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Endothelin-1 and angiotensin II act as progression but not competence growth factors in vascular smooth muscle cells

Hasna Jahan, Sei Kobayashi, Junji Nishimura, Hideo Kanaide *

Division of Molecular Cardiology, Research Institute of Angiocardiology, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan

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Abstract

The direct effects of endothelin-1 and angiotensin II on cell cycle progression were investigated in rat aorta smooth muscle cells in primary culture. The phase of the cell cycle was determined by an immunocytochemical analysis of cell cycle-specific nuclear antigens. The primary cultured cells were synchronized in the G_0 phase (100%) by serum deprivation for 24 h. Endothelin-1 (0.1 μM) or angiotensin II (1 μM) had no effect on the cell cycle of G_0 cells, whereas platelet-derived growth factor (PDGF) stimulated the entry of the G_0 cells into the G_1 phase (100%) without a further progression to the S and M phases. Endothelin-1 or angiotensin II stimulated the progression of the PDGF-pretreated G_1 cells to the S and M phases. Fura-2 microfluorometry revealed that, between the G_0 and G_1 cells, there were no differences in the extent and time course of cytosolic Ca^{2+} elevations induced by endothelin-1 or angiotensin II, which suggested that endothelin-1 and angiotensin II receptors and their signaling pathways regulating cytosolic Ca^{2+} remained intact in these cell phases. We thus conclude that endothelin-1 and angiotensin II require the prior G_0/G_1 transition induced by a competence growth factor such as PDGF to exert their mitogenic effects. These results suggest the important role of endothelin-1 and angiotensin II in atherosclerosis as promoters (progression growth factors), but not as initiators.

Keywords: Endothelin-1; Angiotensin II; Atherosclerosis; Cell cycle progression; Ca^{2+}, cytosolic; Primary culture

1. Introduction

The proliferation of vascular smooth muscle cells induced by various growth factors has been implicated in a wide variety of pathological processes including atherosclerosis, hypertension, and restenosis after balloon angioplasty procedures (Ross, 1986; Jackson and Schwartz, 1992). Growth factors can be grouped into two categories based on the mode of stimulation of the cell cycle (Stiles et al., 1979). Competence growth factors stimulate the cell cycle from the G_0 phase to the competent G_1 phase without further progression to the S and M phases, while progression growth factors have no effect on the cell cycle of G_0 cells, but stimulate cell cycle progression from the competent G_1 phase to the S and M phases. Thus, both competence and progression growth factors are required for cells to complete a cell cycle, although some single growth factors may act as either competence or progression factors. We have recently shown that platelet-derived growth factor (PDGF) is a typical competence growth factor (Kobayashi et al., 1994).

It has been reported that vasoconstrictive agonists, including angiotensin II and endothelin-1, share many common intracellular signaling mechanisms with growth factors (Newby and George, 1993). In addition to the vasoconstrictor role, endothelin-1 and angiotensin II have been also suggested to act as cellular growth factors and to contribute to atherosclerosis and hypertension (Bobik and Campbell, 1993). However, the mode by which endothelin-1 or angiotensin II affect vascular smooth muscle cell growth still remains a matter of controversy (Newby and George, 1993). While in some studies these agonists have been described as

* Corresponding author. Tel.: 81-92-641-1151 (Ext. 2786); fax: 81-92-632-6513.

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having proliferative effects on serum-deprived vascular smooth muscle cells (Komuro et al., 1988; Nakaki et al., 1989; Sachindis et al., 1992), in other reports they have been shown to either have no such proliferative effects (Bobik et al., 1990) or to have hypertrophic effects without any hyperplastic effects on serum-deprived vascular smooth muscle cells (Geisterfer et al., 1988; Chua et al., 1992). It has also been reported that endothelin-1 or angiotensin II promotes mitogenesis when combined either with other growth promoters (Araki et al., 1990; Yeh et al., 1991; Bobik and Campbell, 1993; Newby and George, 1993) or with minimal concentrations of serum (Jackson and Schwartz, 1992; Janakidevi et al., 1992). In all of these previous studies, the proliferative effects of endothelin-1 and angiotensin II have been assessed by DNA synthesis, cell count, or flowcytometry. In these studies the G_0 phase and the G_1 phase were not distinguished and thus the mode of action (whether a competent or a progression effect) on the cell cycle has not been evaluated (Yeh et al., 1991; Chua et al., 1992).

