Epidermal Growth Factor Induction of Phenotype-dependent Cell Cycle Arrest in Vascular Smooth Muscle Cells Is through the Mitogen-activated Protein Kinase Pathway*

Received for publication, September 2, 2003, and in revised form, September 30, 2003
Published, JBC Papers in Press, October 9, 2003, DOI 10.1074/jbc.M3099640200

Yu Gui and Xi-Long Zheng‡
From the Smooth Muscle Research Group, Department of Biochemistry & Molecular Biology, The University of Calgary, Calgary, Alberta T2N 4N1, Canada

The heterogeneity of vascular smooth muscle cells is well established in tissue culture, but their differential responses to growth factors are not completely defined. We wished to identify effects of epidermal growth factor (EGF) on vascular smooth muscle cells in distinct phenotypes, such as spindle and epithelioid. We found that the EGF receptors were abundant in epithelioid cells but not spindle cells. EGF treatment inhibited serum-independent DNA synthesis, which was absent in spindle cells, of epithelioid cells. Additionally, using a pulse-chase assay, we found that bromodeoxyuridine-labeled cells failed to re-enter the S phase in the presence of EGF. These EGF effects were abolished by either inhibiting the EGF receptor tyrosine kinase with AG1478 or inhibiting the mitogen-activated protein kinase pathway with PD98059. In response to treatment with EGF, the EGF receptor was phosphorylated, which was correlated with phosphorylation and activation of p42/44 mitogen-activated protein kinases. Inhibition of EGF receptor phosphorylation and mitogen-activated protein kinase activation resulted in a reversal of the EGF-induced inhibition of bromodeoxyuridine incorporation and cell cycle arrest. Subsequent studies revealed that the activation of the EGF receptor and the mitogen-activated protein kinase pathway in epithelioid cells induced expression of the cell cycle inhibitory protein p27Kip1 but not p21Cip1. Taken together, our data demonstrate that the EGF receptor is abundantly expressed in epithelioid vascular smooth muscle cells and that the activation of this receptor results in cell cycle arrest through activation of the mitogen-activated protein kinase pathway.

The proliferation of vascular smooth muscle cells (VSMCs) plays an important role in vascular diseases, including atherosclerosis and restenosis after angioplasty (1). It is increasingly clear that the blood vessel wall contains heterogeneous smooth muscle cells (2–4). The heterogeneity of arterial VSMCs has been described in many species, such as rats (5), mice (6), dogs (7), pigs (8), and humans (9). During tissue culture, these cells display at least two different morphologies: spindle and epithelioid. Both cell types not only contrast themselves in vitro but also in vivo (2). One main difference, in addition to morphology, is that epithelioid VSMCs have serum-independent growth properties. It has been proposed that epithelioid VSMCs may be the cell type resulting in the formation of intimal thickening in vivo (2). Therefore, we wished to characterize the differential responses of these cells to growth factors. Specifically, we were interested in assessing the effects of epidermal growth factor (EGF), which has been identified in a wide range of biological fluids including blood, and its blood concentration is associated with platelet coagulation (10, 11). Moreover, we and others have found that EGF stimulates contraction of smooth muscle (12, 13). Importantly, we identified that EGF has differential intracellular signaling mechanisms in longitudinal and circular gastric smooth muscle (14). In cultured VSMCs, several studies have shown that the direct or indirect activation of EGF receptor stimulates proliferation of cultured VSMCs (15–17). However, we observed that EGF induces apolipoprotein A1 gene expression in human hepatoma Hep G2 cells (18) and inhibits serum-independent growth of these cells. The EGF inhibition of cell growth has also been found in several other cell systems, such as MDA468 human breast cancer cells (19, 20), TE8 esophageal cancer cells (21), and A431 human epidermoid carcinoma cells (22–24). Therefore, we hypothesized that EGF may differentially affect VSMCs in distinct phenotypes.

Activation of EGF receptor with intrinsic tyrosine kinase activity is known to trigger various intracellular signaling pathways for different cell responses (25–27). The well-characterized pathway would be the mitogen-activated protein kinase (MAPK) pathway (28). Although activation of the MAPK pathway may stimulate cell growth or activate the cell cycle, increasing evidence suggests that stimulation of this pathway inhibits cell proliferation or causes cell cycle arrest of even VSMCs (29, 30). Therefore, we speculated that EGF may phenotype-specifically regulate the cell cycle through activation of the MAPK pathway. In this report, we present data showing that the EGF receptor (EGFR) is abundantly expressed in epithelioid smooth muscle cells and that EGF stimulation activates the MAPK pathway and induces cell cycle arrest of epithelioid VSMCs.

