). This reduction in the quality of life is an important issue for head and neck cancer patients (3, 5).

Many phytochemicals, phenolic phytochemicals in particular, have been shown to have a unique ability to damage malignant cells while protecting normal cells (6). Recently, attention has been paid to the protective role of green tea polyphenols (GTPP), especially (-)-epigallocatechin-3-gallate (EGCG), in epidermal epithelial cells subjected to ultra-violet irradiation (7, 8). EGCG at 20 μ M was found to protect epidermal keratinocytes from UV-B-induced hydrogen peroxide (H_2O_2) formation (9). We previously reported that EGCG causes differential oxidative environments that favor tumor cell destruction and normal cell survival (10). While EGCG induces caspase-mediated apoptosis in oral carcinoma cells, normal human epidermal keratinocytes (NHEK) are protected by EGCG through a separate pathway (11-13). In tumor cells, EGCG at higher (non-physiological) concentrations (100 ~ 200 μ M) is able to selectively promote the formation of intracellular H_2O_2 , which is partially responsible for the induction of apoptosis (14). In contrast, NHEK respond to EGCG by cell differentiation without accumulation of intracellular H_2O_2 (10).

Oral consumption of green tea leads to salivary levels of EGCG, the most abundant GTPP, higher than those found in

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serum. The peak levels of salivary EGCG, observed immediately after green tea consumption, range from 10.5 μ M to 48 μ M (15), within the range where significant differential effects on normal and cancer epithelial cell populations have been observed. Green tea consumption may therefore have a protective effect on salivary glands in healthy individuals, and GTPP may provide a mechanism to protect the salivary glands in individuals undergoing cancer chemotherapy. These possibilities have not been explored.

The current study examined the ability of physiologically achievable salivary concentrations of EGCG (0 ~ 50 μ M) to protect immortalized human salivary gland acinar and ductal cells (an *in vitro* model for salivary glandular tissue) from the cytotoxic effects of ionizing radiation and the anticancer drug cis-platinum(II)diammine dichloride (CDDP). The effects of EGCG on these cells were compared to those on oral squamous carcinoma cells treated with these agents. The effects of EGCG at higher, pharmacological concentrations (100 ~ 200 μ M) were also studied. EGCG was found to afford a degree of protection to salivary gland cell populations, but the differential effect in normal versus cancer cells was realized only by pharmacological concentrations of EGCG.

Materials and Methods

Reagents. EGCG, CDDP and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). EGCG was dissolved in cell culture medium and filter-sterilized immediately prior to use.

Cell lines. Immortalized normal salivary gland (NS-SV-AC and NS-SV-DC) cell lines were provided by Dr. Masayuki Azuma (Tokushima University, School of Dentistry, Tokushima, Japan) and maintained in keratinocyte basal medium-2 (KBM-2, Cambrex Bio Science Inc., Walkersville, MD, USA) (16). These cell lines were isolated after transfection of origin-defective mutant SV40 DNA into primary cultured human salivary gland cells. Characteristics of NS-SV-AC and NS-SV-DC cells were morphologically similar to acinar and duct cells, respectively. Oral squamous cell carcinoma (OSC-2 and OSC-4) cells were previously described (13, 17); they were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 50/50 mix medium (Cellgro, Kansas City, MO, USA) supplemented with 10% (v/v) fetal bovine serum, 100 I.U./ml penicillin, 100 μ g/ml streptomycin and 5 μ g/ml hydrocortisone.

Cell treatments. Cells were pretreated with different doses of EGCG for 24 h, and then were either exposed to a range of doses of γ -rays using a ^{137}Cs source, or were cultured in medium containing a range of doses of CDDP for 48 h. Alternatively, EGCG was added after γ -ray irradiation or CDDP treatment at the time and concentrations indicated.

MTT assay. Cells (0.5×10^4 cells/well) were seeded in a 96-well microplate and treated as described above. After the treatments, the cells in each well were washed with 200 μ l of phosphate-buffered saline (PBS) and incubated with 100 μ l of 2% (w/v) MTT

in a solution of 0.05 M Tris-HCl (pH 7.6), 0.5 mM MgCl_2 , 2.5 mM CoCl_2 and 0.25 M disodium succinate at 37°C for 30 min. Cells were fixed by the addition of 100 μ l of 4% (v/v) formalin in 0.2 M Tris-HCl (pH 7.6) and, after a 5-min incubation at room temperature, liquid was removed and the wells were allowed to dry. Each well was rinsed with 200 μ l water and cells were solubilized by the addition of 100 μ l of 6.35% (v/v) 0.1 N NaOH in DMSO. The colored formazan product was measured by a Thermo MAX micro plate reader (Molecular Devices Corp., Sunnyvale, CA, USA) at a wavelength of 562 nm.

