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Review

EGCG-targeted p57/KIP2 reduces tumorigenicity of oral carcinoma cells: Role of c-Jun N-terminal kinase

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Abstract

The green tea polyphenol epigallocatechin-3-gallate (EGCG) regulates gene expression differentially in tumor and normal cells. In normal human primary epidermal keratinocytes (NHEK), one of the key mediators of EGCG action is p57/KIP2, a cyclin-dependent kinase (CDK) inhibitor. EGCG potently induces p57 in NHEK, but not in epithelial cancer cells. In humans, reduced expression of p57 often is associated with advanced tumors, and tumor cells with inactivated p57 undergo apoptosis when exposed to EGCG. The mechanism of p57 induction by EGCG is not well understood. Here, we show that in NHEK, EGCG-induces p57 *via* the p38 mitogen-activated protein kinase (MAPK) signaling pathway. In p57-negative tumor cells, JNK signaling mediates EGCG-induced apoptosis, and exogenous expression of p57 suppresses EGCG-induced apoptosis *via* inhibition of c-Jun N-terminal kinase (JNK). We also found that restoration of p57 expression in tumor cells significantly reduced tumorigenicity in athymic mice. These results suggest that p57 expression may be an useful indicator for the clinical course of cancers, and could be potentially useful as a target for cancer therapies.
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Keywords: EGCG; MAPK; JNK; p57/KlP2; Oral carcinoma

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Introduction

The *p57* gene product is a p53-independent G1 cyclin/CDK inhibitor (Lee et al., 1995). Normal embryonic development in mice requires p57 expression, and p57-knockout mice exhibit growth retardation and early death (Yan et al., 1997; Takahashi et al., 2000). Elevated expression of p57 has been associated with intestinal cell differentiation (Deschenes et al., 2001) and anti-apoptotic mechanisms in circulating memory T lymphocytes (Vattemi et al., 2000; Li et al., 2004). The most abundant green tea catechin – (−) epigallocatechin-3-gallate (EGCG)– promotes p57 (KIP2, CDKN1C)-mediated differentiation in normal epithelial cells, but induces caspase 3-mediated apoptosis in p57-deficient tumor cells (Hsu et al., 2001, 2002a, 2003a, 2003b, 2005a).

The *p57* gene is located on chromosome 11p15.5, and frequent loss of heterozygosity at this locus in tumors suggests a tumor suppressor function (Henry et al., 1989; Bepler and Garcia-Blanco, 1994; Hatada and Mukai, 1995; Shibagaki et al., 1994; Baffa et al., 1996). Like its p21 counterpart, p57 has a binding domain for proliferating cell nuclear antigen (PCNA), an important DNA replication protein (Watanabe et al., 1998). p57 expression can suppress myc/RAS-mediated transformation (Watanabe et al., 1998), and loss of binding domains for either CDK2 or PCNA reduces this ability. Tumor tissues often express lower levels of p57 protein in comparison to normal tissues, and low levels of p57 often correlate with a poor clinical prognosis (Ito et al., 2000, 2002; Nakai et al., 2002; Nan et al., 2005; Fan et al., 2006). Tumor cells with reduced expression of p57 could result from gene silencing rather than mutation (Shin et al., 2000a). Silencing of p57 has been associated with many types of tumors (Hatada et al., 1996; Thompson et al., 1996; Schwienbacher et al., 2000; Soejima et al., 2004) and may be due to epigenetic modifications including hypermethylation of the p57 promoter region and enhanced histone deacetylation (Shin et al., 2000b; Li et al., 2002). Inhibition of DNA hypermethylation significantly increases p57 expression, leading to growth inhibition of tumor cells (Liu et al., 2004), and also results in senescence of urothelial, lung, and breast cancer cells (Kobatake et al., 2004; Hoffmann et al., 2005). Therefore, re-activation of endogenous p57 or introduction of exogenous p57 expression may be a useful approach to treat cancers of the epithelium.

