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Inhibitory effects of epigallocatechin-3-*O*-gallate on serum-stimulated rat aortic smooth muscle cells via nuclear factor-κB down-modulation

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Abstract

The abnormal growth of vascular smooth muscle cells (VSMCs) plays an important role in vascular diseases, including atherosclerosis and restenosis after angioplasty. Although (−)-epigallocatechin-3-*O*-gallate (EGCG) has antiproliferative effects on various cells, relatively a little is known about precise mechanisms of the inhibitory effects of EGCG on SMCs. In this study, the inhibitory effects of EGCG on attachment, proliferation, migration, and cell cycle of rat aortic SMCs (RASMCs) with serum stimulation were investigated. Also, the involvement of nuclear factor-κB (NF-κB) during these inhibitions by EGCG was examined. EGCG treatment resulted in significant ($p < 0.05$) inhibition in attachment and proliferation of RASMCs induced by serum. While non-treated RASMCs migrated into denuded area in response to serum and showed essentially complete closure after 36 h, EGCG-treated cells covered only 31% of the area even after 48 h of incubation. Furthermore, EGCG treatment resulted in an appreciable cell cycle arrest at both G0/G1- and G2/M-phases. The immunoblot analysis revealed that the constitutive expression of NF-κB/p65 nuclear protein in RASMCs was lowered by EGCG in both the cytosol and the nucleus in a dose-dependent manner. These results suggest that the EGCG-caused inhibitory effects on RASMCs may be mediated through NF-κB down-modulation.

Keywords: Epigallocatechin-3-*O*-gallate; Antiproliferation; Rat aortic smooth muscle cell; Cell cycle arrest; Nuclear factor-κB

Reactive oxygen species (ROS) have been known to play an important role in the pathogenesis of atherosclerosis and several other cardiovascular diseases. It is now apparent that ROS induce endothelial cell damage, vascular smooth muscle cell (VSMC) growth, and cardiac remodeling, which are associated with hypertension, atherosclerosis, heart failure, and restenosis [1–3]. The migration of VSMCs from the tunica media to the subendothelial region is a key event in the development and progression of atherosclerosis and post-angioplasty vascular remodeling. Several lines of evidence have indicated that ROS and mitogen-activated protein kinases (MAPKs) were involved in vascular remodeling under various pathological conditions [4,5]. Moreover, it was also reported that matrix metalloproteinases (MMPs) might play a key role in these processes of SMC migration [6,7].

Epidemiological studies suggest that green tea consumption is associated with a reduced risk of cardiovascular disease. Antioxidative properties of green tea flavonoids, catechins, have been believed to be involved in the antiatherogenic effect of green tea, since catechins inhibit low-density lipoprotein oxidation [8]. Recently, it has been demonstrated that some kinds of catechins have the ability to inhibit the activities of some MMPs and MAPKs [9–11].

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Therefore, inhibition of MAPKs or MMPs by antioxidant treatment may prove to be a therapeutic strategy for cardiovascular diseases. Although this inhibitory activity is believed to be mainly due to the antioxidative and possibly antiproliferative effects of polyphenolic compounds in green tea [12], the precise mechanisms are not clear.

Our earlier studies have already shown that green tea polyphenols were significantly effective in protecting rat calvarial osteoblasts [13], human microvascular endothelial cells [14], and human saphenous vein [15] from ROS-induced oxidative stress. In the present study, the inhibitory effects of EGCG on the attachment, proliferation, migration, and cell cycle of rat aortic SMCs (RASMCs) with fetal bovine serum (FBS) stimulation, and the involvement of nuclear factor-κB (NF-κB) as a mechanism of these inhibitions were investigated. NF-κB, a widely distributed transcription factor, is associated with many physiological processes, including inflammation, cellular proliferation, and cancer [16–18]. NF-κB is present in the cytosol as a heterotrimer usually consisting of its p50 and p65 subunits bound to its inhibitory protein IκB [18]. Upon the phosphorylation and subsequent degradation of IκB, NF-κB activates and translocates to the nucleus. A variety of different stimuli, including cytokines, hydrogen peroxide, and serum, have been identified to cause the activation and nuclear translocation of NF-κB [18]. Our data implicate that EGCG treatment inhibits the attachment, proliferation, and migration of RASMCs stimulated with FBS. EGCG was also found to result in both the G0/G1- and G2/M-phase arrest of cell cycle. Importantly, NF-κB, constitutively expressed in RASMCs, was depleted in the cytosol as well as in the nucleus when the cells were subjected to EGCG treatment. Therefore, this inhibition in FBS-induced SMCs may be associated with the EGCG-mediated suppression of NF-κB expression and activation.