DNA synthesis assay. DNA synthesis was analyzed by a BrdU Cell Proliferation Assay Kit (Oncogene Research Products, Boston, MA, USA). Briefly, cells (0.5×10^4 cells/well) were seeded in a 96-well microplate and treated as described above. After the treatment, cells were labelled with BrdU for 24 h (NS-SV-AC and NS-SV-DC cells) or 12 h (OSC-2 cells) at 37°C and then reacted with anti-BrdU antibody. Unbound antibody in each well was removed by rinsing, and horseradish peroxidase-conjugated goat anti-mouse antibody was added to each well. The color reaction was visualized according to the protocol provided by the manufacturer. The color reaction product was quantified using a Thermo MAX microplate reader (Molecular Devices Corp) at dual wavelengths of 450-540 nm.

Western blot. Control and treated 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 μ g/ml leupeptin, 3 μ g/ml aprotinin and 100 mM phenylmethylsulfonyl fluoride (PMSF). Samples of lysates containing 40 μ g protein were loaded in each lane and electrophoretically separated on a 12% SDS polyacrylamide gel. Following electrophoresis, proteins were transferred to a PVDF membrane (Immobilon™-P, Millipore Corporation, Bedford, MA, USA). The membrane was blocked for 1 h with 5% (w/v) non-fat dry milk powder in PBST (0.1% Tween-20 in PBS) and then incubated for 1 h with anti-human rabbit polyclonal antibodies against p16/INK3a, p21/WAF1/CIP1, cyclin D and cyclin-dependent kinase 4 (CDK4) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The membrane was washed three times with PBST and incubated with peroxidase-conjugated, affinity-purified anti-rabbit IgG (Santa Cruz Biotechnology, Inc.) for 1 h. Following extensive washing, the reaction was developed by enhanced chemiluminescent staining using ECL Western blotting detection reagents (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA).

Statistical analysis. All experiments were performed in quadruplicate and all data are reported as mean \pm SD. Mann-Whitney *U*-test was used to analyze statistical significance. Differences were considered statistically significant at $p < 0.05$.

Results

Comparison of cell viability determined by MTT assay. OSC-2, NS-SV-AC and NS-SV-DC cells showed considerably different susceptibility to EGCG (Figure 1). MTT analysis indicated treatment with EGCG alone at physiological concentrations (12.5 ~ 50 μ M) slightly decreased viability of OSC-2 cells. (Figure 1A). Cell viability of NS-SV-AC cells was

