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Role of p21^{WAF1} in Green Tea Polyphenol-induced Growth Arrest and Apoptosis of Oral Carcinoma Cells

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Abstract. *The cyclin-dependent kinase inhibitor p21^{WAF1} participates in cell growth, differentiation and apoptosis. p21^{WAF1} can be induced by green tea polyphenol EGCG in several cancer cell types, but its role in the oral cancer cell response to EGCG is not known. We found that EGCG up-regulates p21^{WAF1} in an oral carcinoma cell line, OSC2, by cDNA microarray. The current study determined the impact of siRNA-suppressed p21^{WAF1} and its response to EGCG on cell growth, DNA synthesis and apoptosis by RT-PCR, Western blot, BrdU incorporation, MTT and caspase 3 activity assays. Suppression of p21^{WAF1} by siRNA resulted in an accelerated cell growth and DNA synthesis, and increased cell viability. However, caspase 3 activity was not significantly inhibited. The evidence showed that p21^{WAF1} is involved in EGCG-induced growth arrest of OSC2 cells, which may facilitate caspase 3-mediated apoptosis. Thus, expression of functional p21^{WAF1} may promote phytochemical-mediated growth arrest and apoptosis in oral carcinoma cells.*

The multifunctional protein p21^{WAF1}, a key member of the cyclin-dependent kinase inhibitors, serves as an effector for p53-mediated tumor suppression and for p53-independent functions, including growth arrest, differentiation and apoptosis (1). Under normal conditions, a low level of p21^{WAF1} is required for cell proliferation, while induction of p21^{WAF1} results in G1-phase growth arrest. Since the majority of oral squamous cell carcinomas (OSCC) are associated with p53 defects, dysregulation of p21^{WAF1} often results in failure to efficiently regulate the progression of OSCC cells, which makes p21^{WAF1} a potential target for cancer therapies. Induction of p21^{WAF1} in tumor cells may help to restore cell cycle control and sensitize the tumor cells for apoptosis. Retroviral transfection and expression of p21^{WAF1} in human OSCC cells xenografted in athymic mice reduced the size of tumors *in vivo* (2). *In vitro* models showed that the induction of p21^{WAF1} in OSCC was achieved by radiation (3), cyclooxygenase 2 (COX2) inhibitors celecoxib (4) and aspirin (20), the chemopreventive agent apigenin (11), or the differentiation inducer vesnarinone (5). Another chemopreventive agent, green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG), activated p53-dependent and Fas-mediated pathways, associated with p21^{WAF1} up-regulation in a human liver cancer cell line Hep G2, leading to G1 growth arrest and apoptosis (9). In prostate cancer cell lines, EGCG induced growth arrest and apoptosis with elevated p21^{WAF1} (10). The induction of p21^{WAF1} was also achieved in MCF10A breast epithelial cells by EGCG, leading to growth arrest in G1-phase (11). However, whether EGCG regulates p21^{WAF1}, and what role p21^{WAF1} might play in EGCG-induced apoptosis in OSCC, were unknown. We previously demonstrated that EGCG selectively induced apoptosis in a human OSCC cell line OSC2, but not in normal human epidermal keratinocytes (NHEK) (6-8). Our hypothesis is that EGCG inversely regulates gene groups that control cell growth, differentiation and apoptosis in normal *versus* tumor cells, including p21^{WAF1}. The current study examined the role of p21^{WAF1} by determination of its expression patterns in NHEK and a metastatic oral carcinoma cell line, OSC2, in response to EGCG exposure using microarray technology (detailed methods and results will be reported separately), followed by *in situ* "knock down" of p21^{WAF1} mRNA using siRNA technology in OSC2 cells. The results indicate that p21^{WAF1} is involved primarily in EGCG-induced growth arrest and loss of cell viability, which may prime the tumor cells for apoptosis. EGCG may activate both p21^{WAF1}-mediated growth arrest and mitochondria-mediated apoptosis in tumor cells, but suppresses

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Key Words: Green tea, polyphenol, EGCG, oral cancer, p21^{WAF1}, apoptosis.