Materials and methods

Smooth muscle cell culture. RASMCs were purchased from BioBud (Seoul, Korea) and used between passages 3 and 7. The cells were routinely maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 10% FBS (Sigma) and a 1% antibiotic antimycotic solution (including 10,000 U penicillin, 10 mg streptomycin, and 25 μg amphotericin B per ml, Sigma) at 37 °C in a humidified atmosphere of 5% CO_2 in air.

EGCG treatment. EGCG, a major constituent of green tea, was kindly supplied by Pharma Foods International Co. Ltd. (Kyoto, Japan), and its purity exceeded 90%. In order to examine the inhibitory effects of EGCG on attachment, proliferation, migration, and cell cycle in serum-stimulated RASMCs, the cells were seeded into well plates and then incubated in the presence of increasing concentrations (100–400 μM) of EGCG.

Attachment and proliferation assays. The number of viable cells was quantified indirectly using a highly water-soluble tetrazolium salt [WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (Dojindo Lab., Kumamoto, Japan), reduced to formazan dye by mitochondrial dehydrogenases. RASMC attachment and proliferation were found to be directly proportional to the metabolic reaction products obtained in WST-8. Briefly, WST-8 assays were conducted as follows. Each cell culture was incubated with WST-8 for the last 2 h of the culture period for cell attachment (4 h) or proliferation (48 h) at 37 °C in the dark. In order to avoid a direct reaction between antioxidant EGCG and WST-8 to be reduced, the excess EGCG was completely removed and the medium was exchanged before adding WST-8. Parallel sets of wells containing freshly cultured, non-treated cells were regarded as the controls. Absorbance was determined at 450 nm using an ELISA reader (Spectra Max 340, Molecular Device Co., Sunnyvale, CA, USA). At the end of each incubation, the cellular morphologies were observed under an Olympus IX70 inverted microscope (Olympus Optical Co., Osaka, Japan).

Cell migration assay. Using a method similar to one described previously [19], in vitro migration assays were performed. In brief, RASMCs (1×10^5 cells/ml) were seeded in 4-well chambered cover-glass slide and grown to confluence overnight. Monolayers were scraped (denuded) using a 1 ml plastic micropipette tip, and EGCG was treated to the attached cells or not. As described in our previous study [20], the cells were incubated in a self-designed CO_2 mini-incubator placed on the stage of an inverted system microscope (IX70, Olympus Optical Co., Osaka, Japan) and then visualized for the migration of cells into denuded space by a CCD camera (Olympus Optical Co.) attached to the microscope. Migration of cells into denuded areas was monitored for up to 48 h. The average area covered by migrated cells was calculated by using an image-processing software (MATLAB V5.3, Mathwork Inc., Natic, MA, USA).

Cell cycle analysis. For cell cycle analysis, RASMCs, following EGCG treatment for 48 h, were collected and washed with cold phosphate-buffered saline (PBS, pH 7.2). The cells were resuspended in 95% cold methanol for 1 h at 4 °C and then centrifuged at 1100 rpm for 5 min. The resultant pellet was washed twice with cold PBS, suspended in PBS, and incubated with RNase (20 U/ml, final concentration, Sigma) at 37 °C for 30 min. Afterwards, the cells were chilled over ice for 10 min and stained with 100 μg/ml propidium iodide (Sigma) for 1 h. Not less than 10,000 cells were counted through flow cytometry (FACSCalibur, Becton–Dickinson, San Jose, CA, USA), and the data obtained were analyzed using the histogram of ModFit© software, written by Mac-App (Becton–Dickinson).