In primary normal human epidermal keratinocytes (NHEK), p57 activation by EGCG leads to the serial induction of differentiation-related gene products (Hsu et al., 2005a). This phenomenon has also been described during myoblast differentiation (Chang et al., 2003). We reported recently that EGCG induces accelerated differentiation in NHEK that was associated with increased p57 and caspase 14 expression (Hsu et al., 2003a, 2005a). Unlike the other caspases, caspase 14 is not involved in the apoptotic caspase cascade, but rather is associated with barrier formation during terminal differentiation of NHEK (Lippens et al., 2000; Pistritto et al., 2002). In the epidermis, induction of caspase 14 at the transcriptional level facilitates stratum corneum formation (Eckhart et al., 2000), and inhibition of cell differentiation diminishes caspase 14 expression (Rendl et al., 2002). Therefore, caspase 14 is a marker for epidermal differentiation, possibly activating planned cell death and cornification of the epidermis to form the skin barrier.

The EGCG-induced signaling pathways leading to p57 activation are not well understood. MAPK elements have been shown to be essential to EGCG signaling. EGCG-induced activation of p38 transcription may enhance differentiation via a MAPK/ERK kinase (MEK)/p38/activating protein (AP)-1 cascade in NHEK (Balasubramanian et al., 2002, 2005). Conversely, EGCG induction of p57 in NHEK cells leads to inhibition of a majority of (if not all) pro-apoptotic genes (Hsu, 2005). Signaling via another MAPK element, JNK, has been shown to be a mediator of apoptosis in NHEK induced by ultra violet radiation or oxidative stress, and can be inhibited by EGCG (Katiyar et al., 2001; Kim et al., 2005; Wu et al., 2006). In myoblasts, high level expression of p57 suppresses c-Jun N-terminal kinase (JNK) activity (Chang et al., 2003). Thus, p57 may play an important role during cell differentiation by inhibiting JNK-mediated apoptosis, likely as a consequence of the interaction of p57 with JNK (Chang et al., 2003). The failure to block JNK signaling in p57-deficient tumor cells could contribute to their EGCG-induced apoptosis.

In the current study, we investigated the role of MAPK proteins in EGCG induction of p57 and apoptosis. Our results show that 1) the p38 MAPK signaling pathway mediates EGCG-induced expression of p57 in NHEK, 2) JNK signaling mediates EGCG-induced apoptosis in OSC2, and 3) restoration of functional p57 in OSC2 cells results in reduced tumorigenicity *in vivo*.

Methods

Chemicals and antibodies. EGCG was purchased from Sigma-Aldrich (St. Louis, MO). Anti-human p38, pp38, pJNK and pERK antibodies, and anti-human actin (I-19) antibody were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Specific inhibitors for p38 (SB203580), JNK (SP600125) and MEK (PD98059) were supplied by EMD Bioscience, Inc., San Diego, CA.

Cell lines. Pooled normal human primary epidermal keratinocytes were purchased from Cambrex (East Rutherford, NJ) and sub-cultured in growth media (KGM-2) as specified by the manufacturer. The OSC2 cell line was isolated from a cervical metastatic lymph node of a patient with oral squamous cell carcinoma (Osaki et al., 1994). The cells were cultured in DMEM/Ham's F12 medium (50/50 by vol, Cellgro, Kansas City, MO) supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml hydrocortisone. The control vector-transfected OSC2 cell line G6 and the OSC2-derived p57-expressing S2 and S5 cell lines, have been described previously. Both of them express high levels of p57 and caspase 14 mRNA (Hsu et al., 2005a).

Cell assays. Detailed methods for the preparation of cell fractions have been described by Han et al. (1999). Briefly, cells were lysed in the presence of proteinase inhibitors, and mitochondria purified by differential sedimentation through sucrose. The cytosolic fraction was obtained after high-speed (100,000×*g*) centrifugation. Methods for immunoblots have been described previously (Hsu et al., 2005b). EGCG was dissolved in cell culture media as a 100-times concentrated stock immediately prior to use. Dilutions of specific antibodies were: rabbit polyclonal JNK1/2, 1:1000, mouse monoclonal pJNK1/2, 1:1000, rabbit polyclonal ERK, 1:2000, rabbit polyclonal p38, 1:1000, rabbit polyclonal pp38, 1:1000 and goat polyclonal actin, 1:2000. Experiments were repeated three times. The cell growth assay was performed on exponentially growing cultures. Cells