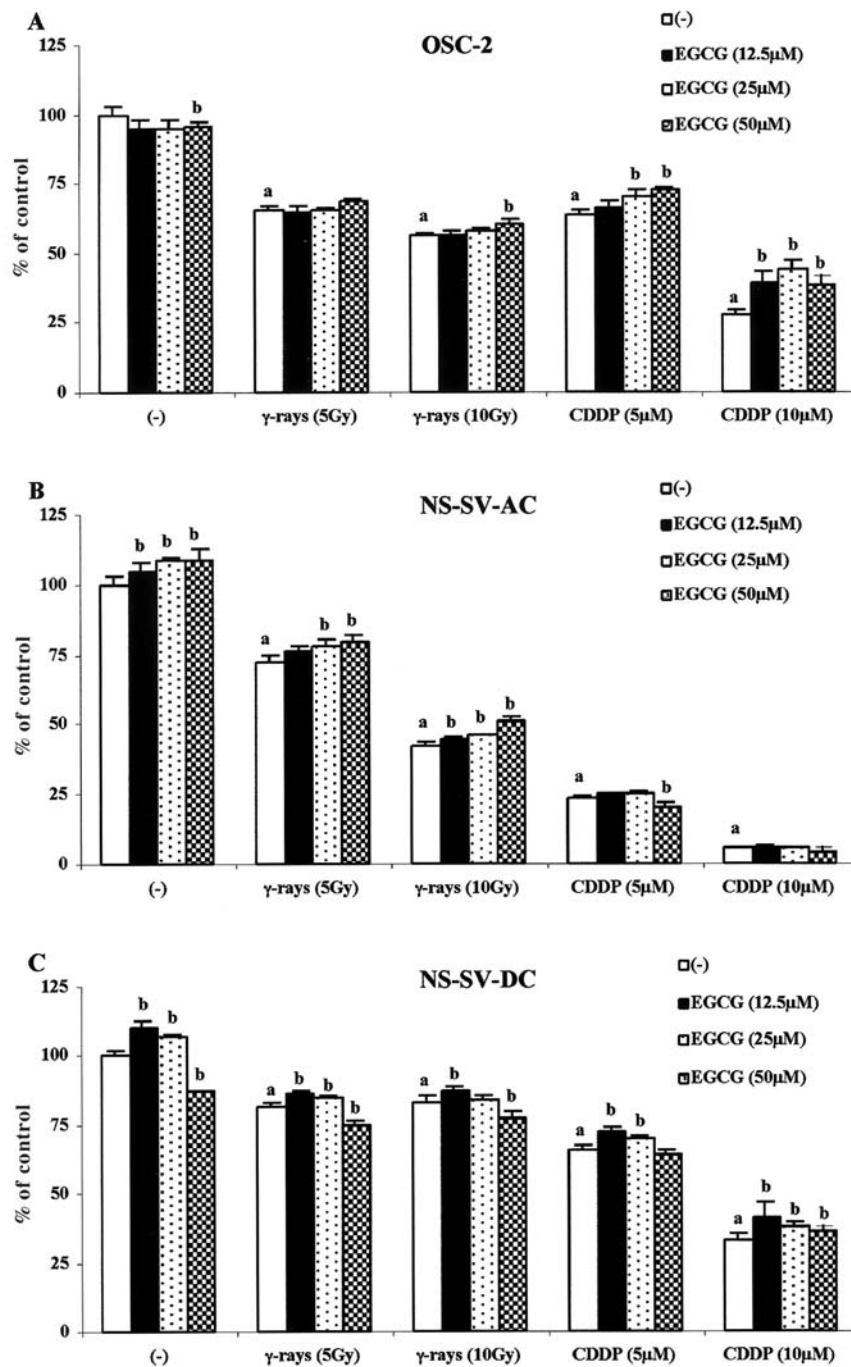


Figure 1. Influence of low concentrations of EGCG on γ -ray- and CDDP-induced cell damage. OSC-2 (A), NS-SV-AC (B) and NS-SV-DC (C) cells were pretreated with the indicated doses of EGCG for 24 h. The cells were then exposed to the indicated doses of γ -rays or cultured in medium containing the indicated doses of CDDP for 48 h, followed by MTT assay. Data are expressed as percentage of untreated cells and error bars represent one standard deviation of the mean. These figures are representative of three independent experiments with similar results. a; $p < 0.05$ against nontreated cells, b; $p < 0.05$ against γ -ray- or CDDP-treated cells without EGCG, by Mann-Whitney U-test.

increased to approximately 105 ~ 108% of the control over the dosage range tested (Figure 1B). In NS-SV-DC cells, treatment with 12 ~ 25 μ M of EGCG increased their viability

to approximately 106 ~ 110% of the control, but 50 μ M of EGCG decreased the viability to $87 \pm 0.4\%$ of the control (Figure 1C).