Western blotting of NF-κB protein. Following the treatment of cells with EGCG for 48 h, the medium was aspirated and the cells were washed twice with cold PBS (10 mM, pH 7.4). Ice-cold lysis buffer (100 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 5 mM DTT, 100 μM aprotinin, 20 μM leupeptin, 1% Triton X-100, 1% deoxy-cholic acid, and 0.1% SDS, pH 8.0) was added to the plates, which were then placed over ice for 10 min. The cells were scraped, and the lysate was collected in a microfuge tube and passed through a 21G needle to break up the cell aggregates. The lysate was cleared by centrifugation at 14,000*g* for 20 min at 4 °C and the supernatant (total cell lysate) was used immediately. The protein concentration was determined by the DC Bio-Rad assay using the manufacturer's protocol (Bio-Rad Laboratories, Inc., Hercules, CA). For immunoblot analysis, 35–40 μg of protein was resolved over 12.5% polyacrylamide–SDS gels and transferred to a PVDF membrane. The blot containing the transferred protein was blocked in blocking buffer (5% non-fat dried milk, 1% Tween 20; in 20 mM TBS, pH 7.6) for 1 h at room temperature followed by an incubation with affinity-purified rabbit NF-κB/p65 polyclonal antibody (Stressgen, Victoria, BC, Canada) at 1:2000 dilution in blocking buffer overnight at 4 °C. This was followed by incubation with anti-rabbit secondary antibody HRP conjugate (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and the protein expression was detected by chemiluminescence using an ECL Plus detection kit (Amersham Biosciences, Buckinghamshire, England) and autoradiography with Hyperfilm (Amersham Biosciences). Densitometric analyses were performed with a Video-Imager (Bio-Rad Laboratories, Inc.).

Statistical analysis. All variables were tested in three independent cultures for each experiment, and each experiment was repeated twice ($n = 6$). The results are reported as means ± SD compared with the non-treated controls. A one-way analysis of variance (ANOVA), which was followed by a Tukey HSD test for the multiple comparisons, was used to detect the inhibitory effects of EGCG on RASMCs. A p value < 0.05 was considered statistically significant.

Results

Inhibitory effects of EGCG on attachment and proliferation of RASCMs

To investigate the EGCG-induced inhibitory effects on FBS-stimulated RASMCs, increasing EGCG concentrations were added to the cells and after incubation their attachment and proliferation were, respectively, determined. Incubating the cells in the presence of micromolar EGCG concentrations resulted in significant ($p < 0.05$) dose-dependent decreases in RASCM attachment and proliferation. After 4 h of treatment with 200 μM EGCG, an approximate 56% decrease of cell attachment was observed with few local attachment (Fig. 1A). When the cells were treated with EGCG concentrations equal to those used in the experiments mentioned above, a significant ($p < 0.05$) dose-dependent reduction (by about 40%) of cell proliferation was observed, suggesting that the EGCG exerts potent antiproliferative activity attributed to its molecular structure with para hydroxyl and gallate groups responsible for antioxidant effects (Fig. 1B).

Inhibitory effects of EGCG on migration of RASCMs

The inhibitory effects of EGCG on RASMCs in response to FBS were then verified by performing in vitro migration assays where a small scrape was made across a confluent monolayer of RASMCs and the process of denuded area closure was monitored. In non-treated cells (Fig. 2A), recover of the scrape was essentially completed by the denuded space covered with migrated cells at 36 h after denuding. The initial denuded area of non-treated cells was $0.83 \pm 0.22\ mm^2$, and about 2% of the area remained uncovered after 36 h of incubation (Fig. 2B). On the contrary, denuded area closure of EGCG-treated cells was appreciably delayed. The denuded area remained unpopulated through 12–48 h, even though cells at the edge of the scrape showed forward movement (Fig. 2A). The first evidence of cell ingrowth did not appear until around 24 h and scrape recover was not complete until some time between 36 and 48 h after denuding. Since the doubling time for these RASMCs with FBS stimulation was about 40–44 h (our unpublished observation), the recover in non-treated cells at 36 h implied that the non-treated cells