© 2003 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.
EXPERIMENTAL PROCEDURES

Materials—Human recombinant EGF was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). AG1478, PD98059, bromodeoxyuridine (BrdUrd), propidium iodide (PI), mouse monoclonal antibodies for smooth muscle α-actin, and α-tubulin were obtained from Sigma. Anti-MAPK was acquired from Calbiochem. Anti-MAPK (phospho-specific), MAPK assay kit, anti-EGFR, and phospho-specific EGFR antibodies were from Cell Signaling Technology. Anti-p27Kip1 and anti-p21Cip1 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Primary Cell Culture, Cell Cloning, and Treatment—Primary culture of VSMCs was carried out using an explant method as described (31). Briefly, the thoracic aortic tissue derived from male Sprague-Dawley rats (250–300 g) was cut into small pieces (1 mm³) and placed on the plastic surface of culture dishes. The tissue explants were maintained in RPMI 1640 medium containing 10% (v/v) fetal calf serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). The culture was incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The culture medium was changed twice a week during the first 2 weeks, and the cells were usually observed to migrate from tissues between days 4 and 7 after explanting. The islets of outgrowing cells were then trypsinized with a 0.25% trypsin, 0.25 mM EDTA solution (Invitrogen), followed by subculture. The cells were then characterized by detecting the expression of several differentiation marker proteins, such as smooth muscle α-actin and h1-calponin. The cells from passages 6–8 were used for cloning purpose. Spindle and epithelioid VSMCs were cloned using a dilution cloning method as described (32). The cloned cells were then examined for typical morphologies and serum dependence of cell growth. For cell starvation, the cultured cells were maintained in RPMI 1640 medium containing 0.5% serum for 2 days. The cells were treated with EGF (10 ng/ml) for the time indicated in the figure legends. For the experiments with pharmacological inhibitors, all of the inhibitors were administered into the medium 30 min before the addition of EGF.

Staining for Incorporated BrdUrd in Nuclei—VSMCs cultured on coverslips were labeled with BrdUrd (10 µM), a thymidine analog, at 37 °C for 60 min. After being washed twice with PBS, the cells were fixed with 80% ethanol for 20 min and 100% ethanol for another 20 min. The cells were then permeablized with 0.25% Triton X-100 in PBS for 30 min. The cells were blocked with PBS containing 0.5% bovine serum albumin and 0.1% Tween 20 for 20 min, followed by incubation with anti-BrdUrd antibodies, anti-mouse, and anti-rabbit secondary antibodies labeled with Alexa Fluor 647 (Molecular Probes). After secondary antibody incubation (40 min), the cells were incubated with PBS containing PI (5 µg/ml) for 20 min. The coverslips were mounted to microscopic slides using 10 µl of mounting solution (75% glycerol, 25% PBS with 5 µg/ml PI) and sealed with nail polish, followed by laser scanning cytometry (LSC) analysis.

Laser Scanning Cytometry—DNA analysis and BrdUrd incorporation in response to various treatments were performed with LSC (CompuCyte, Cambridge, MA) as previously described (34). Briefly, the cell monolayers were grown on coverslips and immunostained with anti-BrdUrd antibody and the Alexa Fluor 647-conjugated secondary antibody, followed by counterstaining with PI for nuclear DNA as described above. The cells mounted on microscopic slides were located visually with a CCD camera attached to the LSC instrument. Detailed information for LSC methodology has been published elsewhere (34, 35). The
slides were scanned under 20× objectives using both argon and helium lasers to excite the PI and Alexa Fluor 647 fluorochromes. Each slide was scanned randomly for three fields, and at least 1000 cells in each field were scanned. The scanning data were acquired and analyzed with WinCyte acquisition software (Version 3.4, CompuCyte). Multiple data parameters were displayed in a series of data windows through software. We contoured each nucleus as a single event through PI staining as described previously (34, 35). All of the scanned cells were used to generate the DNA histograms and the scattergrams for BrdUrd incorporation. The BrdUrd-positive cells were shown to have higher total fluorescence in their nuclei as demonstrated in the scattergram of total fluorescent intensity (Alexa Fluor 647 Integral) versus total DNA content (PI Integral). Because DNA contents reflect the positions of the cell cycle, we were able to observe cells in different cell cycle phases by detecting their DNA contents. Therefore, the cells, after being labeled with BrdUrd, could be monitored for their movement in the cell cycle by detecting the incorporated BrdUrd.

The BrdUrd Pulse-Chase Assay—VSMCs were grown on coverslips to a confluence of ~70% using the RPMI medium containing 10% serum. The cells were then starved for 2 days using the medium containing 0.5% serum, followed by a pulse labeling with BrdUrd (10 μM) for 60 min at 37°C in a CO2 incubator (36, 37). At this time point, the cells in the S phase, which actively synthesize DNA, would become BrdUrd-positive. Thereafter, the medium was aspirated, and the cells were washed three times with sterile PBS. The cells were then cultured with fresh medium with 0.5% serum in the presence or absence of EGF (10 ng/ml). Every 2 h up to 24 h, a fraction of cells with or without treatment with EGF was removed and fixed with 80% ethanol and stored at −20°C. The fixed cells were subsequently processed for immunostaining and detection of BrdUrd using LSC as described. In the interval between the BrdUrd labeling and sampling, the BrdUrd-labeled cells under a normal cell cycle would progress through the S and G2/M phases into the G0/G1 phase of the next generation. Therefore, this set of experiments would demonstrate the cell cycle movement of these BrdUrd-labeled cells in the presence or absence of EGF.