(2×10^4) were seeded into each well of a 6-well cell culture plate. After overnight incubation, the cells were exposed to 100 μM EGCG, with or without inhibitors, for 24 h. Cells were quantified using a hemacytometer and Trypan blue exclusion. The Caspase 3 Apoptosis Detection Kit was purchased from Santa Cruz Biotech. Inc. Cells were plated at 2.5×10^5/well in a 24-well tissue culture plate. The caspase 3 activity assay was performed as described previously after 48 h of incubation with EGCG (Hsu et al., 2005c). For the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, cells were seeded at 10^4/well in 96-well plate format. Following 48 h of incubation with EGCG, the assay was performed as described previously (Hsu et al., 2005c).

Animals and xenograft. Female athymic mice at the age of 4–6 weeks (*nu/nu*) were purchased from Harlan (Indianapolis, IN). At random, seven nude female mice were chosen for each group. The cell lines were administered to the mice according to the following groups: 1) OSC2 (left shoulder), S2 (right shoulder); 2) G6 (left shoulder), S5 (right shoulder). Xenografts from each cell line were injected into the mice subcutaneously at a concentration of 10 million cells in 100 μl PBS. In each group, one cell type was introduced just above the left scapula while the other was introduced just above the right scapula. 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 by employing the following equation: $\text{Volume} = \text{width}^2 \times \text{length}/2$ (mm^3). Any animal that began to show paralysis was immediately euthanized. The tumor incidence between the p57 transfectant subclones and p57-null OSC2 or G6 was analyzed by Chi-square analysis.

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

Results

Induction of p57 by EGCG in NHEK is mediated by the p38 MAPK pathway

EGCG at 50 μM has been shown previously to efficiently induce p57 expression in NHEK cells (Hsu et al., 2001). During the 24 h following the addition of 50 μM EGCG, p57 was induced in NHEK, but not in OSC2 cells (Fig. 1A). Higher levels of EGCG (up to 200 μM) failed to induce p57 in OSC2 cells (Hsu et al., 2001). To determine the role of p38 in EGCG-induced p57 expression, NHEK were treated with 50 μM EGCG for 24 h with or without a 30-min pretreatment of a p38 inhibitor (SB203580), MEK inhibitor (PD98059), or JNK inhibitor (SP600125). After 24 h, p57 expression was measured. Inhibition of p38 blocked EGCG-induced p57 expression in a dose-dependent manner. In contrast, MEK or JNK inhibitors had no effect on the induction of p57 by EGCG, even at the highest concentration tested (Fig. 1B). These concentrations have been shown to be highly effective for MAPK inhibition in other systems (Ramos et al., 2006). Thus, the p38 MAPK pathway appeared essential to EGCG-induced p57 activation.

EGCG-induced OSC2 growth inhibition and apoptosis is mediated by the JNK MAPK pathway

As reported previously, EGCG induces growth inhibition and caspase 3-mediated apoptosis in the OSC2 cell line (Hsu et al., 2003, 2003b, 2005b). To determine the role played by MAPK pathway components in these events, OSC2 cells were treated with a MEK, p38 or JNK inhibitor (as above), then exposed to 100 μM EGCG, the minimum dose found to induce significant caspase 3 activity (Hsu et al., 2005b). Inhibition of JNK signaling restored cell growth to control levels and reduced caspase 3 activity to approximately 50% of the level induced by EGCG alone (Fig. 2A). Immunoblots verified that EGCG induced an increase in phosphorylation of JNK proteins in OSC2 cells (Fig. 2B). Both pJNK1 and pJNK2 are increased after 60 min, and elevated levels persist for at least one hour (Fig. 2B). Thus, in the p57-deficient OSC2 cell line, EGCG activates the proapoptotic JNK signaling pathway, leading to caspase 3-mediated apoptosis.