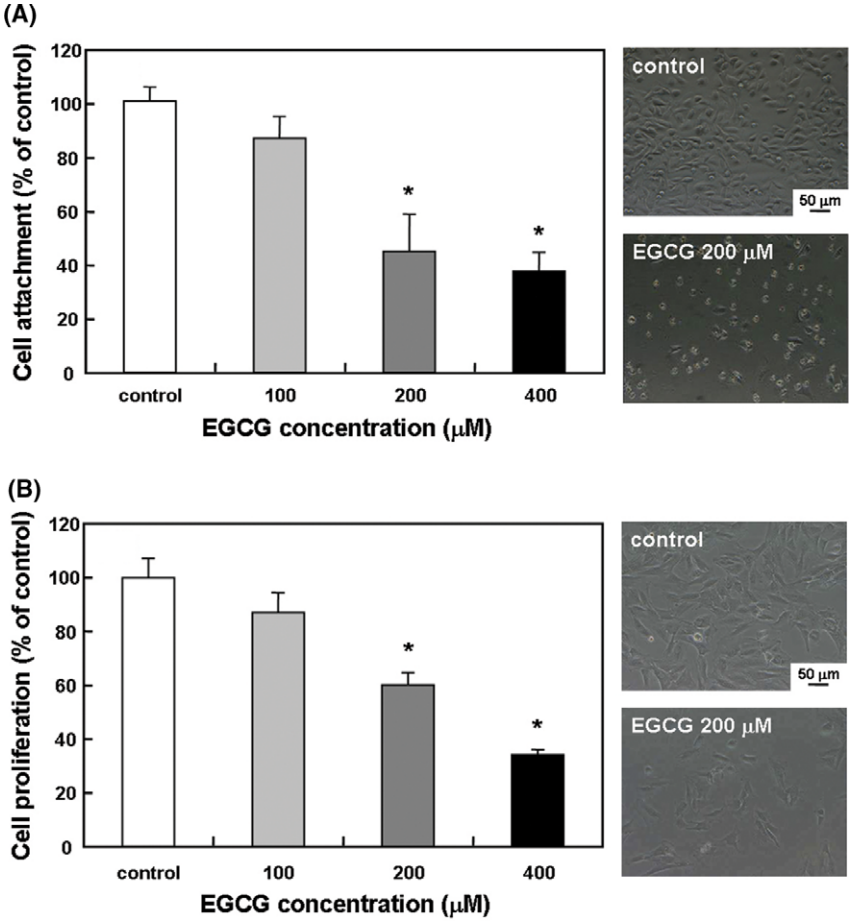

Fig. 1. Effects of EGCG on FBS-stimulated RASMC attachment and proliferation. Cells were incubated without or with EGCG (100–400 μM) for the predetermined time periods. The cell attachment (A) and proliferation (B) were, respectively, measured by WST-8 assay, as described in Materials and methods. The results are reported as means ± SD ($n = 6$). The data are analyzed by a Tukey HSD test. The values marked with asterisks are significantly different from the control (*$p < 0.05$). The photographs (100×) shown in this figure are representative of six independent experiments, showing similar results.