Determination of Cell Growth—Both spindle and epithelioid cells were maintained in 10% serum. The cells were trypsinized, counted to determine cell concentration, and seeded in 35-mm Petri dishes with 0.5 × 10^5 cells in each dish with medium containing 10% serum for 24 h. The cells were then cultured with the medium containing 0.5% serum for 2 days, followed by harvesting and counting cells as day 0. Three dishes of cells from both spindle and epithelioid cells were harvested for cell count determination using a hemocytometer every day up to day 5.

Western Blotting—VSMCs grown in 100-mm Petri dishes were washed with PBS and lysated with 1 ml of ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2.5 mM EDTA, 2.5 mM EGTA, 10 mM β-mercaptoethanol, 1 mM NaF, 0.1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). The cell lysate was then passed through a 20-gauge needle three times, and cellular debris was removed by centrifugation (10,000 × g for 10 min). Supernatants were collected for protein measurement by the Bradford assay. Equal amount of protein of each sample (40 μg) was separated by 11% SDS-PAGE gel and transferred to nitrocellulose membrane. Antibodies against MAPK, phospho-specific MAPK, p27<sup>kip1</sup>, and p21<sup>cip1</sup> at 1:2000 dilution were used as primary antibodies to detect respective proteins. To detect EGFR, cell lysate protein of 60 μg from each sample was loaded for separation on SDS-PAGE gel, and antibodies (1:1000 dilution) against total EGFR, phospho-EGFR (Tyr<sup>1068</sup>), phospho-EGFR (Tyr<sup>1084</sup>), and phospho-EGFR (Tyr<sup>1145</sup>) (Cell Signaling Technology) were applied, respectively. Horseradish peroxidase-coupled secondary antibodies (1:2500 dilution) were then used, and peroxidase activity was detected using an ECL detection kit (Amersham Biosciences) as previously described (18).

MAPK Activity Assay—MAPK activity was analyzed with the p44/42 MAPK assay kit (Cell Signaling Technology) according to the manufacturer’s recommendations. Briefly, cultured cells were treated with and without EGF (10 ng/ml) for various times followed by extraction of protein from cell lysates as described in Western blotting. Equal amounts of protein (200 μg) from each cell lysate sample were incubated with 15 μl of immobilized phospho-p44/42 MAPK (Thr<sup>202</sup>/Tyr<sup>204</sup>) monoclonal antibody with gentle rocking at 4°C overnight. The immune complex was collected by centrifugation at 8000 × g for 30 s, followed by a wash with lysis buffer, twice with kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiobiotol, 0.1 mM Na3VO4, and 10 mM MgCl2). The complex was then incubated with 50 μl of kinase buffer containing 200 μM ATP and 2 μg of EIK-1 fusion protein at 30°C for 30 min. The reaction was terminated by adding 12.5 μl of 5× SDS sample buffer. The samples were boiled for 5 min and briefly microcentrifuged. Equal volumes (25 μl) of the supernatant from each sample were separated on 11% SDS-PAGE gel for Western immunoblot detection with phospho-EIK-1 antibody (dilution 1:1000). The second antibody was coupled to horseradish peroxidase, which was detected by chemiluminescence using a Phototop<sup>®</sup> horseradish peroxidase Western detection kit (Cell Signaling Technology).

Data Analysis—The data are expressed as the means ± S.E. The number of replicates (n) represents the number of cell samples used in the studies. The differences between the means were evaluated by the Student’s t test (paired or independent) when two groups were compared and by analysis of variance followed by Bonferroni’s correction when three or more groups were compared. p < 0.05 or p < 0.01 was considered significant. All of the statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS, version 10, Chicago, IL).