In the present study, we directly determined the effects of endothelin-1 and angiotensin II on the cell cycle progression of vascular smooth muscle cells at the single-cell level by employing a newly developed method of immunocytochemical analysis of cell cycle-specific nuclear antigens (Kobayashi et al., 1994). The novel finding of this study is that endothelin-1 and angiotensin II are progression growth factors for vascular smooth muscle cells, but do not have any competent effects on G_0 cells. Therefore, the competent state at the G_1 phase, as obtained by competence growth factors such as PDGF, is essential for endothelin-1 and angiotensin II to exert their mitogenic effects on vascular smooth muscle cells. Comparative studies with primary cultured and passaged vascular smooth muscle cells in this study suggest that the discrepancies in the previous reports concerning the proliferative effects of these vasoconstrictors on serum-deprived cells may be due to some variations in the competent state in passaged cultured cells. We show that the elevations of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by endothelin-1 and angiotensin II are not affected by pretreatment with PDGF, which stimulates the competence proliferation from the G_0 to G_1 phase in vascular smooth muscle cells.

2. Materials and methods

2.1. Primary cell culture

Vascular smooth muscle cells were enzymatically dispersed from the aortic media of Wistar rats (Yamamoto et al., 1983) and seeded either on ultra-thin cover glass slips (No. 1, Matsunami, Japan) coated with fibronectin (Iwaki, Japan) or on chamber slides (No. 4804, Lab-Tek), as previously described (Kobayashi et al., 1994). The vascular smooth muscle cells in primary culture were grown in Dulbecco's modified Eagle's medium (Gibco) containing 10% heat-inactivated fetal bovine serum (Flow) and antibiotics. Electron microscopic studies and immunofluorescent staining of native smooth muscle actin and myosin revealed that the cells were not contaminated with either fibroblasts or endothelial cells (Yamamoto et al., 1983; Kobayashi et al., 1985). The cells in primary culture showed an increase and a decrease in $[Ca^{2+}]_i$ in response to various kinds of vasoconstrictors and vasorelaxants, respectively (Kobayashi et al., 1986, 1994; Kanaide et al., 1988; Kanaide, 1990). During each experimental procedure, high cell viability (> 99%) was maintained, as assessed by the trypan blue exclusion test (Kobayashi et al., 1985, 1994).

2.2. Subculture and established cell line

The confluent cells in primary culture were subcultured by trypsinization and seeded on the chamber slides. Subcultures at 3–6 passages were used. A-10 (established cell line of rat aorta) cells were trypsinized and seeded on the chamber slides. Both the subculture and A-10 cell line were grown in the same growth medium described above.

2.3. Determination of the cell cycle

The phase of the cell cycle of each single cell was determined by an immunocytochemical analysis of monoclonal antibodies against cell cycle-specific nuclear antigens, as previously described (Kobayashi et al., 1994). Briefly, the cells on a chamber slide were rapidly cryo-fixed in 70% ethanol (−20°C) and were incubated with monoclonal antibodies (fluorescein isothiocyanate isomer 1 (FITC)-conjugated anti-proliferating cell nuclear antigen (PCNA) with 1:20 dilution and *R*-phycoerythrin (RPE)-conjugated Ki-67 antigen with 1:10 dilution) for 30 min at 30°C. Double-labeled immunofluorescent staining was then observed under a fluorescent microscope (Axioskop, Zeiss, Germany) equipped with an appropriate filter combination (BP485, FT520; Zeiss, Germany). The color pattern of the nucleus produced by double staining makes possible the following differentiation of phases of the cell cycle: G_0 phase (no specific nuclear fluorescence); G_1 phase (green); S phase (yellow); G_2 phase (orange); and M phase (red). To assess the cell population in each treatment, the number of cells in each individual phase of the cell cycle was counted from 7–10 microscopic fields, and expressed as a percentage, assuming the total number of cells to be 100%.