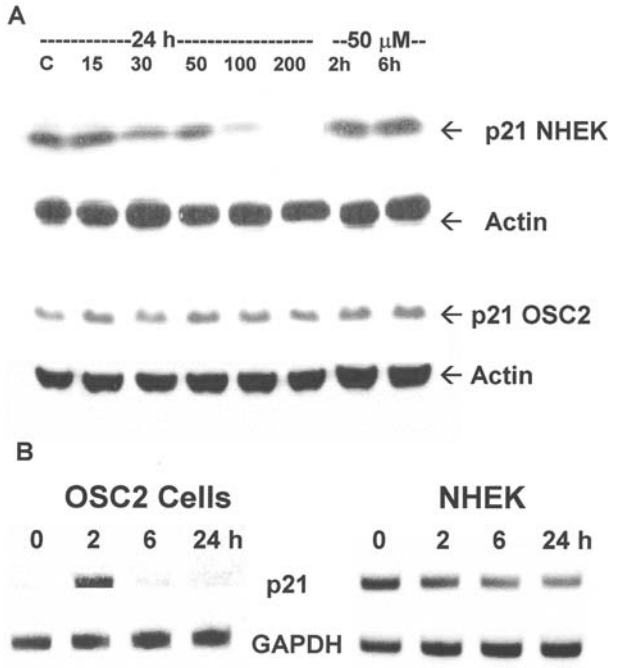

Figure 1. *EGCG inversely regulates the expression of p21^{WAF1} in NHEK versus OSC2 cells A. Protein expression patterns of NHEK vs. OSC2 at indicated exposure times and EGCG concentrations. B. RT-PCR result showing inverse regulation of p21^{WAF1} mRNA levels by 100 μM EGCG.*

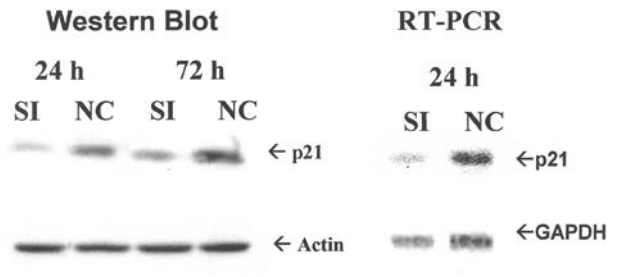

Figure 2. *siRNA for p21^{WAF1} reduced p21^{WAF1} mRNA and protein levels in OSC2 cells. Left. Western blot showing reduced levels of p21^{WAF1} after siRNA was expressed in OSC2 cells at the indicated time. Right. mRNA levels of p21^{WAF1} were determined by RT-PCR after the siRNA vector was transfected for 24 h.*

p21^{WAF1} in NHEK, thus inducing differential response in normal *versus* tumor cells.

Materials and Methods

Chemicals and reagents. 3-(4,5-Dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and EGCG were purchased from Sigma Aldrich (St. Louis, MO, USA). EGCG was dissolved in cell culture media immediately prior to use. Antibodies against human p21^{WAF1} and actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell lines and cell culture. The pooled normal human primary epidermal keratinocytes were purchased from Cambrex (East Rutherford, NJ, USA) and sub-cultured in the specific growth media (KGM-2) provided by the manufacturer. The OSC2 cell line was isolated from a cervical metastatic lymph node of a patient with oral squamous cell carcinoma, and p21^{WAF1} can be induced by exogenous signals such as TGF β1 in these cells (12, 13). The cells 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 μg/ml streptomycin and 5 μg/ml hydrocortisone.

Western analysis. Cell lysates containing 30 μg protein were separated on a 15% SDS polyacrylamide gel. The method for Western analyses was previously described (17). The resulting bands were visualized by enhanced chemiluminescent staining using ECL Western blotting detection reagents (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA).

siRNA transfection. The SureSilencing™ Human CDKN1A siRNA Kit was purchased from Super Array Bioscience Corp (Frederick, MD, USA). A vector expressing siRNA for p21^{WAF1} (SI) and empty vector (NC) were transfected into OSC2 cells using Lipofectamine 2000 (Invitrogene, Carlsbad, CA, USA) according to the manufacturer's protocol.

Total RNA extraction and semi-quantitative reverse-transcription PCR (RT-PCR). Total RNA was extracted using an RNeasy total RNA isolation system (QIAGEN, Valentia, CA, USA). RT reaction and PCR reaction were performed according to the manufacturer's protocol (Super Array Bioscience Corp).

Growth rate determination by cell counting. Cell growth assay was performed on exponentially growing OSC2 cells in complete DMEM/Ham's F12 medium. Cells at 2 X 10^4 were initially plated in each well of a 6-well cell culture plate at time 0. Cell quantification was achieved by cell counting using a hemacytometer and Trypan blue exclusion at 24, 48 and 72 h.