RESULTS

EGF Inhibition of Serum-independent Growth of Epithelioid VSMCs—From primary cultured rat aortic VSMCs, we had cloned both spindle and epithelioid VSMCs. Consistent with the findings by other laboratories (2, 3, 5), spindle and epithelioid VSMCs not only displayed distinct morphologies (Fig. 1, A and B) but also demonstrated differential growth properties. In
FIG. 3. EGF induction of cell cycle arrest in epithelioid VSMCs. The cells, after starvation for 2 days, were pulse-labeled with BrdUrd for 60 min, followed by culturing with fresh medium containing 0.5% serum without (A, C, E, and G) or with (B, D, F, and H) EGF (10 ng/ml) treatments for the indicated time, followed by fixation and detection of BrdUrd-positive cells as described under “Experimental Procedures.” BrdUrd-positive cells are shown in red in the upper panels of quadrants of the scattergrams for BrdUrd incorporation (Alexa Fluor 647 integral) versus PI integral (total DNA in each cell). The inset in each panel (A–H) shows the DNA histogram of scanned cells, in which region 1 indicates the G₀/G₁ phase. The data represent three independent experiments. I shows the percentage of BrdUrd-positive cells that re-enter the S phase (cells in quadrant region 2) compared with the total BrdUrd-positive cells (cells in both quadrant regions 1 and 2). The asterisk indicates that the percentage of epithelioid VSMCs re-entering the S phase in the presence of EGF is significantly lower compared with the control (n = 3, p < 0.01).
the presence of 0.5% serum for 2 days, very few spindle cells underwent DNA synthesis (Fig. 1, C and D). In response to 60 min of BrdUrd labeling, only ~5% of cells were BrdUrd-positive, as shown with red in the upper two quadrants of Fig. 1D. In contrast, the epithelioid cells had up to 20% BrdUrd incorporation rate in the same conditions (Fig. 1, E and F). Consistently, in the presence of 0.5% serum, the cell number of spindle cells increased slightly, but epithelioid cells replicated themselves with a significant increase in cell number (Fig. 1G). In keeping with the findings by others (5, 7, 8, 38), we also observed that in epithelioid VSMCs the smooth muscle differentiation marker protein, smooth muscle α-actin, was much lower than that in spindle cells (Fig. 1G, inset). Taking advantage of those two cell systems, we wished to examine the effect of EGF on the smooth muscle cell cycle. To do so, we first examined the expression of EGFRs in these two cell subtypes. As detected by Western blot (Fig. 2A), the expression of EGFRs in epithelioid cells was significantly higher than that in spindle cells. However, there was no significant difference in the abundance of α-tubulin protein detected (Fig. 2A). Based on this finding, we hypothesized that these two VSMC subtypes might respond to EGF differently. As expected, treatment with EGF (10 ng/ml) for 48 h significantly reduced the serum-independent BrdUrd incorporation rate in epithelioid cells (Fig. 2B). However, EGF neither stimulated nor inhibited the BrdUrd incorporation of spindle cells. This might be due to a lower expression of EGFR in these VSMCs. Although we observed significant inhibition of BrdUrd incorporation by EGF in epithelioid cells, we did not observe inhibition of cell proliferation in the presence of EGF for several days compared with the control epithelioid cells without EGF treatment (data not shown). This result was likely due to the presence of apoptosis in the control cells and the antiapoptotic effect of EGF. Nevertheless, we wished to further characterize the inhibitory effect of EGF on cell cycle progression in the presence of 0.5% serum.

**Cell Cycle Arrest of Epithelioid VSMCs by EGF**—Because we found that epithelioid VSMCs actively synthesized DNA in the starvation medium containing 0.5% serum and that the treatment with EGF inhibited DNA synthesis but did not inhibit cell proliferation, we wanted to examine whether the epithelioid VSMCs indeed underwent a normal cell cycle with cell division and whether EGF inhibited cell cycling under the same conditions. We labeled the S phase cells, which were actively synthesizing DNA, with BrdUrd (10 μM) for 60 min so that these BrdUrd-positive cells, when entering another phase of the cell cycle, could be detected through the immunostaining using monoclonal antibody against BrdUrd as described under “Experimental Procedures.” In the scattergrams of the BrdUrd incorporation, the cells in quadrant regions 1 and 2 were defined as BrdUrd-positive (Fig. 3, red). According to the DNA content and cell distribution pattern in the DNA histogram (Fig. 3, right panels of A and B), cells in either quadrant region 1 or 3 were considered in the G0/G1 phase, and those in quadrant region 2 or 4 were considered in the S and G2/M phases. One of cell cycle features of epithelioid cells in the presence of 0.5% serum was that there were few cells in the G2/M phase (~3% of total cells). The positions of the BrdUrd-positive cells were displayed in red in the scattergrams and the DNA histograms (Fig. 3, A and B, right panels, or C–H, insets) through WinCyte software (Version 3.4, CompuCyte). Right after pulse labeling (0 h), as anticipated, the BrdUrd-positive cells were typically located in the S phase (Fig. 3, right panels of A and B). These labeled cells were then maintained in culture and observed for their cell cycle positions at different time points in the presence (Fig. 3, D, F, and H) or absence (Fig. 3, C, E, and G) of EGF (10 ng/ml). Using this BrdUrd pulse-chase assay as described under “Experimental Procedures,” we found that the BrdUrd-positive cells (as shown in red) exited the S phase and moved to the G2/M phase after ~6 h (Fig. 3, C and D). Some of these labeled cells underwent cell division to produce daughter cells, which could be recognized through their location in the G0/G1 phase as shown in quadrant region 1 of panel C or D of Fig. 3, and in region-1 (the G0/G1 peak) of the DNA histograms in the insets of panels C and D. This location change of the BrdUrd-positive cells was more significant after 12 h with and without the presence of EGF. After 12 h, most BrdUrd-positive cells (red) were located in the G0/G1 phase as shown in quadrant region 1 of the scattergrams and region 1 of the DNA histograms (Fig. 3, E and F). At this time point, we observed some BrdUrd-positive cells (red) started to appear in quadrant region 2 or re-enter the cell cycle in the absence of EGF (Fig. 3E) but not in the presence of EGF (Fig. 3E).

More significantly, at the time point of 24 h, we noticed that ~30% of these BrdUrd-positive daughter cells re-entered the S phase of the cell cycle in the absence of EGF (Fig. 3G). However, in the presence of EGF, only a few of cells were observed to re-enter the cell cycle (Fig. 3H). The data from the control cells suggested that epithelioid cells indeed underwent cell division or cell cycling in the starvation medium containing 0.5% serum. In contrast, in the presence of EGF, the BrdUrd-positive daughter cells failed to re-enter the S phase, as indicated by few BrdUrd-positive daughter cells in quadrant region 2 after 24 h (Fig. 3H), suggesting cell cycle arrest. The accumulated data were shown as the percentages of the BrdUrd-positive cells re-entering the S phase or the cell cycle in quadrant region 2 with and without the presence of EGF (Fig. 3I). All of these data demonstrated that EGF, in the presence of 0.5% serum, induced cell cycle arrest of epithelioid VSMCs in the G0/G1 phase. Next, we wished to identify the underlying signaling mechanisms.