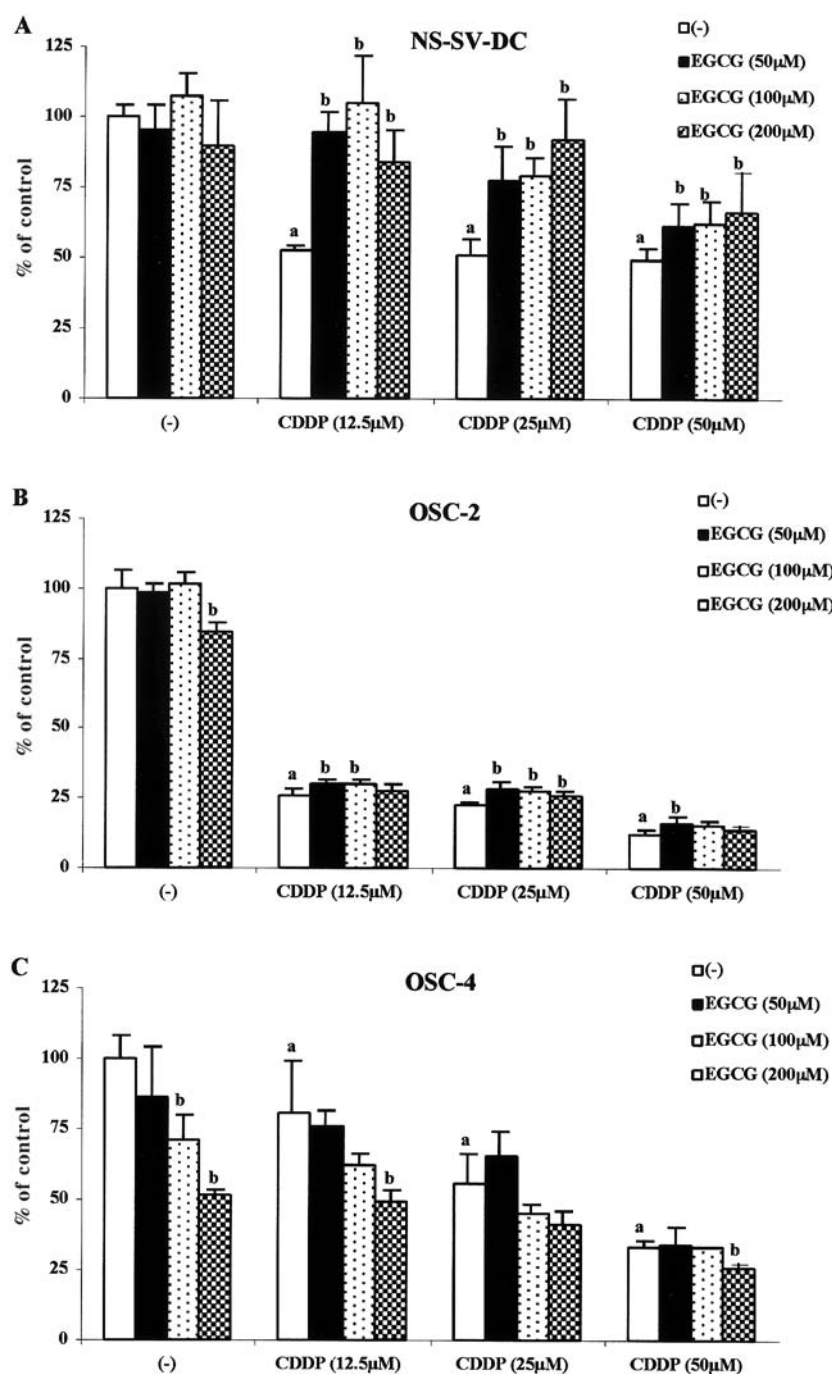


Figure 2. Influence of high concentrations of EGCG on CDDP-induced cell damage. NS-SV-DC (A), OSC-2 (B) and OSC-4 (C) cells were pretreated with the indicated doses of EGCG for 24 h. The cells were then cultured in medium containing the indicated doses of CDDP for 48 h, followed by MTT assay. Data are expressed as percentage of untreated cells and error bars represent one standard deviation of the mean. These figures are representative of three independent experiments with similar results. a; $p < 0.05$ against nontreated cells, b; $p < 0.05$ against γ -ray- or CDDP-treated cells without EGCG, by Mann-Whitney U-test.

OSC-2 and NS-SV-AC cells exhibited a dose-dependent decline in viability when γ -irradiated at 5 Gy ($65.5 \pm 1.8\%$ and $72.5 \pm 1.9\%$, respectively, of the controls) and 10 Gy ($56.4 \pm 1.0\%$ and $42.4 \pm 1.3\%$, respectively, of the controls).

NS-SV-DC cells were more resistant to γ -ray treatment: at 5 Gy viability had declined to $81.2 \pm 1.9\%$, and did not decline further ($83.0 \pm 2.8\%$ of the control) at 10 Gy. NS-SV-DC cells also behaved differently in response to γ -ray-irradiation

following EGCG pretreatment. Both OSC-2 and NS-SV-AC cells showed a slight, but significant increase in viability relative to non-pretreated, γ -irradiated cells at either 5 or 10 Gy when the concentration of EGCG was increased. In contrast, the pretreatment with 12.5 ~ 25 μ M of EGCG increased cell viability of NS-SV-DC cells after γ -ray-irradiation, but at the highest EGCG dose tested (50 μ M), NS-SV-DC cells showed a modest decrease in viability relative to non-pretreated cells (5 Gy; $74.9 \pm 1.3\%$ vs. $81.2 \pm 1.9\%$, 10 Gy; $77.0 \pm 2.5\%$ vs. $83.0 \pm 2.8\%$).

All three cell lines showed a significant, dose-dependent decline in cell viability following treatment with CDDP, although they differed in susceptibility. For OSC-2, NS-SV-AC, and NS-SV-DC cells, viabilities at 5 μ M were $64.1 \pm 1.4\%$, $23.3 \pm 1.4\%$ and $65.6 \pm 1.9\%$, respectively, and the viabilities at 10 μ M were $27.5 \pm 1.6\%$, $5.3 \pm 0.2\%$ and $32.8 \pm 2.3\%$, respectively, relative to untreated cells. In OSC-2 cells, pretreatment with 25 and 50 μ M of EGCG significantly reduced the cytotoxicity of 5 μ M CDDP to $70.5 \pm 2.4\%$ and $72.7 \pm 1.1\%$, respectively (CDDP alone; $64.1 \pm 1.4\%$). For NS-SV-AC cells, EGCG had no protective effect on CDDP-induced cytotoxicity at any concentration of EGCG or CDDP tested and 50 μ M of EGCG enhanced 5 μ M of CDDP-induced cytotoxicity. Pretreatment of NS-SV-DC cells with EGCG gave significant increases in viability relative to cells treated with 5 or 10 CDDP μ M alone, except for the combination of 50 μ M EGCG and 5 μ M CDDP.