Caspase activity assay. The Caspase 3 Apoptosis Detection Kit was purchased from Santa Cruz Biotech. Inc. Cells were plated at 2.5X10^4/well in a 24-well tissue culture plate. Caspase 3 activity assay was performed as described previously after 48 h incubation with EGCG (8).

MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Cells were seeded at 10^4/well in a 96-well plate. Following 48-h incubation with EGCG, the assay was performed as previously described (8).

BrdU assay. The BrdU cell proliferation kit was purchased from Oncogene Research Products (Boston, MA, USA). Cells were cultured in 96-well plates at the density of 5X10^3 cells/well. After 48-h incubation with EGCG, the cells were labeled with BrdU for 4 h followed by the BrdU assay procedure previously described (15).

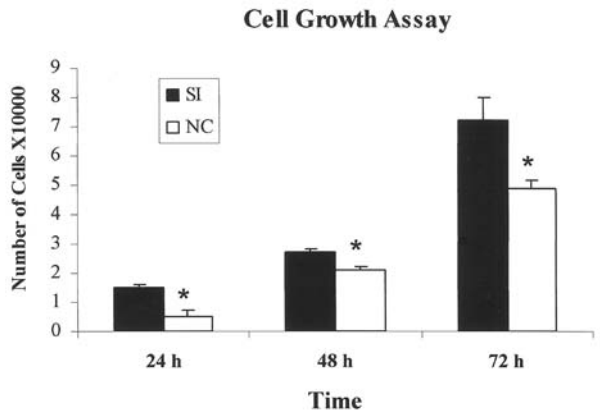

Figure 3. *Differential growth rate between siRNA-transfected and empty vector-transfected cells. OSC2 cells transfected with the p21^{WAF1} siRNA (SI) displayed a remarkable difference in the growth rate when compared to OSC2 cells transfected with empty vector (NC). Suppression of p21^{WAF1} led to accelerated cell growth. The SI cells were 25% and 30% greater in cell number at 48 and 72h than the NC cells, respectively. Asterisks indicate significant differences between SI and NC-transfected cells (t test, 2-sided, α=0.05, n=2).*

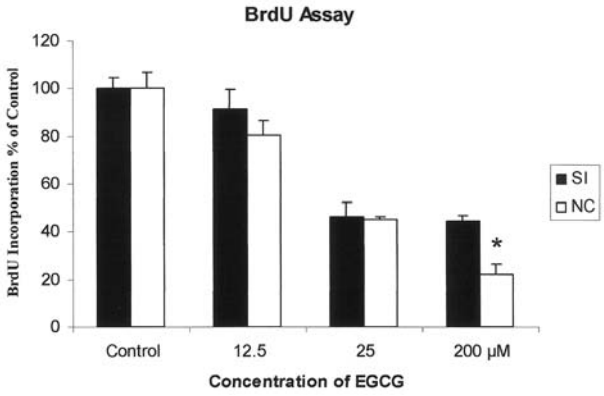

Figure 4. *Suppression of p21^{WAF1} resulted in significant resistance to 200 μM EGCG. Decrease in cell viability was detected in both SI and NC cells after a 48-h exposure to 200 μM EGCG measured by MTT assay. However, while NC cell viability decreased by 75%, SI cells only lost approximately 40% of viability 48 h after exposure to 200 μM EGCG. Asterisk indicates significant difference between SI and NC-transfected cells (t test, 2-sided, α=0.05, n=3).*

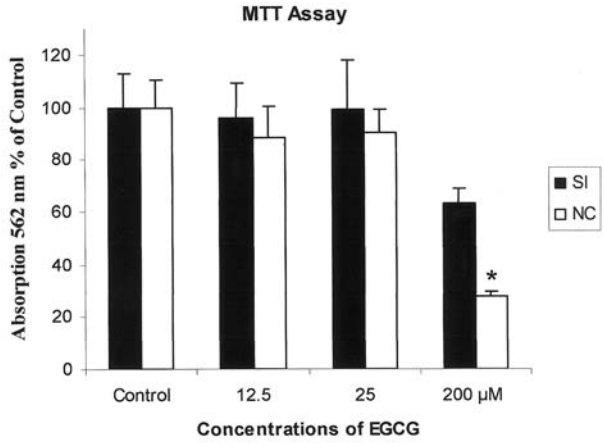

Figure 5. *Suppression of p21^{WAF1} resulted in resistance of EGCG-induced inhibition of DNA synthesis. BrdU incorporation, a measure of new DNA synthesis, was performed in siRNA p21^{WAF1}-transfected cells (SI) and empty vector-transfected cells (NC). SI cells still showed a significant rate of DNA synthesis (40%) after 48-h exposure to 200 μM EGCG, in comparison with NC (20%). Asterisk indicates significant difference between SI and NC-transfected cells (t test, 2-sided, α=0.05, n=3).*