2.4. Measurement of $[Ca^{2+}]_i$

Changes in $[Ca^{2+}]_i$ were monitored using the microfluorometry of fura-2, as previously described (Kobayashi et al., 1994). Briefly, cells in primary culture on an ultra-thin cover glass were loaded with fura-2 by incubating them with 5 μM fura-2/AM (acetoxymethyl ester) in the growth medium for 60 min at 37°C. After stabilization (at least 30 min) in normal physiological salt solution (normal PSS) at 25°C, fluorescence (500 nm) intensities at alternating (400 Hz) excitation (348 nm and 380 nm) and the ratio ($F348/F380$) were continuously measured using an inverted fluorescent microscope (TMD 56, Nikon, Japan) equipped with a spectrophotometer (CAM 220, Japan Spectroscope Co., Japan). The fluorescence measurements were carried out at 25°C to prevent any leakage and/or sequestration of the dye at a higher temperature. At this temperature there was no significant leak of the dye, while the cytosolic concentration of the dye was stable in the presence and in the absence of extracellular Ca^{2+} (Kobayashi et al., 1986). There was no significant difference in the $[Ca^{2+}]_i$ responses between 37°C and 25°C. Since the responses to repeated depolarization with 100 mM K^+ were reproducible up to 90 min, the 100 mM K^+-induced $[Ca^{2+}]_i$ transient was used as a reference response for normalization of the separate measurements. The changes in the fluorescence ratio were expressed as a percentage, assuming that the values at normal PSS and in 100 mM K^+ PSS are 0% and 100%, respectively. The absolute $[Ca^{2+}]_i$ values, as calculated according to Grynkiewicz et al. (1985), at normal PSS and in 100 mM K^+ PSS were 97.96 ± 16.4 nM ($n = 11$) and 203.6 ± 10.79 nM ($n = 11$) in the serum-deprived G_0 cells, respectively, and were not significantly different from those obtained for PDGF-pretreated G_1 cells ($P > 0.05$): 96.6 ± 16.5 nM ($n = 5$) and 201 ± 16.31 nM ($n = 5$), respectively. The composition of the normal PSS was: NaCl (135 mM); KCl (5 mM); $CaCl_2$ (1 mM); $MgCl_2$ (1 mM); glucose (5.5 mM); Hepes (10 mM, pH 7.4 at 25°C). For the 100 mM K^+ solution, 100 mM KCl was substituted for 100 mM NaCl, isosmotically.

2.5. Drugs and chemicals

Synthetic endothelin-1 and angiotensin II were purchased from the Peptide Institute Co. (Osaka, Japan). The recombinant human BB isomer of PDGF came from Biomedical Technologies (USA). PDGF was dissolved in 5 mM of acetic acid solution containing 2 mg/ml of bovine serum albumin. Endothelin-1 was dissolved in 0.1% acetic acid solution containing 2 mg/ml bovine serum albumin. The vehicle solutions had no effect on $[Ca^{2+}]_i$ levels and cell phases. The monoclonal antibodies (FITC-conjugated anti-PCNA and RPE-conjugated anti-human Ki-67) were purchased from Dako Japan (Japan). Fura-2/AM was from Dotite (Japan), and the A-10 cells were from Dainippon Seiyaku (Japan).

2.6. Data analysis

The data are presented as the means ± standard deviation (S.D.). The values were statistically analyzed by the unpaired Student's t-test. Differences were considered significant at a value of $P < 0.05$.

3. Results

3.1. The effects of angiotensin II and endothelin-1 on the cell cycle in vascular smooth muscle cells in primary culture

Serum deprivation of vascular smooth muscle cells in primary culture (24 h) resulted in a synchronization of the cell cycle in the G_0 phase of all of the cells and even after 72 h of serum deprivation the cells were still in the G_0 phase (Fig. 1-A1 and 1B). Treatment with 0.1 μM endothelin-1 or with 1 μM angiotensin II (up to 72 h) had no stimulatory effect on the cell cycle of the serum-deprived G_0 cells (Fig. 1-A2, 1-A3, and 1B), whereas 1 nM PDGF stimulated the cell cycle of all the cells from the G_0 phase to the G_1 phase within 24 h without any further progression of the cell cycle to the S and M phases, even after prolonged (48–72 h) incubation (Fig. 1-A4 and 1B), as also described previously (Kobayashi et al., 1994). When the PDGF-pretreated G_1 cells were exposed to 0.1 μM endothelin-1 for 24 h after washing out of PDGF, the cell cycle was stimulated from the G_1 phase to the S phase (8.11 ± 4.22%) and the M phase (10.45 ± 4.09%) ($P < 0.01$). When the PDGF-pretreated G_1 cells were exposed to 1 μM angiotensin II for 24 h after the PDGF was washed out, the cell cycle was stimulated from the G_1 phase to the S phase (8.03 ± 5.82%) and the M phase (9.41 ± 5.48%) ($P < 0.01$). Endothelin-1 (0.1 μM) and angiotensin II (1 μM) had equal potency to stimulate the cell cycle progression of vascular smooth muscle cells in primary culture ($P > 0.05$). The same results were obtained when the PDGF-pretreated G_1 cells were exposed to 0.1 μM endothelin-1 or to 1 μM angiotensin II for 24 h in the presence of 1 nM PDGF (data not shown).