**Inhibition of EGF Receptor Activation Reversed the EGF-induced Cell Cycle Arrest**—To examine whether EGF-induced cell cycle inhibition of epithelioid VSMCs was through activation of the EGFR, we first tested the effect of an inhibitor for EGFR tyrosine kinase, AG1478. In the presence of AG1478 (5 μM), the EGF-induced reduction in BrdUrd incorporation was completely inhibited (Fig. 4A). After these pharmacological studies, we wished to further characterize the roles of EGFRs in this cell system. Because activation of the EGFR involves receptor dimerization and autophosphorylation, tyrosine phosphorylation of EGFRs in response to EGF will indicate the activation of this receptor. Therefore, we detected phosphorylated EGFR and total EGFR in response to EGF (10 ng/ml) stimulation using specific antibodies (Fig. 4B). Our results showed that EGF stimulation increased phosphorylation of EGFR at tyrosine residue 1068 (Y1068), with the maximal phosphorylation occurring at 10 min (Fig. 4B). We also observed that the total amount of EGFR was reduced in response to EGF treatment as shown in the immunoblotting results (Fig. 4B, inset), which was likely due to its rapid degradation after phosphorylation and/or internalization. However, we did not detect increased phosphorylation of EGFR at tyrosine residues 864, 992, and 1045 using phospho-specific antibodies for EGFR (data not shown). In addition, the presence of AG1478 completely inhibited the phosphorylation of EGFR in response to EGF treatment for 10 min (Fig. 4C).

**The MAPK Pathway Is Involved in the EGF Reduction of BrdUrd Incorporation**—Because several intracellular signaling pathways, such as the PI3-kinase pathway, the protein kinase C, and the Ras-MAPK pathway, can be activated in response to EGFR activation, we wished to identify which pathway was involved. We examined the effects of LY294002 and wortmannin (for PI3-kinase), CP118,556/PP1 (for nonreceptor tyrosine kinase, Src), GF109203X (for protein kinase C),
show that AG 1478 inhibited the EGF-induced increase in phosphorylation of EGFR.

The phosphorylation of EGF receptor (Tyr1068) stimulated by EGF treatment. The cells were treated with EGF for the indicated time (the x axis), and same amount of total cell lysate protein (60 μg) from each time point were subjected to Western blot analysis using anti-EGFR and a phospho-specific antibody against phosphorylated EGFR (Tyr1068), respectively. The y axis indicates the fold increase of phosphorylation compared with the control at 0 min. The data represent the mean values of densitometry measurements from four independent experiments. The asterisk indicates a significant difference when compared with the control at 0 min (p < 0.01). The insets present Western blot results for EGFR (upper panels) and phosphorylated EGFR (Tyr1068) (lower panels) in response to EGF treatment at the indicated time. A, inhibition of EGFR phosphorylation by AG1478. The cells were treated with EGF for 10 min in the presence or absence of AG1478, and phosphorylated EGFR were detected in the cell lysates. The representative Western blot results (inset) and mean data (n = 6, p < 0.01) of densitometry measurements (histograms) show that AG 1478 inhibited the EGF-induced increase in phosphorylation of EGFR.

and PD98059 (for MEK, MAPK kinase) on EGF inhibition of cell cycle progression. We found that PD98059, but not the other inhibitors, completely blocked the EGF-stimulated reduction in BrdUrd incorporation (Fig. 5A). Therefore, these data suggested that the activation of MAPKs might be required for EGF inhibition of the cell cycle in epithelioid VSMCs. We next examined the activation of the MAPKs in response to EGF in this epithelioid cell system and further characterized the role of MAPKs in EGF responses.

Time-dependent Activation of MAPKs in Epithelioid Cells by EGF Stimulation—To characterize the activation of MAPKs in response to EGF treatment, we first detected phosphorylated MAPKs with phospho-specific antibody. As shown by a representative Western blot in Fig. 5B, in response to EGF (10 ng/ml) treatment, phosphorylated MAPKs were markedly increased in a time-dependent manner during the first 15 min. The phosphorylation of MAPKs reached the maximal level at ∼15 min (Fig. 5B). In the presence of PD98059 (20 μM), a MEK inhibitor, the phosphorylation of MAPKs in response to EGF treatment for 15 min was completely inhibited (Fig. 5B). We next measured the MAPK activity using a nonradioactive kinase assay with Elk1 as a substrate. Consistent with the patterns of phosphorylation of MAPKs, the activity of MAPKs were also time-dependently increased in response to EGF treatment with the maximal increase at 15 min (Fig. 5C). The MAPK activity dramatically decreased after 30 min but was still maintained above the basal level after several hours. The addition of the MEK inhibitor, PD98059, which inhibited the phosphorylation of MAPKs and reversed the EGF inhibition of BrdUrd incorporation, completely blocked the increase in MAPK activities. All of these data clearly demonstrated that the MAPK pathway was involved in the EGF-induced cell cycle arrest. Because of its transient activation of this pathway, it is likely that EGF might induce expression of some other gene product(s) that could subsequently exert an inhibitory effect on the cell cycle. Given that cell cycle inhibitory proteins p21Cip1 and p27Kip1 have been identified to play critical roles in cell cycle regulation of VSMCs (39, 40), we hypothesized that in response to the activation of the MAPK pathway, p21Cip1 and/or p27Kip1 might be induced in the presence of EGF.