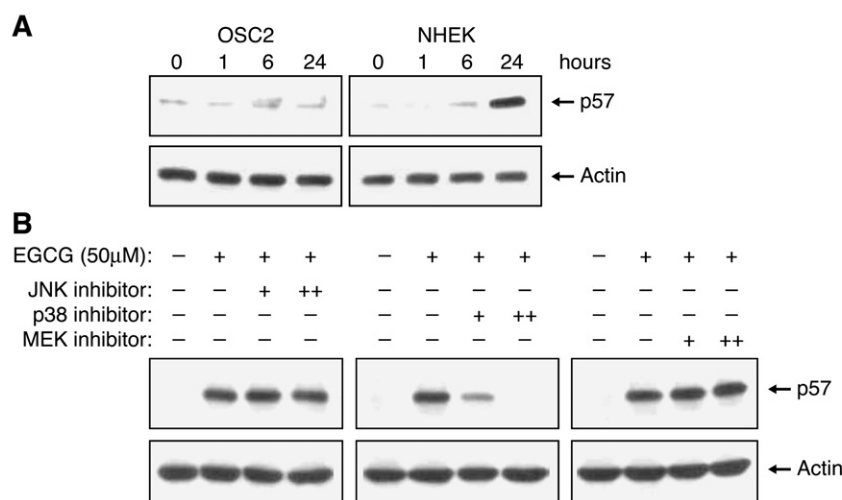

Fig. 1. Role of MAPK pathway elements in mediating effects of EGCG on p57 expression. (A) p57 is induced by EGCG in NHEK, but not OSC2 cells. Both OSC2 and NHEK cells were treated with 50 μM EGCG for the indicated time period. EGCG was added to replicate flasks at different times and then the cells were harvested at the same time (i.e., at the same stage of growth) to give the indicated exposure times. Only NHEK responded to EGCG by producing large amount of p57 protein at 24 h. (B) p38 inhibitor specifically blocked the EGCG-induced p57 expression. NHEK cells were treated with 50 μM EGCG for 24 h with or without specific inhibitors of p38 (SB203580), MEK (PD98059) or JNK (SP600125). Induction of p57 by EGCG was blocked specifically by the p38 inhibitor, but not by the JNK or MEK inhibitors. (Concentration of inhibitors: $^{+}$10 μM, $^{++}$30 μM, added to the cell culture medium 30 min prior to EGCG exposure.)

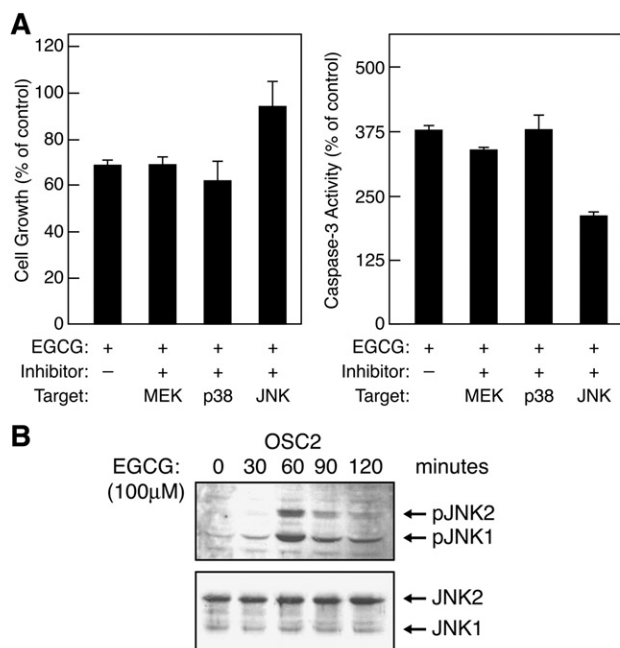

Fig. 2. Role of MAPK elements in mediating effects of EGCG on growth and apoptosis in OSC2 cells. (A) EGCG-induced growth arrest and caspase 3 activity were blocked by JNK inhibitor. Inhibitors of MAPK elements MEK (PD98059), JNK (SP600125) and p38 (SB203580), were added to the cell culture medium of OSC2 cell cultures for 30 min, followed by exposure to 100 μM EGCG for 24 h prior to the assay analyses (concentrations of inhibitors: 10 μM SB203580, 100 μM PD98059, 25 μM SP600125, dissolved in DMSO as stocks). Only the JNK inhibitor blocked the effects of EGCG by restoring the cell growth and decreasing caspase 3 activity. (B) 100 μM EGCG induced JNK phosphorylation of both JNK1 and JNK2 proteins in OSC2 cells. JNK1/2 were hyper-phosphorylated at 60 min and phosphorylation remained elevated through 120 min.