3.2. The effects of angiotensin II and endothelin-1 on the cell cycle in vascular smooth muscle cells in subculture and in A-10 cells

Since it has been reported that endothelin-1 and angiotensin II have mitogenic effects in serum-de-

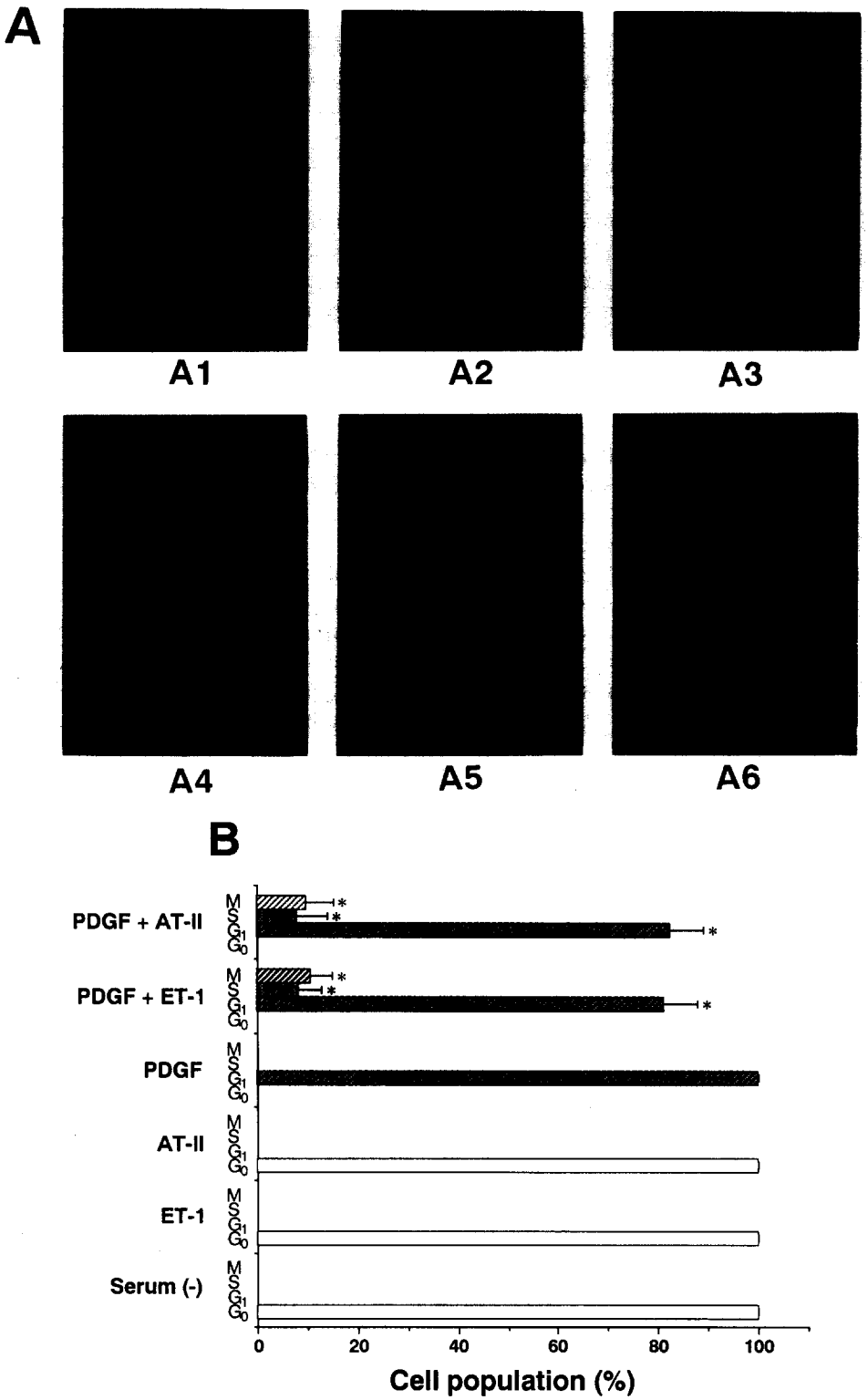

Fig. 1. The effects of endothelin-1, angiotensin II, and PDGF on the cell cycle in vascular smooth muscle cells in primary culture. The cell cycle of vascular smooth muscle cells was synchronized in the G_0 phase by serum deprivation for 24 h before starting the experiment. (A) Immunofluorescence microphotographs of the double-stained (PCNA and Ki-67) cells. The serum-deprived cells (A1, control) were treated with 0.1 μM endothelin-1 (A2), 1 μM angiotensin II (A3), or 1 nM PDGF (A4) for 24–48 h. In A1–A3, the cells had no specific nuclear fluorescence (the G_0 phase) (up to 72 h). The nucleus of the cells treated with PDGF (A4) showed a green fluorescence (the G_1 phase). The PDGF-treated G_1 cells (A4) were subsequently treated with 0.1 μM endothelin-1 (A5) or 1 μM angiotensin II (A6) in the absence of PDGF for 24 h. In A5 and A6, the nuclei of the cells emitted various colors of fluorescence, including green (G_1 phase), yellow (S phase), and red (M phase). (B) Summary of the repeated experiments as shown in (A). The data were obtained from 7–10 independent experiments and are presented as the mean of the cell population (%) in each phase of the cell cycle in the microscopic fields: G_0 phase, G_1 phase, S phase, and M phase). The horizontal bars indicate S.D. * $P < 0.01$, as compared to the cells treated with only PDGF.