Induction of Cell Cycle Inhibitory protein p27Kip1 by EGF Treatment—To investigate further the underlying mechanism for cell cycle arrest by EGF and the activation of the MAPK pathway, we examined whether the activation of the MAPK cascade stimulated the expression of cell cycle inhibitory proteins, specifically, p21Cip1 and p27Kip1, using Western blotting analysis. We found that the treatment of epithelioid VSMCs with EGF caused an ∼5-fold increase in p27Kip1 (Fig. 6). Importantly, the induction of p27Kip1 was completely inhibited by the presence of either the EGFR inhibitor, AG1478, or the MEK inhibitor, PD98059 (Fig. 6). However, the expression of p21Cip1, which was barely detected from the same amount of total cell lysate protein as that for p27Kip1 detection, did not increase in response to treatment with EGF (data not shown).

**DISCUSSION**

The novel finding in this report is that EGF selectively inhibits the cell cycle of epithelioid VSMCs. Our studies to analyze the underlying mechanisms have revealed that the EGFR is abundant in epithelioid cells but not in spindle cells, and the activation of EGFRs and the MAPK pathway contributes to the EGF-induced cell cycle arrest in epithelioid cells. Another novel finding
is that in response to EGF and MAPK activation, the cell cycle inhibitory protein \( p27^{\text{Kip1}} \) is induced, which likely contributes to cell cycle arrest. All of these data have provided a novel model for the actions of growth factors, which prompts us to re-evaluate the effects of growth factors on VSMCs with the consideration of VSMC phenotypic heterogeneity.

Our conclusion that EGF inhibits cell cycle progression of epithelioid cells in the presence of 0.5% serum is based on the following evidence. First, the presence of EGF significantly inhibits DNA synthesis as detected by BrdUrd incorporation using LSC (Fig. 1), suggesting the inhibition of the cell cycle. However, in our studies, we observed that in the presence of EGF for several days, cell number was slightly increased compared with the control cells in the absence of EGF. This might result from an inhibition of apoptosis by the presence of EGF, because serum starvation could induce smooth muscle apoptosis, and EGF has been observed to inhibit apoptosis in other cells (41, 42). To prove this in epithelioid VSMCs, more studies are warranted. Second, we have directly observed using LSC in a pulse-chase assay that in the presence of EGF, BrdUrd-labeled epithelioid VSMCs were able to exit the S phase and undergo mitosis. However, unlike those from the control cells, the daughter cells in the presence of EGF were unable to re-enter the S phase after 24 h. Therefore, cells have been arrested in the G0/1 phase (Fig. 3). This result has been further supported by our observation that EGF treatment induced expression of the cell cycle inhibitory protein \( p27^{\text{Kip1}} \).

We established in our system that the signaling mechanism for the EGF-induced cell cycle arrest is through the EGFR and MAPK pathway. First, we found that EGFR is abundant in epithelioid VSMCs, but very low abundance of EGFRs is de-

![PD98059, an MEK inhibitor, blocked EGF-induced reduction in BrdUrd incorporation and MAPK activity.](image)

**Fig. 5.** PD98059, an MEK inhibitor, blocked EGF-induced reduction in BrdUrd incorporation and MAPK activity. A, epithelioid cells, stimulated with EGF with or without the presence of PD98059 for 2 days, were analyzed for BrdUrd incorporation as described under “Experimental Procedures.” In the presence of PD98059 (20 \( \mu \)M), the EGF-induced reduction in the BrdUrd-positive cells was completely reversed. The asterisk indicates a significant difference between the EGF-treated groups and the groups treated with both EGF and PD98059 (\( p < 0.05, n = 6 \)). B, epithelioid cells were treated with EGF for the indicated time. Total cell lysates (40 \( \mu \)g of protein) were separated by 11% SDS-PAGE and immunoblotted for total MAPKs (p44/p42) and phosphorylated MAPKs (phospho-p44/p42). PD98059 + EGF, the cells were pretreated with PD98059 for 30 min before addition of EGF for 15 min. The data represent similar Western blot results from four experiments. C, the MAPK activities stimulated by EGF at various time points were measured using Elk1 as a substrate and detected with phospho-Elk1 antibody, as described under “Experimental Procedures.” A typical activity assay result is shown in the inset. The accumulated data are shown as a histogram, in which the \( x \)-axis shows MAPK activities (% of control), and the times are indicated on the \( x \)-axis. The asterisk indicates a significant difference when compared with the control at 0 min (\( p < 0.01 \)). The double asterisks indicate that MAPK activity in response to EGF (15 min) was significantly reduced by the presence of PD98059.