Exogenous p57 expression in OSC2 cells inhibits EGCG-induced translocation of BAX and cytochrome c, nd phosphorylation of JNK

Immunoblots of p57-stably transfected OSC2 subclones (S1–S5) expressed high levels of p57 protein in comparison to untransfected and control vector-transfected (G6) OSC2 cells (Fig. 3A). All five subclones (but not non-transfected OSC2 or control vector-transfected G6 cells) also expressed caspase 14 mRNA (Hsu et al., 2005a). However, only the S2 subclone had significant levels of caspase 14 protein (Fig. 3A). The S5 subclone expressed the lowest level of caspase 14. Therefore, the S2 and S5 subclones were selected for further study, as representative of the high and low extremes of caspase 14 protein expression, to allow an evaluation of the role of caspase 14 in tumorigenicity. Control vector-transfected (G6) and p57-expressing (S2) cells were exposed to 100 μM EGCG (the minimum concentration found previously to induce caspase 3 activity in 24 h) prior to isolation of cytosolic and mitochondrial protein fractions (Han et al., 1999). G6 cells showed a significant release of cytochrome *c* into the cytosol, peaking at 12 h after the addition of EGCG (Fig. 3B). In contrast, S2 cells showed no increased release. Further, mitochondrial translocation of BAX, a pro-apoptotic Bcl-2 family protein, was observed only in G6 cells (Fig. 3B). Thus, these results suggest that exogenous p57 expression significantly limited EGCG induction of two classical indicators of apoptosis in the S2 subclone. As measured by the MTT assay, EGCG has relatively little effect on the growth of NHEK (Hsu et al., 2003a). In contrast, EGCG significantly reduces the growth of OSC2 cells (Hsu et al., 2003) and control GFP-transfected OSC2 cells (G6, data not shown). In the S2 subclone, p57 expression decreased the effect of EGCG on cell growth (data not shown). We next examined if exogenous expression of p57 had an effect on EGCG-induced JNK activation. Control vector-transfected OSC2 cells (G6) exhibited an activation of JNK1 and JNK2 30 min after EGCG