prived vascular smooth muscle cells in subculture and established cell lines of vascular smooth muscle cells (Nakaki et al., 1989; Harris et al., 1990; Gibbons et al., 1992), we also studied the effects of these agonists on the cell cycle in subcultured vascular smooth muscle cells and in cells of the A-10 cell line. In contrast to vascular smooth muscle cells in primary culture, serum-deprived (up to 72 h) subcultured vascular smooth muscle cells were synchronized only partially (22.10 ± 6.90%) in the G_0 phase, and cells in the G_1 (65 ± 11%), S (6.20 ± 3.30%), M (7.16 ± 4.81%) phases were observed (Fig. 2A and 2C). The exposure of the serum-deprived subcultured vascular smooth muscle cells to 0.1 μM endothelin-1 and to 1 μM angiotensin II for 24 h resulted in an increase in the cell population in the S phase ($P < 0.05$) and M phase ($P < 0.01$) and a corresponding decrease in the cell population in the G_1 phase ($P < 0.01$ and 0.05, respectively), without affecting the cell population in the G_0 phase ($P > 0.05$), indicating that 0.1 μM endothelin-1 or 1 μM angiotensin II stimulates cell cycle progression from the G_1 to the S and M phases, but not from the G_0 to the G_1 phase. The serum-deprived (up to 72 h) A-10 cells were never synchronized in the G_0 phase (0%), whereas cells in the G_1 (82.13 ± 8.05%), S (9.85 ± 5.02%), M (8.02 ± 4.68%) phases were observed (Fig. 2B and 2D). Exposure of the serum-deprived A-10 cells to 0.1 μM endothelin-1 and to 1 μM angiotensin II for 24 h resulted in an increase in the cell population in the S phase ($P < 0.01$) and M phase ($P < 0.05$) and a corresponding decrease in the cell population in the G_1 phase ($P < 0.01$), which was compatible with the function of these agonists as progression growth factors. Endothelin-1 at the concentration of 0.1 μM and angiotensin II at the concentration of 1 μM had equal potency to stimulate cell cycle progression at any cell phase in vascular smooth muscle cells in subculture and in A-10 cells ($P > 0.05$).