The effects of pretreatment with pharmacological EGCG concentrations were tested on OSC-2, OSC-4, and NS-SV-DC cells at higher CDDP doses (Figure 2). Even the highest dose of EGCG (200 μ M) had no effect on the viability of NS-SV-DC cells (Figure 2A). In contrast, in both OSC-2 and OSC-4 cells, the cell viability was significantly decreased to $84.5 \pm 2.8\%$ and $51.5 \pm 1.4\%$, respectively, of the controls by 200 μ M of EGCG (Figure 2B and 2C). For OSC-4 cells, pretreatment with 200 μ M EGCG followed by treatments with CDDP resulted in a reduction in viability greater than treatment with either agent alone *i.e.* the effects of the agents were additive. For OSC-2 cells, EGCG pretreatment had no additional effect on cytotoxicity when followed by treatments with CDDP. In contrast, NS-SV-DC cells responded to pretreatment with high EGCG doses with a significantly increased viability: 100 μ M EGCG restored cell viability to the untreated control levels when cells were exposed to 12.5 μ M CDDP; 200 μ M EGCG gave almost 100% protection against 25 μ M CDDP. Although the protective effect of EGCG declined when CDDP doses were increased to 50 μ M, the overall protective effect of EGCG was significant.

Comparison of DNA synthesis rate measured by BrdU incorporation assay. OSC-2, NS-SV-AC and NS-SV-DC cells showed similar patterns of changes in BrdU incorporation

when exposed to γ -rays or CDDP, either alone or in combination with EGCG (Figure 3). γ -irradiation at 10 Gy decreased BrdU incorporation to $75.8 \pm 6.0\%$, $68.4 \pm 9.7\%$ and $57.2 \pm 2.2\%$ of the controls in OSC-2, NS-SV-AC and NS-SV-DC cells, respectively. EGCG pretreatment significantly reduced the inhibitory effects of γ -irradiation or CDDP on DNA synthesis. In OSC-2 and NS-SV-AC cells, 12.5 μ M of EGCG completely reversed the γ -irradiation-induced inhibition of DNA synthesis (Figure 3A and 3B), while NS-SV-DC cells required more than 12.5 μ M of EGCG for complete reversal (Figure 3C). A combination of 50 μ M EGCG pretreatment with 10 Gy of γ -irradiation, accelerated BrdU incorporation in all three cell lines: in OSC-2, NS-SV-AC and NS-SV-DC cells, DNA synthesis was increased to $111.8 \pm 3.4\%$, $105.4 \pm 1.2\%$ and $114 \pm 0.4\%$, respectively, of the controls.

BrdU incorporation in all three cell lines was inhibited to less than 30% by 5 μ M of CDDP. Physiological concentrations of EGCG partially reversed the CDDP-induced inhibition of DNA synthesis in a dose-dependent manner. Pretreatment with 50 μ M of EGCG increased BrdU incorporation (as a percent of the untreated control) from $27.0 \pm 2.0\%$ to $76.4 \pm 10.4\%$ in OSC-2 cells, $12.7 \pm 6.5\%$ to $57.4 \pm 2.6\%$ in NS-SV-AC cells, and $20.7 \pm 7.3\%$ to $49.1 \pm 14.9\%$ in NS-SV-DC cells.

Changes in cell cycle regulator protein levels detected by Western blot. EGCG did not quantitatively affect the protein levels of cyclin D and CDK4 (Figure 4A). In contrast, the expression of p21/WAF1 was decreased by pretreatment with 50 μ M of EGCG in NS-SV-DC cells but constant in OSC-2 and NS-SV-AC cells (Figure 4B). p21/WAF1 expression was induced by 10 Gy γ -irradiation in NS-SV-AC cells. Pretreatment with EGCG inhibited p21/WAF1 expression in NS-SV-DC cells, indicating a pro-survival role by preventing growth arrest and apoptosis. However, γ -ray-induced p21/WAF1 expression was enhanced by the pretreatment with EGCG in OSC-2 and NS-SV-AC cells. p21/WAF1 expression was not induced by CDDP in all cell lines. Pretreatment with EGCG suppressed p21/WAF1 expression in NS-SV-DC cells but enhanced p21/WAF1 levels in OSC-2 cells. Expression levels of p16 in NS-SV-AC and NS-SV-DC cells were not significantly changed by EGCG or anti-cancer agents, and lack of p16 in OSC-2 cells was noticed (Figure 4B).