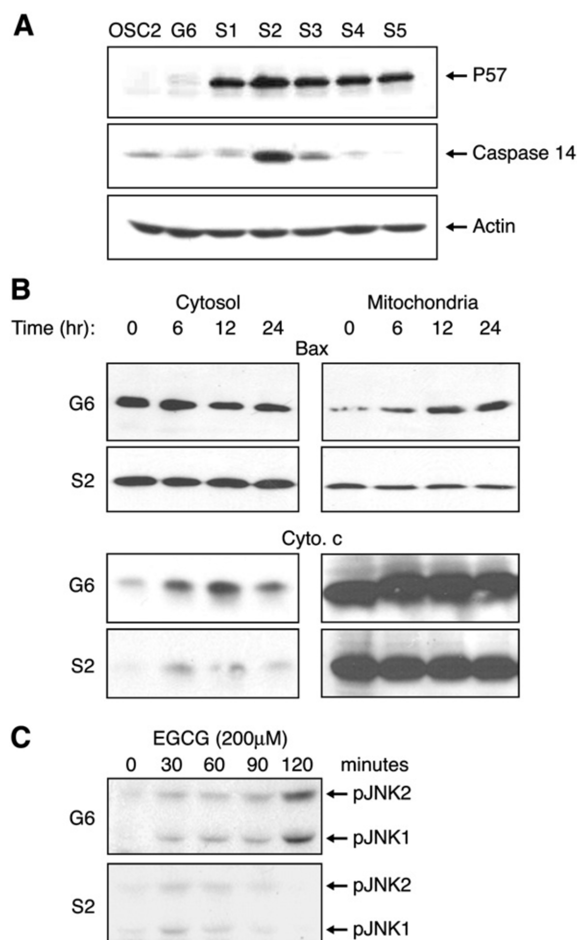

Fig. 3. Comparison of p57 stably transfected (S2) and control vector-transfected (G6) OSC2 cells. (A) Protein levels of p57 and caspase 14 in OSC2, G6 and S subclones determined by Western blot. Basal levels of p57 and caspase 14 were detected in both OSC2 and G6 cells, while all the S subclones express high levels of p57 protein. Only the S2 subclone produces large amount of caspase 14 protein. (B) Western blot results of the mitochondrial and cytosolic fractions of G6 and S2 cells exposed to 100 μM EGCG. p57-expressing S2 cells did not exhibit translocation of BAX from the cytosol to the mitochondria, while BAX in control vector-transfected G6 cells translocated to the mitochondria (upper). S2 cells also failed to show cytochrome *c* release, while G6 cells showed extensive cytochrome *c* release into the cytosol (lower). (C) Western blot results of hyper-phosphorylated JNK in G6 and S2 cells after EGCG exposure. G6 cells showed JNK hyper-phosphorylation throughout the 120 min-period, while JNK phosphorylation was not sustained in S2 cells. Cells were incubated with 100 μM EGCG for time periods as indicated prior to Western analysis for phosphorylated JNK1 and JNK2.