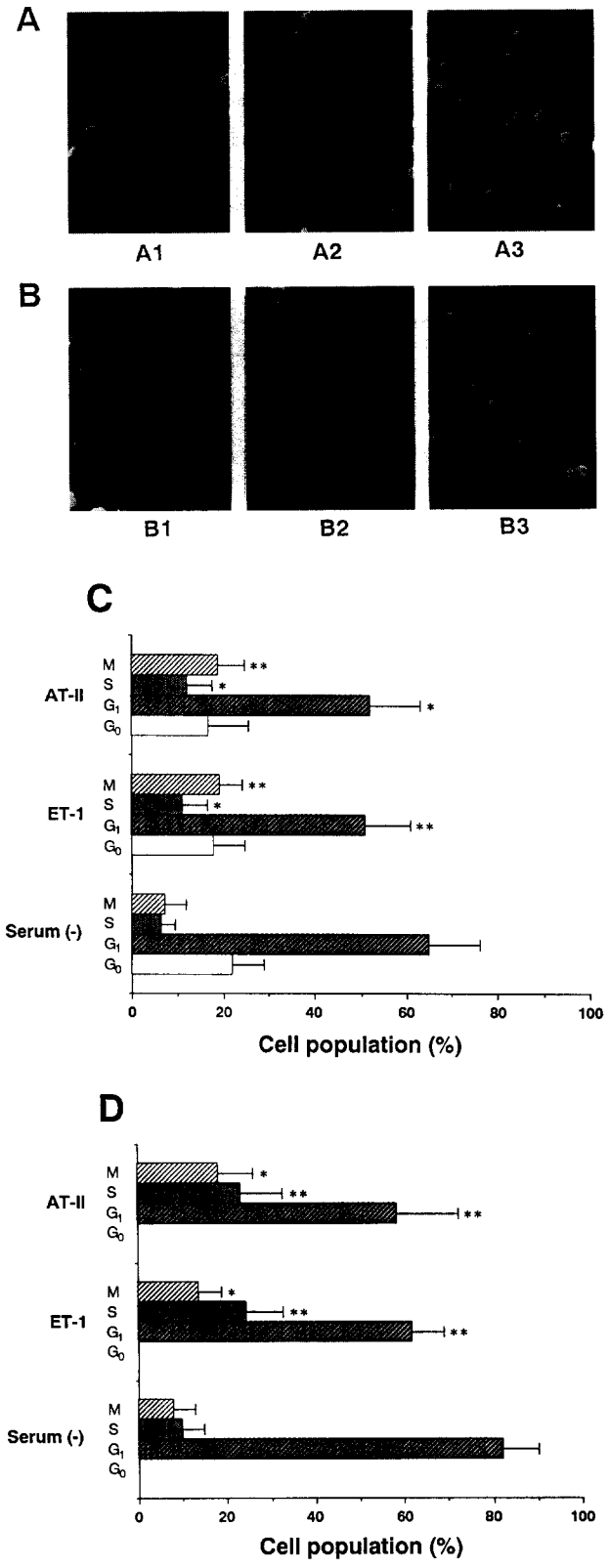

Fig. 2. Effects of endothelin-1 and angiotensin II on the cell cycle in vascular smooth muscle cells in subculture (A and C) and in A-10 cells (B and D). (A and B) Immunofluorescence microphotographs of double-stained (PCNA and Ki-67) vascular smooth muscle cells in subculture (A) and in A-10 cells (B). The cells were cultured in a serum-free medium for 24 h (A1 and B1), and then were subsequently treated with either 0.1 μM endothelin-1 (A2 and B2) or 1 μM angiotensin II (A3 and B3) for 24 h. The nuclei of the cells emitted various colors of fluorescence, including green (G_1 phase), yellow (S phase), and red (M phase). (C and D) Summary of the repeated experiments as shown in (A) and (B), respectively. The data were obtained from 7–10 independent experiments and are presented as the mean of the cell population (%) in the each phase of the cell cycle: G_0 phase, G_1 phase, S phase, and M phase. The horizontal bars indicate S.D. ** $P < 0.01$ and * $P < 0.05$, as compared to the serum-deprived cells.

3.3. The effects of angiotensin II and endothelin-1 on $[Ca^{2+}]_i$ in vascular smooth muscle cells in primary culture

Endothelin-1 (0.1 μM) and angiotensin II (1 μM) induced an initial transient (1st component) and a