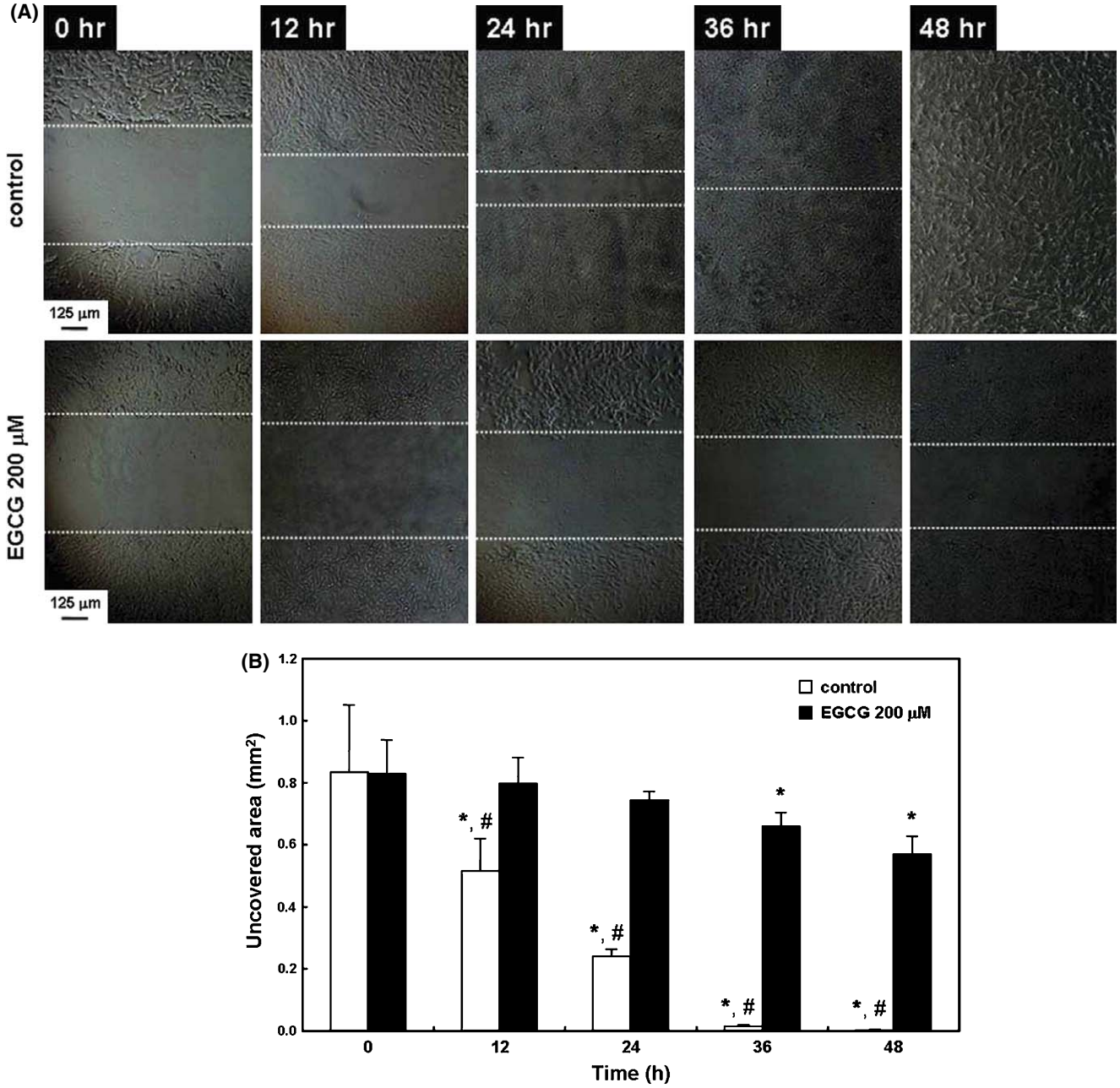

Fig. 2. Effect of EGCG on FBS-induced RASMC migration. (A) Microscopic photographs (40×) of cells migrating into denuded areas. A small scrape was made across a confluent monolayer and the process of denuded area closure was monitored for up to 48 h in the presence or absence of EGCG (200 μM). The photographs shown in this figure are representative of six independent experiments, showing similar results. (B) Uncovered area was calculated by an image-processing software, as described in Materials and methods. The results are reported as means ± SD ($n = 6$). The data are analyzed by a Tukey HSD test. The values marked with asterisks are significantly different from 0 h ($^{*}p < 0.05$). The values marked with sharps are significantly different between EGCG-treated and non-treated at the same time ($^{\#}p < 0.05$).

had extensively migrated from the edge of the scrape into the denuded space prior to the onset of cell proliferation. However, EGCG-treated cells did not show any migration prior to the onset of cell proliferation and ingrowth was delayed even after the onset of cell proliferation. The EGCG-treated cells covered only 31% of the initial scraped area even at 48 h (Fig. 2B). There was significant ($p < 0.05$) difference in the uncovered area between the non-treated and EGCG-treated cells at each time point. These phenomena would seem to be closely related to the anti-atherogenic activities of EGCG, which exhibits a strong antioxidant activity.