Discussion

Radiation and chemotherapy are commonly applied to oral cancer patients. Ionizing irradiation such as γ -rays is used in γ -knife surgery and radiation therapy. Salivary gland cells undergo growth arrest and cell death following exposure to γ -irradiation (18-20). Likewise, CDDP, which exerts its

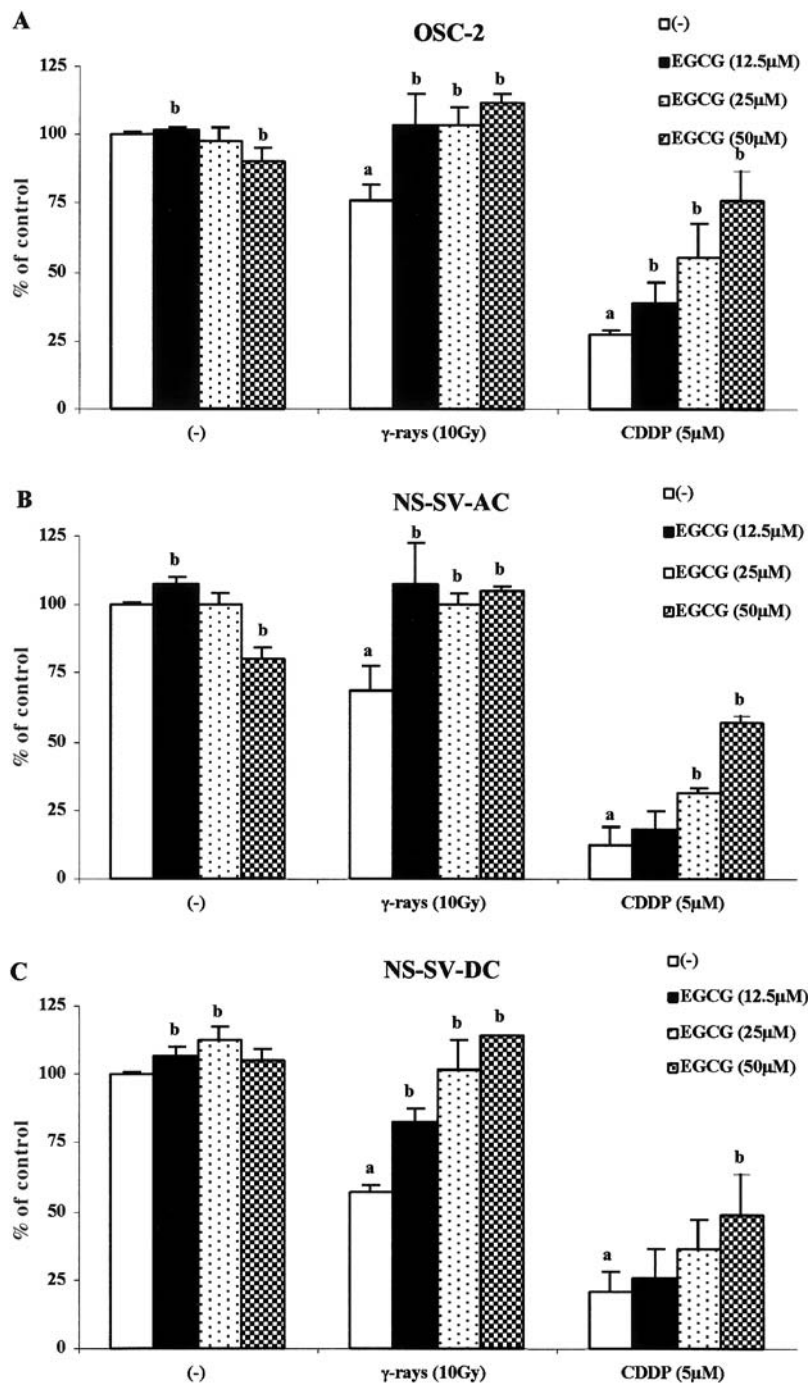


Figure 3. Influence of EGCG on γ -ray- and CDDP-induced inhibition of DNA synthesis. OSC-2 (A), NS-SV-AC (B) and NS-SV-DC (C) cells were pretreated with the indicated doses of EGCG for 24 h. The cells were then exposed to 10 Gy of γ -rays or cultured in medium containing 5 μ M of CDDP for 48 h, followed by MTT assay. Data are expressed as percentage of untreated cells and error bars represent one standard deviation of the mean. These figures are representative of three independent experiments with similar results. a; $p < 0.05$ against nontreated cells, b; $p < 0.05$ against γ -ray- or CDDP-treated cells without EGCG, by Mann-Whitney U-test.

cytotoxic effect by impairing DNA synthesis and transcription, and thus inhibits salivary gland functions, is commonly used as a chemotherapeutic drug for oral cancer

patients (21). Radiation- and chemotherapy-induced xerostomia brings a variety of problems to patients of head and neck cancers, which negatively affect their quality of