![EGF induction of cell cycle inhibitory protein p27Kip1 in epithelioid cells.](image)

**Fig. 6.** EGF induction of cell cycle inhibitory protein \( p27^{\text{Kip1}} \) in epithelioid cells. Epithelioid cells, treated without EGF or with EGF in the presence or absence of AG1478 (5 \( \mu \)M) or PD98059 (20 \( \mu \)M) for 2 days, were analyzed for protein expression of \( p27^{\text{Kip1}} \) by Western blot (inset). The histograms show \( p27^{\text{Kip1}} \) expression levels determined by densitometry analysis. The asterisk indicates a significant difference in \( p27^{\text{Kip1}} \) expression stimulated by EGF compared with the control (\( p < 0.01, n = 4 \)). The double asterisks indicate \( p27^{\text{Kip1}} \) expression in response to EGF is significantly reduced by the presence of either AG1478 or PD98059 (\( p < 0.01, n = 4 \)).
EGF Inhibits the Cell Cycle of VSMCs

EGF Inhibits the Cell Cycle of VSMCs

Finally, previous studies by others showed EGF stimulation of cell growth (15–17), which may not necessarily contradict with our findings. As mentioned earlier, we also observed that incubation of cells with EGF slightly increased cell number compared with the controls. Another important difference is that homogenous cell populations have been used in our studies. The mixed population of VSMCs may result in differential cell responses to EGF or other growth factors, but further investigations are necessary.

In summary, our data have provided a novel model for the actions of EGF and potentially other growth factors in vascular smooth muscle. To precisely analyze the effect of growth factors on VSMCs, cell heterogeneity must be taken into consideration.

Acknowledgments—We are grateful to Dr. Michael Walsh for providing us with a laser scanning cytometer through Canada Research Chairs Fund (Tier I).

REFERENCES

1. Ross, R. (1993) Nature 362, 801–809
2. Hao, H., Gabbiani, G., and Bochaton-Piallat, M. L. (2003) Arterioscler. Thromb. Vasc. Biol. 23, 152–159
3. Orlandi, A., Ehrlich, H. P., Ropraz, P., Spagnoli, L. G., and Gabbiani, G. (1994) Arterioscler. Thromb. 14, 982–989
4. Walker, L. N., Bowen-Pope, D. F., Ross, R., and Reidy, M. A. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 7311–7315
5. Bochaton-Piallat, M. L., Ropraz, P., Gabbiani, F., and Gabbiani, G. (1996) Arterioscler. Thromb. Vasc. Biol. 16, 815–820
6. Ehler, E., Jat, P. S., Noble, M. D., Citi, S., and Draeger, A. (1995) Circulation 92, 3258–3265
7. Holfield, B., Helgason, T., Jennekens, S., Taylor, A., Navran, S., Allen, J., and Seidel, C. (1996) J. Clin. Invest. 97, 814–825
8. Hao, H., Ropraz, P., Verin, V., Camenzind, E., Geinzo, A., Pepper, M. S., Gabbiani, G., and Bochaton-Piallat, M. L. (2002) Arterioscler. Thromb. Vasc. Biol. 22, 1093–1099
9. Li, S., Fan, Y. S., Chow, H. L., Van Den, D. C., Van, D., V, Sims, S. M., and Pickering, J. G. (2001) Circ. Res. 89, 517–525
10. Nexo, E., Jorgensen, E., and Hansen, M. R. (1992) Regul. Pept. 42, 75–84
11. Oka, Y., and Orth, D. N. (1983) J. Clin. Invest. 72, 249–259
12. Berk, B. C., Brock, T. A., Webb, R. C., Taubman, M. B., Atkinson, W. J., Gimbrone, M. A., Jr., and Alexander, R. W. (1985) J. Clin. Invest. 75, 1083–1086
13. Zheng, X. L., Renaux, B., and Hollenberg, M. D. (1998) J. Pharmacol. Exp. Ther. 285, 325–334
14. Akiyama, M., Yamada, O., Kanda, N., Aki, S., Kawano, T., Ohno, T., Mizoguchi, H., Eto, Y., Anderson, R. K., and Yamada, H. (2002) Cancer Lett. 178, 187–197
15. Kuchi, S., Numaguchi, K., Iwasaki, H., Matsutomo, T., Yamakawa, T., Usunohiya, M., Motley, E. D., Kawakatsu, H., Owada, K. M., Hirota, Y., Marumo, F., and Inagami, T. (1998) J. Biol. Chem. 273, 8860–8866
16. Matsuura, M., Gama, S., Shimizu, N., and Yoshida, Y. (1994) Arterioscler. Thromb. Vasc. Biol. 14, 1364–1371
17. Yamamoto, M., and Yamamoto, K. (1994) Exp. Cell Res. 212, 62–68
18. Zheng, X. L., Matsubara, S., Diao, C., Hollenberg, M. D., and Wong, N. C. (2001) J. Biol. Chem. 276, 13822–13829
19. Xie, W., Su, K., Wang, D., Paterson, A. J., and Koff, A., and Mendelsohn, J. (1995) J. Gastroenterol. Hepatol. 10, 2627–2633
20. Prasad, K. A., and Church, J. G. (1991) Exp. Cell Res. 195, 20–26
21. Ichiba, M., Miyazaki, Y., Kitamura, S., Kiyohara, T., Shinomura, Y., and Matsuzawa, Y. (2002) J. Gastroenterol. 37, 497–503
22. Barnes, D. W. (1983) J. Cell Biol. 93, 1–4
23. Barnes, D. W. (1984) Adv. Exp. Med. Biol. 172, 49–66
24. Fan, Z., Lu, Y., Wu, X., DeBlasio, A., Koff, A., and Mendelsohn, J. (1995) J. Cell Biol. 131, 235–242
25. Schlessinger, J. (2002) Cell 110, 669–672
26. Harris, R. C., Chung, E., and Coffey, R. J. (2003) Exp. Cell Res. 284, 2–13
27. Jorgensen, E. R., Walker, F., Pouliot, N., Garrett, T. F., Ward, C. W., and Burgess, A. W. (2003) Exp. Cell Res. 284, 31–53
28. Chang, L., and Kari, M. (2001) Nature 410, 37–40
29. Bornfeldt, K. E., Campbell, J. S., Koyama, H., Argast, G. M., Leslie, C. C., Raines, E. W., Krebs, E. G., and Ross, R. (1997) J. Clin. Invest. 100, 875–885
30. Pumiglia, K. M., and Decker, S. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 448–452
31. Chamley-Campbell, J., Campbell, G. R., and Ross, R. (1979) Physiol. Rev. 59, 1–61
32. Blouin, M. C., and Daniel-Lamazier, J. M., Michaille, J. J., Andujar, M., and Covacho, C. (1991) In Vitro Cell. Dev. Biol. 27A, 725–734
33. Gratzer, H. G. (1982) Science 218, 474–475
34. Kamenetsky, L. A., and Buss, D. E., Greshman, R. J., Kamenetsky, L. D., and Luther (1997) Acta Cytol. 41, 123–143
35. Darzyynkiewicz, Z., Bedner, E., Li, X., Gorczyca, W., and Melamed, M. R. (1999) Exp. Cell Res. 248, 1–12
36. Crissman, H. A., Oishi, N., and Habbersett, R. (1994) Methods Cell Biol. 41, 341–349
37. Higashikubo, R., Ragouzis, M., and Roti Roti, J. L. (1996) Cell Prolif. 29, 43–57