Effects of EGCG on cell cycle distribution in RASMCs

To investigate the effect of EGCG on cell cycle distribution, DNA cell cycle analysis was performed employing the growing RASMCs with FBS stimulation. As shown by the

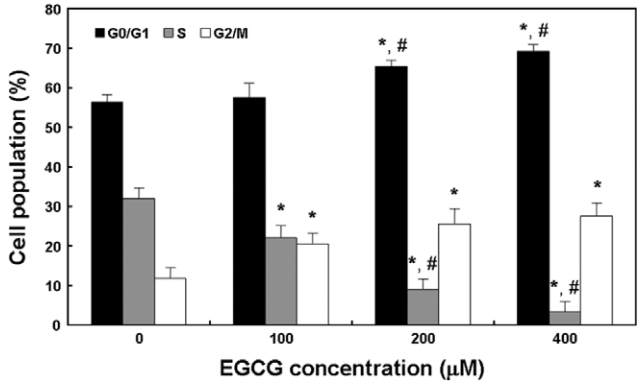

Fig. 3. Effect of EGCG on cell cycle distribution in FBS-induced RASMC. Cells were incubated without or with EGCG (100–400 μM) and analyzed by flow cytometry, as described in Materials and methods. The percentages of cells in the G0/G1-, S-, and G2/M-phases were calculated using ModFit© software with histograms. The results are reported as means ± SD ($n = 6$). The data are analyzed by a Tukey HSD test. The values marked with asterisks are significantly different from the control ($^{*}p < 0.05$). The values marked with sharps are significantly different from the cells treated with 100 μM EGCG ($^{\#}p < 0.05$).

data in Fig. 3, the EGCG treatment resulted in an appreciable increase in the G0/G1-phase of the cell cycle in RASMCs (57.5%, 65.4%, and 69.2% at 100, 200, and 400 μM, respectively), with a concomitant decrease of cell population in the S-phase. Accordingly, the RASMCs could not enter the S-phase during the EGCG treatment and EGCG might induce a G0/G1-phase arrest in the cell cycle. In this experiment, the G0/G1 cell population of controls at the specified doses did not change and ranged between 53.5% and 58.7%, probably because growing (unsynchronized) cells were employed in these experiments. Interestingly, the data also showed that EGCG resulted in an appreciable increase in the population of G2/M-phase of the cell cycle. These results imply that the increases in the number of the cells and DNA synthesis during the treatment period may be suppressed by EGCG.

Effects of EGCG on NF-κB expression in cytosol and nucleus of RASMCs

To evaluate the effects of EGCG on the constitutive NF-κB expression in both the cytosol and the nucleus in FBS-induced RASMCs, an antibody directed against the p65 subunit of NF-κB was employed. As shown in Fig. 4, EGCG treatment resulted in a significant decrease in NF-κB/p65 protein expression in the cells. In the cytosol (Fig. 4A), a very strong inhibition was observed even at the lowest dose of 100 μM, and at the higher doses (200 and 400 μM), almost complete inhibition of NF-κB expression occurred. In the nucleus of the cells (Fig. 4B) also, the immunoblot analysis demonstrated a pattern of down-modulation of NF-κB/p65 protein expression similar to that shown in the cytosol. When compared to the cytosol, a strong EGCG-mediated inhibition of NF-κB expression occurred at 200 μM in the nucleus. These results suggest that either IκB phosphorylation and degradation or later NF-κB activation and translocation may be blocked by EGCG in RASMCs.