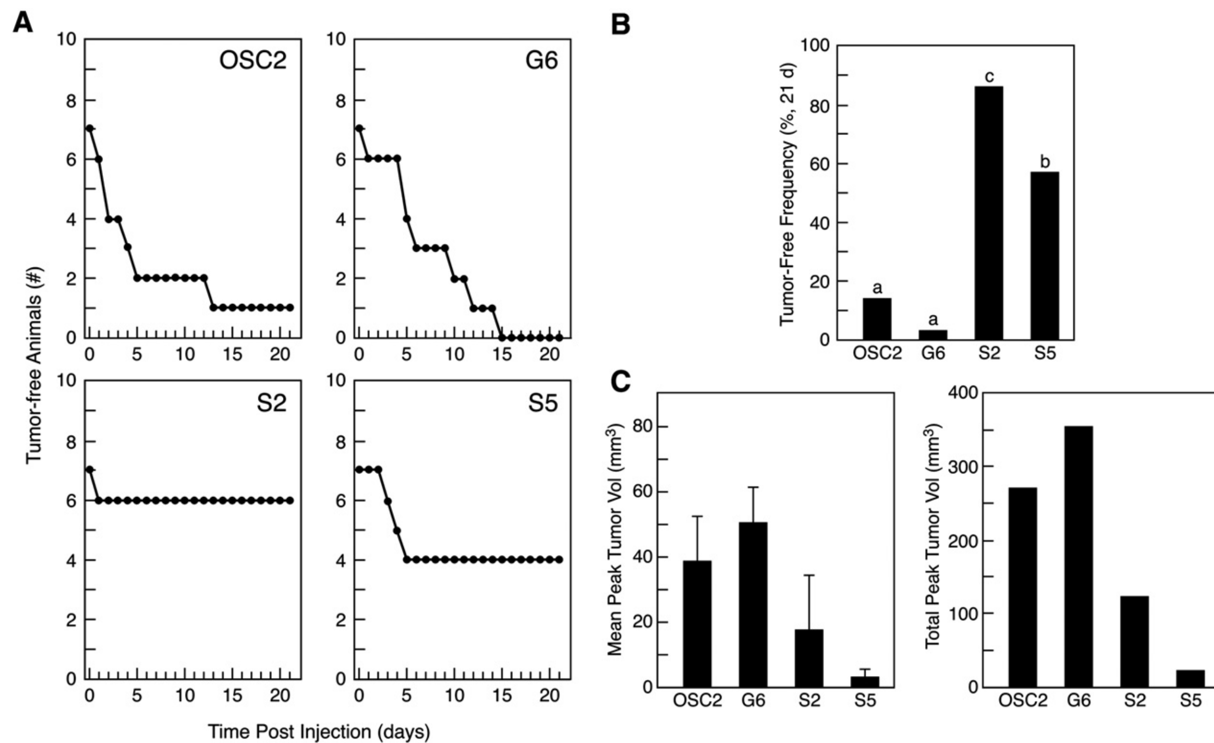

Fig. 4. Xenograft of transfected OSC2 cells in nude mice and tumor development. (A) Result of xenograft experiments using OSC2 derived cells either expressing exogenous p57 (S2 and S5) or GFP (G6), plus the parental cells. Tumors developed in all but one parental OSC2-xenografted animals and all GFP-expressing G6-xenografted animals. In contrast, of the seven S2 xenografted animals, six were tumor-free, and only three out of seven S5-xenografted animals developed tumors. Each animal received 10 million cells subcutaneously and was monitored over a 3-week period. Among OSC2 and G6 cell-induced tumors, some of them invaded into the bone, verified by pathological analyses (data not shown). S2 and S5 cells did not form metastases (data not shown). (B) Frequencies of tumor-free animals xenografted with the cell lines. The tumor incidence was lower in the p57 transfectant subclones compared to OSC2 or G6 (χ^2 analysis, $p<0.025$). (C) The mean peak volumes and the total peak tumor volumes from each group of animals (tumor-free animals received a 0 as tumor value) resulting from different cell lines.

treatment, with the highest levels seen after 2 h. In contrast, p57-expressing cells (S2) showed only a weak elevation of JNK phosphorylation at 30 min, followed by a decline to control levels (Fig. 3C). This failure to activate JNK is similar to the lack of activation of JNK seen in p57-expressing NHEK cells when they were treated with EGCG and apoptosis inducers (Katiyar et al., 2001; Kim et al., 2005).

Expression of p57 in tumor cells reduces tumorigenicity in athymic mice

The above results suggested that p57 expression in oral epithelial tumor cells could reduce tumorigenicity by promoting a more differentiated phenotype (as observed in NHEK cells following p57 induction by EGCG). We used an athymic mouse model to assess if exogenous expression of p57 limited OSC2 cells tumor development in vivo. Xenografts of p57-transfected cells (S2 and S5) formed fewer tumors than mice grafted with OSC2 or control vector-transfected (G6) cells (Fig. 4A). The appearance of these tumors was rapid with the OSC2 cell xenograft; tumors were visible within 1–2 days of injection. There was a gradual increase in the size of the tumor during the first 2 weeks, eventually peaking by the end of the third week (not shown). In the fourth week after injection, there was an unexpected decline in the size of the tumors. However, this decline coincided with the manifestation of multiple tumors at sites other than the xenograft sites. All G6 cell xenografts also resulted in tumor growth, and G6 cells displayed an almost identical pattern to the OSC2 cells with similar mean tumor frequency, mean peak volume, and total tumor peak volume (Figs. 4A, B). In contrast, xenografts of the S2 line caused only one tumor (Fig. 4A), and xenografts of S5 cells only resulted in three small tumors (Figs. 4A, B). Thus, the p57-expressing OSC2 stable transfectants were less tumorigenic (χ^2 analysis, $p<0.025$).

Discussion

We have reported previously that EGCG exposure leads to caspase 3-dependent apoptosis in OSC2 cells, while NHEK undergo terminal differentiation (Hsu et al., 2003, 2003a, 2005b). EGCG also induces p57 expression in NHEK, but not in OSC2 cells (Hsu et al., 2001, 2002a, 2003, 2005a, and Fig. 1A). The MAPK proteins p38, ERK and JNK were found to be associated with EGCG signaling, either during induction of tumor cell apoptosis or normal epithelial cell differentiation (Stratton et al., 2000; Balasubramanian et al., 2002; Afaq et al., 2003; Singh et al., 2003; Vayalil et al., 2003). Based on previous work reported by ourselves and others (Hsu et al., 2005b), we hypothesized that the p38 MAPK pathway is essential for EGCG-induced p57 expression. The current study showed that EGCG induction of p57 in NHEK requires p38 activity, but is