Detected in spindle cells. This is a novel finding for these two cell types, which may serve as a biochemical marker to distinguish the two cell types (2). This finding has provided solid evidence to characterize the effect of EGF in this cell system. Second, we detected an increase in tyrosine phosphorylation of EGFR in response to EGF treatment. The phosphorylation of EGFRs was transient, reached the maximal level at ~10 min, and then returned toward the basal level after ~15 min (Fig. 4). Interestingly, we consistently observed that in response to EGF treatment, the abundance of total EGFR decreased (Fig. 4). It is likely that this decrease results from a rapid degradation of the EGFR in response to the EGF ligand binding, but more studies are warranted. Nevertheless, the transient phosphorylation or receptor activation of EGF is involved in the EGFr-induced cell cycle arrest, because the addition of the EGFR tyrosine kinase inhibitor AG1478 inhibited the receptor tyrosine phosphorylation and reversed EGF inhibition of BrdUrd incorporation (Fig. 4). Importantly, in the presence of AG1478, EGF failed to induce the expression of the cell cycle inhibitory protein p27kip1 (Fig. 6). It is well known that EGF receptor activation induces receptor autophosphorylation, which is able to trigger several intracellular signaling pathways (25), for example, the PI 3-kinase pathway, the protein kinase C pathway, and the Ras-MAPK pathway. Our studies using their pharmacological inhibitors, such as LY294002 and wortman-
38. Frid, M. G., Aldashev, A. A., Dempsey, E. C., and Stenmark, K. R. (1997) Circ. Res. 81, 940–952
39. Tanner, F. C., Yang, Z. Y., Duckers, E., Gordon, D., Nabel, G. J., and Nabel, E. G. (1998) Circ. Res. 82, 396–403
40. Tanner, F. C., Boehm, M., Akyurek, L. M., San, H., Yang, Z. Y., Tashiro, J., Nabel, G. J., and Nabel, E. G. (2000) Circulation 101, 2022–2025
41. Gibson, S., Tu, S., Oyer, R., Anderson, S. M., and Johnson, G. L. (1999) J. Biol. Chem. 274, 17612–17618
42. Sibilia, M., Fleischmann, A., Behrens, A., Stingl, L., Carroll, J., Watt, F. M., Schlessinger, J., and Wagner, E. F. (2000) Cell 102, 211–220
43. Marra, D. E., Simoncini, T., and Liao, J. K. (2000) Circulation 102, 2124–2130
44. Bravo, R. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 4848–4850
45. Benditt, E. P., and Benditt, J. M. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 1753–1756
46. Pearson, T. A., Dillman, J. M., Soder, K., and Heptinstall, R. H. (1978) Am. J. Pathol. 90, 93–116