Discussion

SMC proliferation and neo-intima formation are important events in the pathophysiological course of atherosclerosis and restenosis after balloon angioplasty. After endothelial cell activation, locally produced growth factors and cytokines mediate an inflammatory response within the vessel wall, which involves monocyte recruitment, stimulation of macrophage proliferation, migration of SMCs from the medial layer of the vessel, and finally deposition of collagen and other extracellular matrix proteins leading to the formation of a fibrous cap [21]. EGCG has been shown to have protective effects on the cardiovascular system, including anti-atherosclerotic and anti-hypercholesterolemic effects [22,23]. A number of studies have shown that EGCG inhibits SMC proliferation and hypertrophy and blocks the stimulation by serum or growth factors [24,25]. However, the inhibitory mechanism of EGCG on serum-induced SMC behaviors remains poorly, or if any partly, understood. This study concentrated on investigating the involvement of NF-κB as the mechanism of the antiproliferative and pro-apoptotic responses of EGCG against RASMCs stimulated with FBS. NF-κB is believed to be among the key players mediating the growth, proliferation, hypertrophy, migration, and survival responses of vascular cells and functions as an important mediator in the pathogenesis of vascular lesions, such as atherosclerosis [26,27].

In the present studies, it has been demonstrated that EGCG inhibits the attachment, proliferation, and migration of RASMCs and results in cell cycle arrest. Although earlier investigations in vascular or aortic SMCs had reported that EGCG exerted the antiproliferative, anti-invasive, and anti-metalloproteinase activities [6–11], similar work in RASMCs had not been conducted. The attachment and proliferation of FBS-stimulated RASMCs following EGCG treatment showed almost an identical pattern, a significant dose-dependent inhibition (Fig. 1). A number of reports have shown that these inhibitory effects of EGCG can be closely related to its molecular structure with abundant hydroxyl groups and gallate rings [28,29]. As an index of migration in FBS-stimulated RASMCs after EGCG treatment, the denuded area closure indicated that neither cell migration nor ingrowth occurred prior to and even after the onset of cell proliferation (Fig. 2). These results suggest that EGCG may be effective in inhibiting the migration and invasion of SMCs, as EGCG would appear to bind to specific sites and thus interrupt the exogenous signals required for the proliferation and growth of cells. It was also shown that EGCG led to cell cycle arrest at both G0/G1- and G2/M-phases, whereas the population of cells in the S-phase was significantly decreased in the FBS-stimulated RASMCs