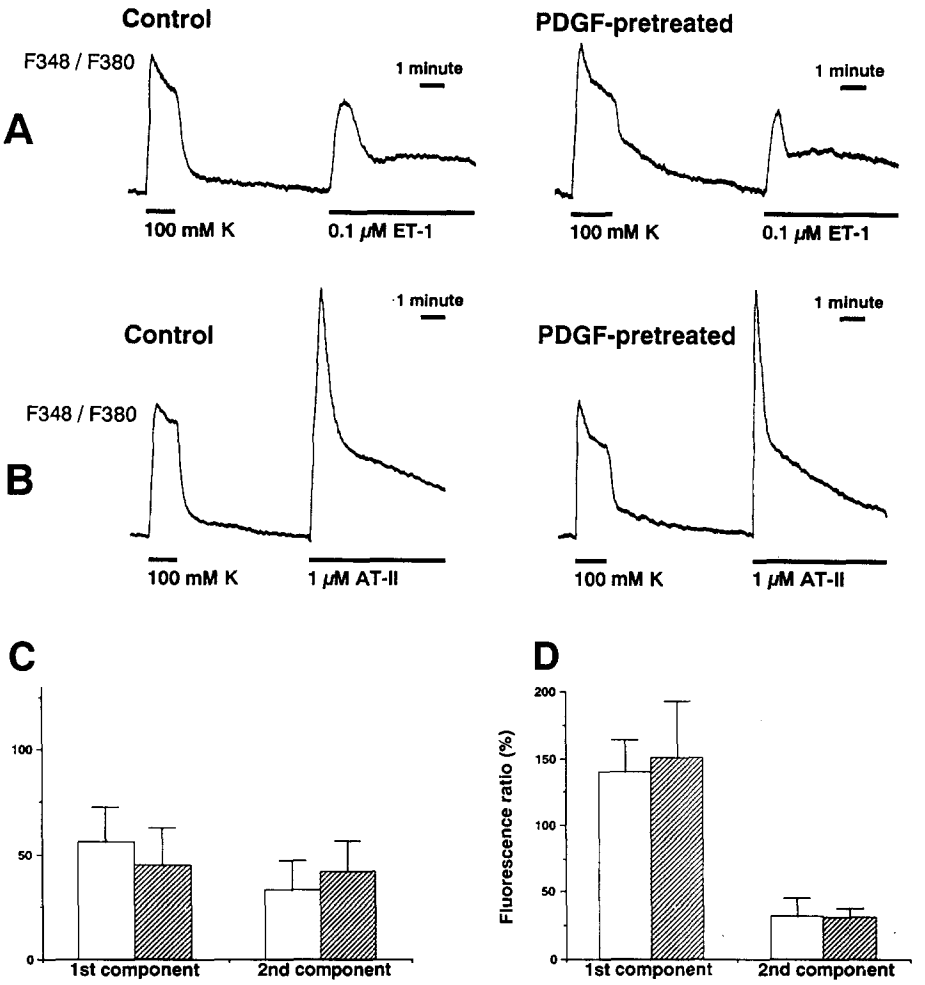

Fig. 3. The effects of endothelin-1 (A and C) and angiotensin II (B and D) on $[Ca^{2+}]_i$ in vascular smooth muscle cells in primary culture. (A and B) Representative recordings of $[Ca^{2+}]_i$ elevations induced by 0.1 μM endothelin-1 (A) and by 1 μM angiotensin II (B) in the serum-deprived (24 h or 48 h) cells (left panels, control) and in the PDGF-pretreated (24 h or 48 h) cells (right panels). The $[Ca^{2+}]_i$ was assessed by measuring changes in the fluorescence ratio ($F348/F380$) of fura-2. Each recording was obtained from different cells in separate dishes. Since the absolute $[Ca^{2+}]_i$ values at normal PSS and in 100 mM K^+ PSS were not significantly different between the serum-deprived G_0 cells and the PDGF-pretreated G_1 cells, the 100 mM K^+-induced $[Ca^{2+}]_i$ elevation was used as a reference response for the normalization of separate measurements in the G_0 cells and G_1 cells (see the details in Materials and methods). After recording the $[Ca^{2+}]_i$ elevation induced by 100 mM K^+ depolarization (100% response), either endothelin-1 or angiotensin II was applied in the presence of extracellular Ca^{2+} (1 mM). (C and D) Summary of the repeated experiments as shown in (A) and (B), respectively. The data were obtained from 6–7 independent experiments in the serum-deprived cells (open columns, control) and in the PDGF-pretreated cells (hatched columns). The data are presented as the mean $[Ca^{2+}]_i$ values (%) at the peak of the initial $[Ca^{2+}]_i$ elevations (1st component) and at the subsequent lower steady-state $[Ca^{2+}]_i$ elevations (2nd component, 7 min after the application of the drugs). The vertical bars indicate S.D.

subsequent lower steady-state or slowly declining (2nd component) elevation of $[Ca^{2+}]_i$ in primary cultured vascular smooth muscle cells, synchronized either in the G_0 phase by serum deprivation (24 h) or in the G_1 phase by 24-h treatment with 1 nM PDGF (Fig. 3A and 3B). These time courses of $[Ca^{2+}]_i$ elevations are typical for those of Ca^{2+} transients induced by endothelin-1 and angiotensin II, consisting of the 1st and 2nd components mainly due to the release of intracellular Ca^{2+} and the influx of extracellular Ca^{2+}, respectively, as described previously (Capponi et al., 1985; Miasiro et al., 1988; Kai et al., 1989). Between the G_0 and the G_1 cells, there was no significant difference in the $[Ca^{2+}]_i$ levels of the 1st and 2nd components following stimulation with endothelin-1 and with angiotensin II ($P > 0.05$) (Fig. 3C and 3D). These results indicate that the lack of a stimulatory effect of endothelin-1 and angiotensin II on the cell cycle in G_0 cells may not be due to changes in the number and characteristics of the endothelin-1 and angiotensin II receptors in these cells.

4. Discussion

In the present study, using an immunocytochemical analysis of the cell cycle at the single-cell level, we demonstrated for the first time that both endothelin-1 and angiotensin II are progression growth factors, but not competence growth factors for vascular smooth muscle cells in primary culture. In primary cultured vascular smooth muscle cells, even after a prolonged (up to 72 h) incubation, endothelin-1 and angiotensin II had no competent effect on the serum-deprived G_0 cells (Fig. 1). The present and previous study (Kobayashi et al., 1994) showed that, even after a prolonged (up to 72 h) incubation, PDGF stimulates the cell cycle from the G_0 phase to the competent G_1 phase without any further progression to the S and M phases, which indicates that PDGF is a competence growth factor for vascular smooth muscle cells in primary culture. Endothelin-1 and angiotensin II can stimulate progression of the cell cycle from the competent G_1 phase to the S and M phases. Therefore, a transition from the G_0 phase to the competent G_1 phase in advance, as stimulated by a competence growth factor such as PDGF, is essential for endothelin-1 or angiotensin II to exert their mitogenic effects in vascular smooth muscle cells in primary culture. It is unlikely that the lack of proliferative effects of these vasoconstrictors on G_0 cells may be due to a possible deterioration of or a decrease in the number of receptors in G_0 cells, because both agonists induced $[Ca^{2+}]_i$ transients in G_0 cells, and the extent and duration of these $[Ca^{2+}]_i$ transients were comparable to those observed in G_1 cells (Fig. 3). This finding suggested that endothelin-1 and angiotensin II receptors and their signaling pathways regulating cytosolic Ca^{2+} were maintained in the G_0 and G_1 phases of the cell cycle.