Statistical analysis. All data are reported as mean ± SD. A one-way ANOVA and unpaired Student's *t*-tests were used to analyze statistical significance. Differences were considered statistically significant at $p<0.05$.

Results

Inverse regulation of p21^{WAF1} in OSC2 cells vs. NHEK. Data from gene array analysis showed that EGCG at 100 μM suppressed p21^{WAF1} mRNA levels at early time points in NHEK (74% at 2 h and 54% at 6 h), and these mRNA levels recovered to the control levels by 24 h. Conversely, in OSC2 cells, the mRNA levels of p21^{WAF1} were significantly increased (378%) when incubated with 100 μM EGCG at 2 h, and remained slightly elevated (140%) from 6 h to 24 h post EGCG addition (data not shown). RT-PCR analysis demonstrated that the message of p21^{WAF1} in OSC2 cells increased 50-fold at the 2 h time point prior to decline; and that p21^{WAF1} message gradually decreased in NHEK, in response to EGCG exposure (Figure 1B). Western analyses also demonstrated opposing expression changes of p21^{WAF1} in OSC2 and NHEK in response to EGCG (Figure 1A).

Suppression of p21^{WAF1} by siRNA resulted in decreased levels of p21^{WAF1} mRNA and protein in OSC2 cells. RT-PCR analysis demonstrated that p21^{WAF1} message was suppressed in p21^{WAF1} siRNA-transfected OSC2 cells (SI) when compared with empty vector-transfected OSC2 cells (NC). Western analysis showed less p21^{WAF1} protein was present in both the 24-h and 72-h post-transfection of SI cells. In contrast, NC cells showed significantly higher levels of p21^{WAF1} protein at both 24h and 72h post transfection (Figure 2).

siRNA-transfected cells accelerated OSC2 cell proliferation. OSC2 cells transfected with the p21^{WAF1} siRNA (SI) displayed a remarkable difference in the growth rate when compared to OSC2 cells transfected with empty vector

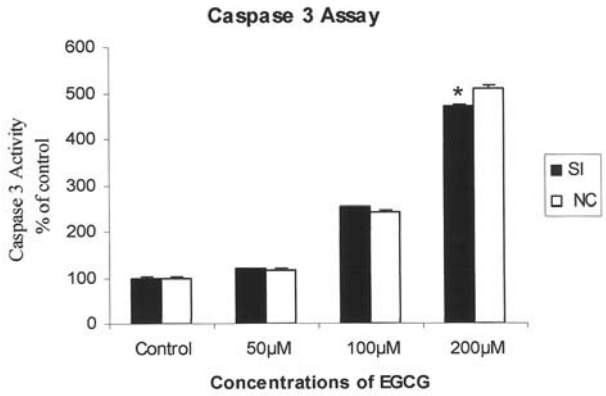

Figure 6. *Caspase 3 activities in SI and NC cells treated with different concentrations of EGCG. EGCG at 100 μM activated caspase 3 by approximately 2.5-fold in both SI and NC. Small but significant differences in caspase 3 activities were observed when SI and NC cells were exposed to 200 μM EGCG for 48 h (6.6-fold vs. 7.1-fold). Asterisk indicates significant difference between SI and NC-transfected cells (t test, 2-sided, $\alpha=0.05$, $n=3$).*

(NC). The SI cell numbers were 25% higher at 48 and 72h than the NC cells (Figure 3).

MTT assay. A significant decrease in cell viability was detected in both SI and NC cells after a 48-h exposure to 200 μM EGCG as measured by MTT assay (Figure 4). However, while NC cell viability decreased by 75%, SI cell viability decreased by less than 60%.

BrdU assay. BrdU incorporation was affected by EGCG in a dose-dependent fashion. The major difference in BrdU incorporation was detected at 48-h exposure to 200 μM EGCG. The $p21^{WAF1}$ siRNA-transfected SI cells still possessed approximately 40% of DNA synthesis activity, but NC cells only possessed 20% of DNA synthesis activities (Figure 5).