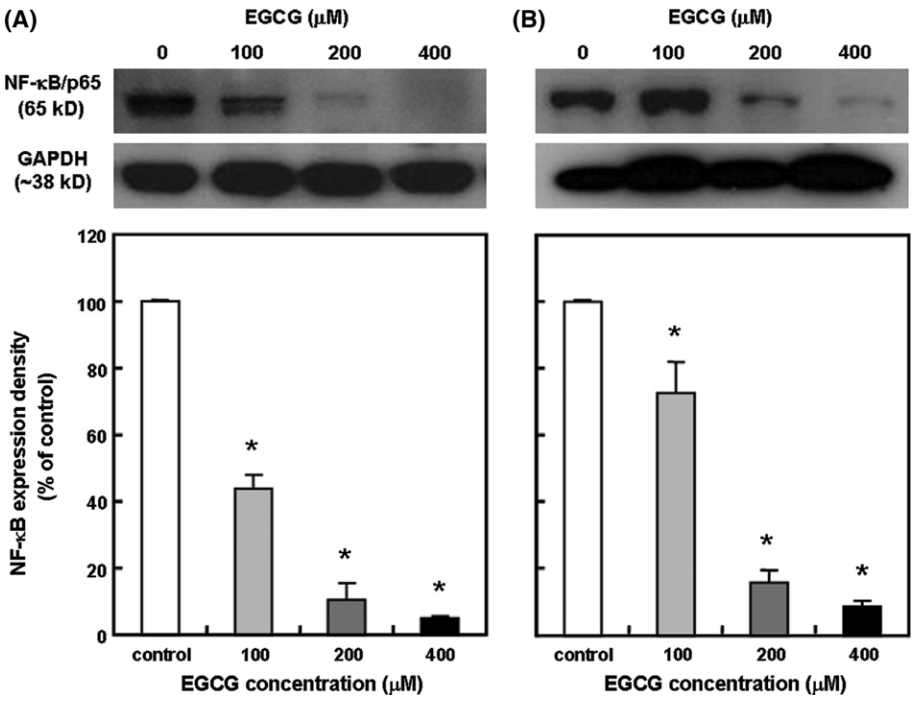

Fig. 4. Effect of EGCG on NF-κB expression in the cytosol and the nucleus of FBS-induced RASMC. NF-κB/p65 protein levels in the cytosol (A) and the nucleus (B) were detected by immunoblot analysis. Cells were incubated without or with EGCG (100–400 μM), and Western blot analysis was performed with antibodies specific for NF-κB/p65, as detailed under Materials and methods. Results from representative experiments were normalized to GAPDH expression by densitometry. The quantitative results are shown in the lower panel and indicated values are means of two triplicate experiments ($n = 6$). The data are analyzed by a Tukey HSD test. The values marked with asterisks are significantly different from the control ($^*p < 0.05$).

(Fig. 3). This is consistent with other studies showing that EGCG induces G1 cell cycle arrest in several cell lines [30–32]. There was enough evidence that the induction of a G0/G1-phase arrest in the cell cycle was due to the adsorption of EGCG or blocking at specific sites on the cellular membrane or DNA that effected the proliferation of cells and DNA replication [33,34]. Furthermore, recent study has demonstrated that expression of the metastasis-associated 67-kDa laminin receptor might confer EGCG responsiveness to cancer cells at physiologically relevant concentrations [35].

Finally, it was attempted to determine whether EGCG inhibits FBS-stimulated NF-κB expression in RASMC, because the expression of NF-κB plays important roles in the pathogenesis of arteriosclerosis and restenosis after vascular injury [25,26]. Of considerable interest in this study was the marked decrease in FBS-induced NF-κB expression in the cytosol of RASMC following treatment with EGCG as determined by an immunoblot analysis (Fig. 4A). Under the same experimental conditions, EGCG treatment also inhibited the expression of NF-κB in the nucleus (Fig. 4B). The down-modulation of NF-κB in the cytosol and the nucleus was likely due to EGCG-mediated blocking of either degradation of its cytoplasmic inhibitor IκB or its translocation to the nucleus where it binds κB target sequences, which leads to growth and migration inhibition as well as cell cycle arrest in RASMCs. Another possibility is that the down-modulation of NF-κB in both the cytosol and nucleus of RASMCs might be related to apoptotic activity of NF-κB. Many other reports have shown that the suppression of NF-κB in the cytosol and the nucleus is associated with apoptosis [31,36]. In view of these reports and our data showing an inhibition of NF-κB, it is suggested that the antiproliferative and cell cycle-arresting effects of EGCG on cells may be resulting from the down-modulation of constitutive NF-κB in the cytoplasm and the nucleus. However, it is not clear in the present study because data supporting this possibility were not included. Recent study has shown that EGCG treatment resulted in up-regulation of Bax via stabilization of p53 and a parallel down-regulation of Bcl-2 via negative regulation of NF-κB, which might ultimately initiate the activation of the caspase cascade leading to apoptosis in prostate carcinoma LNCaP cells [37]. On the contrary, EGCG has been shown to induce NF-κB by up-regulating the MAPK signaling pathway in proliferating VSMCs, which leads to apoptosis of them via activation of p53 [38]. The effects of EGCG on NF-κB activity appear cell-type specific and likely reflect differences in intersecting signaling pathways [39].

Although much attention has been paid to the inhibitory effect of EGCG on serum-induced SMCs, the molecular and cellular mechanisms underlying the inhibition by EGCG in FBS-stimulated RASMCs remain to be examined. Additionally, multiple cytosolic signals such as cyclic AMP, MAPKs, and ERK are involved in the regulation of SMC growth [40,41]. These multiple cytosolic signals might converge on NF-κB [26,42]. Collectively,

the findings provide support to a scenario in which the suppression of NF-κB expression plays a key role in the inhibition of proliferation and migration as well as G0/G1 arrest of cell cycle, in response to EGCG in RASMC. However, a limitation in this study is that the post-transcriptional mechanism of NF-κB on EGCG-treated RASMC has not been clarified. Furthermore, these results do not rule out the possibility of other transcription factors in the EGCG-mediated inhibition of SMCs induced by various stimuli and the exact mechanism needs to be elucidated.

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