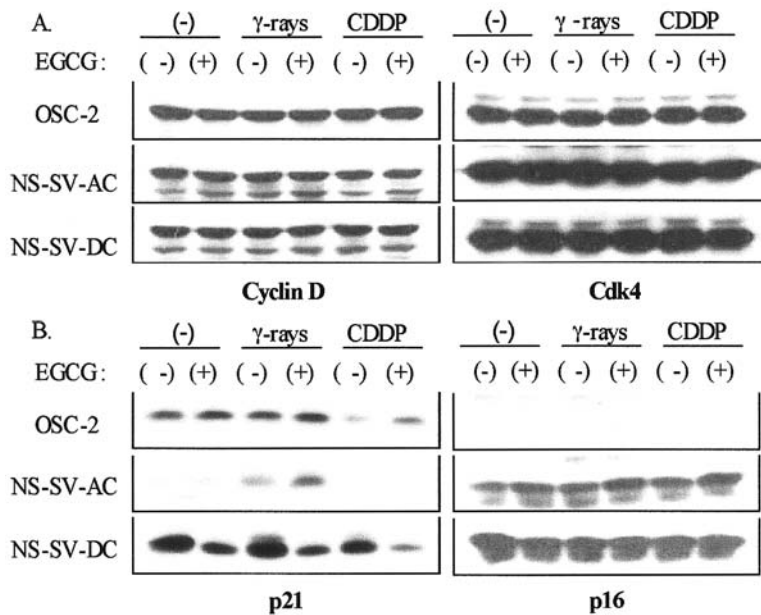


Figure 4. Western blot of whole cell lysates from γ -ray-irradiated or CDDP-treated OSC-2, NS-SV-AC and NS-SV-DC cells. OSC-2, NS-SV-AC and NS-SV-DC cells were pretreated with 50 μ M of EGCG for 24 h. The cells were then exposed to 10 Gy of γ -rays or cultured in medium containing 5 μ M of CDDP for 48 h, followed by Western blot analysis as described in Materials and Methods.

life. Therefore, it is important to identify nontoxic/natural agents that may reduce the adverse effects of radiation and chemotherapy on salivary gland function. In the current study, salivary gland cells were protected by physiologically achievable EGCG concentrations from CDDP- and γ -ray-induced cytotoxicity. NS-SV-AC exhibited a small reversal of γ -ray-induced cell viability loss when the cells were pretreated with 25 ~ 50 μ M of EGCG (Figure 1B). However, NS-SV-AC cells exhibited extreme sensitivity to CDDP treatment, and the pretreatment with any doses of EGCG was not able to protect the cells from CDDP-induced cell damage. CDDP at 10 μ M almost eliminated their cell viability as measured by MTT assay, suggesting CDDP could cause significantly more damage to NS-SV-AC cells than other cell types (Figure 1). This sensitivity of NS-SV-AC cells to chemically-induced damage may be one of the causal factors for age-related xerostomia, that may result from chronic exposure to chemicals/drugs (3). This result is consistent with previous findings in animal models, which showed a significant reduction of fluid secretion by acinar cells following a single dose of 10 Gy γ -irradiation (23). In contrast, cell viability of NS-SV-DC cells, was increased by pretreatment with physiological concentrations of EGCG under identical therapeutic conditions (Figure 1C).

Both NS-SV-AC and NS-SV-DC cells restored the BrdU incorporation rates to control levels when 10 Gy of γ -irradiation was combined with pretreatment of EGCG (Figure 3B and 3C). This result could be significant for repopulation of salivary gland cells from surviving stem cells

post cancer therapy. Nevertheless, OSC-2 cells also were protected by EGCG from γ -irradiation or CDDP-induced inhibition of DNA synthesis (Figure 3A). These data suggest that, although EGCG at physiological concentrations would protect salivary gland cells from γ -irradiation- and chemotherapeutic drug-induced damage by stimulating DNA synthesis, application of EGCG prior to or during cancer therapy also may result in protection of tumor cells by accelerating cell cycle.

Cell cycle progression is regulated by cyclins, CDKs and CDK inhibitors such as the CIP/KIP and INK4 protein families. EGCG inhibited p21/WAF1 expression with or without γ -irradiation and CDDP in NS-SV-DC cells but not in OSC-2 and NS-SV-AC cells (Figure 4). Since p53 expression in all cell lines was not altered by EGCG, γ -rays, or CDDP (data not shown), and the p53 gene in OSC-2 cells is mutated, changes in p21/WAF1 expression by EGCG, γ -rays, or CDDP could be p53-independent. These findings indicate that EGCG may down-regulate p21/WAF1 expression in a p53-independent manner. Expression of cyclin D, CDK4, and p16 in all cell lines was not changed by EGCG, γ -rays, or CDDP, whereas p16 was not expressed in OSC-2 cells (Figure 4), suggesting that EGCG regulates cell cycle by regulating CDK inhibitor, especially p21/WAF1 but not by modulating cyclin or CDK. Since mutation in the p16 gene is uncommon, the lack of p16 expression in OSC-2 cells may be due to hypermethylation of the promoter region of the p16 gene or changes in histone deacetylation.