independent of JNK or MEK activity (Fig. 1B). EGCG has been shown previously to not activate JNK in NHEK cells (Katiyar et al., 2001). In contrast, in p57-deficient OSC2 cells, EGCG treatment induced rapid activation of JNK, and subsequent tumor cell apoptosis. Inhibition of JNK activation abolished EGCG-induced growth inhibition and reduced caspase 3-dependent apoptosis in OSC2 cells (Fig. 2). Thus, the differential effect of EGCG between NHEK and OSC2 cells could be caused in part by interaction of EGCG-induced p57 and JNK in NHEK cells; this interaction inhibits the phosphorylation of JNK proteins and blocks any subsequent proapoptotic signaling (Chang et al., 2003). In parallel, EGCG-induced p57 promotes differentiation (Hsu et al., 2003a; Hsu, 2005, Hsu et al., 2005a). In OSC2 cells, EGCG-activated JNK signaling is not blocked by p57, leading to apoptosis.

To assess the role of p57 in OSC2 cell behavior, we have previously introduced exogenous p57 cDNA using a retroviral expression system (Hsu et al., 2005a). p57 significantly reduced OSC2 cell growth, potentially by the inhibitory activity of p57 on the cell cycle. The expression in the S subclones of caspase 14 mRNA (a terminal differentiation marker specific for epidermal keratinocytes) was unexpected, but consistent with the prodifferentiation effects of p57 (Hsu et al., 2005a). The current study examined both p57 and caspase 14 protein expression in OSC2, G6 (control vector-transfected), and the S (p57 expressing) subclones, and demonstrated that while all S subclones express high levels of p57, only the S2 subclone expressed high levels of both p57 and caspase 14 proteins (Fig. 3A). The reason(s) for the discordance between mRNA and protein expression is unclear, and may reflect post-transcriptional controls. Regardless, S2 cells possess two hallmarks of differentiating NHEK. In addition, S2 cells exhibited inhibition of EGCG-induced cytochrome *c* release and BAX translocation (Fig. 3B), growth arrest (data not shown), and JNK activation (Fig. 3C). None of these effects were observed in the G6 subclone. These results suggest that loss of endogenous p57 function may be associated with OSC2 transformation, and thus introduction of exogenous p57 in OSC2 cells could reverse some aspects of tumor phenotype.

This potential role of p57 in tumorigenicity was evaluated in the current investigation using p57-expressing subclones S2 and S5 xenografted into athymic mice. The majority of the S2/S5 animals did not develop tumors. Among the seven p57-expressing xenografts, S2 cells only produced one tumor; and S5 cells produced three small tumors. In contrast, all G6 cell xenografts, and six of the seven OSC2 xenografts, developed tumors (Fig. 4A). When the mean peak tumor volumes were compared, the p57-expressing xenografts produced significantly smaller tumors than OSC2 and G6 cells (Fig. 4B). We reported previously that S5 cells have a low growth rate, which could account for the very small tumor sizes (Hsu et al., 2005a). These results indicate that expression of exogenous p57 in highly metastatic human oral carcinoma cells reduced the tumor occurrence and tumor size. There were no significant differences between OSC2 and G6 cells with regard to tumor-free frequency and the mean peak tumor volumes, suggesting that the transfection of GFP cDNA failed to alter the tumorigenicity *in vivo*. After the 21-day observation period, the majority of the OSC2 or G6 animals exhibited metastasis. Indeed, pathological analyses demonstrated bone metastasis from OSC2 and G6 cells (data not shown), which could be the cause of paralysis in some animals.

In conclusion, EGCG-induced p57 expression in normal epithelial cells is dependent on p38 MAPK activity. In tumor cells that lack p57, introduction of exogenous p57 reduced their tumorigenicity, and was associated with inhibition of both JNK phosphorylation and caspase 3 activation in response to EGCG. These results suggest that the EGCG-targeted *p57* gene regulation is an important modulator of epithelial cell differentiation or apoptosis. Thus, p57 may be useful as a diagnostic marker for tumors, and a good candidate target for novel therapeutic strategies.

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