Caspase 3 activity assay showed a small difference between SI and NC cells. A dose-dependent response in caspase 3 activity was detected in both transfectants; 50 μM EGCG did not induce significant caspase 3 activity; while 100 μM EGCG increased the activity of caspase 3 by approximately 2.5-fold in both SI and NC. Small but significant differences of caspase 3 activities were observed when SI (6.6-fold) and NC cells (7.1-fold) were exposed to 200 μM EGCG for 48 h (Figure 6).

Discussion

We previously reported that EGCG or a mixture of green tea polyphenols induced differential impacts in normal *versus* tumor cells by activating different pathways (14, 15) and creating opposite oxidative environments (16, 17). The gene array analyses further confirmed this unique property of green tea polyphenols. While NHEK responded to EGCG by undergoing a differentiation pathway (15), $p21^{WAF1}$ was dose-dependently inhibited on both mRNA and protein levels (Figure 1). In contrast, OSC2 cells, which commit to caspase 3-mediated apoptosis when exposed to EGCG (8), elevated $p21^{WAF1}$ mRNA at 2 h and increased protein levels (Figure 1). These observations suggest that $p21^{WAF1}$ is involved in EGCG-induced growth arrest and apoptosis in OSC2 cells; however, in NHEK, suppression of $p21^{WAF1}$ by EGCG is associated with an inhibitory mechanism for caspase 3-mediated apoptosis.

Induction of $p21^{WAF1}$ was previously found to be a downstream event of p53 stabilization by EGCG (18). Our results indicate that EGCG also activates $p21^{WAF1}$ in a p53-independent pathway. Clinicopathological data indicated that the expression of p53 and $p21^{WAF1}$ in oral dysplasia and OSCC were not correlated (19). However, when expressions of both p53 and $p21^{WAF1}$ were considered for cumulative patient survival rate, "p53-positive and $p21^{WAF1}$-positive" represent the lowest survival rate among patients in comparison to all other combinations (19). In fact, OSC2 cells are "p53-positive and $p21^{WAF1}$-positive" and extremely metastatic, and are able to infiltrate bone soon after xenograft into athymic mice and cause paralysis and animal death (data not shown, manuscript in preparation). However, when $p21^{WAF1}$ was considered as a single factor, a ten-year clinical study of OSCC found that patients with tumors expressing high levels of $p21^{WAF1}$ had increased disease-specific survival time, suggesting that OSCC cells expressing $p21^{WAF1}$ could respond more effectively to anticancer therapies or agents such as EGCG (21). Thus, EGCG-induced apoptosis in OSCC cells may be more efficient if the cells are arrested at G1 caused by elevated $p21^{WAF1}$ expression. In OSC2 cells, induction of $p21^{WAF1}$ by EGCG is associated with growth arrest, which may favor the mechanism of caspase 3-mediated apoptosis, either parallel or downstream to growth arrest. Suppression of $p21^{WAF1}$ by siRNA led to accelerated cell growth of OSC2 cells without EGCG (Figure 3). This activation of proliferation could be a major factor for SI cells to be more resistant to EGCG in comparison to the cell viability with NC cells (Figure 4). In addition, SI cells were significantly more active in DNA synthesis during EGCG treatment, suggesting $p21^{WAF1}$ could be a crucial factor for EGCG-induced growth arrest pathways in tumor cells (Figure 5). On the other hand, caspase 3 was activated in both SI and NC cells following EGCG exposure (Figure 6). The only significant difference in caspase 3 activity was from 200 μM EGCG-treated samples. However, this difference was not proportionally correlated with cell proliferation rate, MTT and BrdU assay results.

In conclusion, we have observed that green tea polyphenol EGCG inversely regulates the expression of $p21^{WAF1}$ in NHEK *versus* OSC2 cells; and EGCG-induced $p21^{WAF1}$ is involved primarily in the growth arrest pathway with minimal impact on caspase 3-mediated apoptosis. This finding is consistent with clinical data that patients with tumors expressing $p21^{WAF1}$ showed increased survival time. The evidence suggests that EGCG-induced cytotoxicity and apoptosis could more significantly impact tumor cells with elevated levels of $p21^{WAF1}$ than tumor cells with suppressed $p21^{WAF1}$.

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