It was reported that EGCG at physiological concentrations protects cells from DNA/chromosomal damage, and high concentrations of EGCG cause DNA/chromosomal damage by producing reactive oxygen species (ROS) (24). In the oral cavity, salivary EGCG concentrations can reach 50 μM by drinking green tea, which is considered to be the physiological salivary EGCG concentration; however, EGCG concentrations can be significantly increased by simple high-dose delivery systems. In a human study, holding high concentrations of green tea extract in the oral cavity resulted in salivary EGCG concentrations reaching 370 μM (14). We previously hypothesized that cells in frequent contact with plant-derived polyphenols, such as cells found in the epidermis, oral mucosa and digestive tract, have developed mechanism(s) to mitigate the toxicity of these phytochemicals and benefit from the protective effects of the compounds (12). In comparison, EGCG, when applied in high doses, is cytotoxic to other cells that lack this tolerance and to cancer cells that have lost these protective mechanisms (10). Thus, only an EGCG concentration range that selectively protects salivary gland cells but not cancer cells should be considered for application in combination with cancer therapies.

After OSC-2 cells were incubated with 5 μM of CDDP for 48 h, cell viability was reduced to approximately 60% (Figure 1A), and DNA synthesis was decreased to 30% (Figure 3A), indicating the development of a severe CDDP-induced cytotoxicity. However, it is surprising that EGCG, a potent apoptosis inducer of tumor cells, reduced the cytotoxicity of CDDP in OSC-2 cells. EGCG at 50 μM restored cell viability to almost 70% and DNA synthesis rate to over 75% (Figures 1A and 3A). This observation suggests that green tea oral consumption while under cancer therapy may reduce the effectiveness of the therapy, since the salivary EGCG concentration may not reach 50 μM after regular green tea consumption. We previously reported that EGCG at higher concentrations (100 ~ 200 μM) imposed additive damage to OSC-2 cells by promoting the formation of intracellular ROS (13). In OSC-2 cells, EGCG at 200 μM caused ROS production equivalent to 50 μM H_2O_2 , but at 50 μM , EGCG did not accelerate ROS formation (25). Thus, EGCG concentration could be a key factor to determine whether EGCG serves to protect or to damage tumor cells during cancer therapies.

To evaluate the possible concentration difference, two oral squamous cell carcinoma cell lines, OSC-2 and OSC-4 cells, and NS-SV-DC cells were used for the high EGCG concentration experiment; NS-SV-AC cells were not included because they are extremely sensitive to high doses of CDDP. While EGCG alone at any concentration did not induce significant cell damage in NS-SV-DC cells (Figure 2A), EGCG higher than 100 μM significantly induced cytotoxicity in both oral cancer cell lines. At 200 μM , EGCG reduced cell viability in OSC-2 and OSC-4 cells to

approximately 80% and 50%, respectively (Figures 2B and 2C). NS-SV-DC cells were protected by EGCG from the cytotoxicity induced by CDDP, regardless of EGCG concentration or CDDP concentration, indicating that EGCG is able to neutralize, at least in part, the damaging effect of CDDP (Figure 2A). Conversely, EGCG at 200 μM not only failed to protect tumor cells from CDDP-induced cytotoxicity, but also enhanced its effectiveness (Figures 2B and 2C). This differential effect of EGCG may be due to the oxidative stress imposed on the tumor cells, and reduced ROS in NS-SV-DC cells (13). Therefore, we report here, for the first time, that EGCG is able to protect salivary gland cells from damage caused by chemotherapeutic drug and radiation therapy. On the other hand, EGCG also may protect tumor cells under therapeutic conditions when EGCG concentrations are at physiological levels.

In conclusion, frequent oral consumption of green tea or green tea-containing products in healthy individuals may provide protection from medication and/or age-related dysfunction of salivary glands such as xerostomia. The protective property of EGCG in green tea is able to reduce ROS, and thus neutralize the damaging effects of chemicals/medicine and radiation in salivary gland cells, especially in salivary ductal cells, which showed down-regulation of p21/WAF1 upon exposure to EGCG. However, our results suggest that cancer patients undergoing chemo- or radiation therapy should not consume green tea systematically in order to avoid a possible decrease in therapeutic effectiveness caused by EGCG. If topical EGCG treatment is sought to protect the salivary glands during oral cancer therapy, high-dose delivery of EGCG (>200 μM) should be considered pending additional information from future studies.

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