In contrast to the lack of proliferative effects on the serum-deprived primary cultured vascular smooth muscle cells, as observed in the present and previous study (Bobik et al., 1990), endothelin-1 and angiotensin II had a proliferative effect on serum-deprived vascular smooth muscle cells in subculture and in cells of the A-10 cell line in the present study, which is compatible with previous observations in which endothelin-1 and angiotensin II induced DNA synthesis (corresponding to the S phase) and mitosis (at the M phase) in serum-deprived passaged vascular smooth muscle cells (Nakaki et al., 1989; Harris et al., 1990; Hamada et al., 1990; Yeh et al., 1991; Yu et al., 1991; Gibbons et al., 1992). However, these results do not necessarily indicate that these agonists stimulate cell cycle progression from the G_0 phase to the S and M phases. In the present study, we found that G_0 synchronization by serum deprivation was complete (100%) in vascular smooth muscle cells in primary culture, while it was only partial ($\leq 20\%$) in passaged cells even after prolonged (72 h) serum deprivation. Especially in the A-10 cell line, no cell was synchronized in G_0 phase by serum deprivation. Under such conditions, endothelin-1 and angiotensin II stimulated the cell cycle of the preexisting G_1 cells to the S and M phases, without affecting the cell cycle of the G_0 cells. Therefore, this finding for vascular smooth muscle cells in subculture and for cells of the A-10 cell line is consistent with our conclusion for primary cultured vascular smooth muscle cells, namely that endothelin-1 and angiotensin II are progression growth factors, but not competence growth factors. Since the competent state is essential for endothelin-1 and angiotensin II to stimulate cell cycle progression, various competent states in passaged vascular smooth muscle cells and A-10 cells may contribute to the apparently discrepant results concerning the mitogenic effects of endothelin-1 and angiotensin II on serum-deprived cells. Endothelin-1 and angiotensin II would not have a proliferative effect if serum-deprived cells could be synchronized completely in the G_0 phase, as expected and as observed in the primary culture in the present study. In contrast, if serum deprivation were to result in only a partial synchronization in the G_0 phase, these agonists would have a proliferative effect on the G_1 cells, but not on the G_0 cells. In addition, incomplete G_0 synchronization in passaged vascular smooth muscle cells may also suggest the presence of an autocrine secretion of growth factors into the serum-free medium from passaged vascular smooth muscle cells. Thus, the application of endothelin-1 and angiotensin II to passaged vascular smooth muscle cells would produce variable results, depending on the amount and the kind of growth factors secreted by the passaged vascular smooth muscle cells, since it has been reported that endothelin-1 and angiotensin II have synergistic effects with various kinds of growth factors (Bobik et al., 1990; Araki et al., 1990; Yeh et al., 1991; Jackson and Schwartz, 1992; Janakidevi et al., 1992; Newby and George, 1993; Bobik and Campbell, 1993). Although the present study demonstrates the advantage of primary cultured vascular smooth muscle cells over passaged vascular smooth muscle cells in terms of completeness of G_0 synchronization by serum deprivation, it should be noted that the effects on the cell cycle can be evaluated directly at the single-cell level by immunocytochemical analysis of PCNA and Ki-67, either in primary cultured or passaged vascular smooth muscle cells, since all phases of the cell cycle, especially the G_0 and G_1 phases, can be clearly distinguishable by this technique.

It has been reported that angiotensin II stimulates the basal secretion of autocrine growth factors, such as PDGF-AA, TGF-β1, basic fibroblast growth factor (bFGF) from serum-deprived vascular smooth muscle cells in subculture (Naftilan et al., 1989; Gibbons et al., 1992; Itoh et al., 1993). TGF-β1 has been suggested to

have an antiproliferative effect, whereas PDGF-AA and bFGF have been suggested to cause proliferation. However, in the present study, since angiotensin II had no proliferative effects on the serum-deprived vascular smooth muscle cells in primary culture (Fig. 1), it is unlikely that the primary cultured cells produce autocrine growth factors in response to angiotensin II.

Although the importance of $[Ca^{2+}]_i$ elevations in cell proliferation has been proposed (Yu et al., 1988; Block et al., 1989; Tucker et al., 1989; Dilbert et al., 1990), we have recently demonstrated that $[Ca^{2+}]_i$ transients are not required for PDGF to induce a cell cycle progression from the G_0 to the G_1 phase in vascular smooth muscle cells (Kobayashi et al., 1994). When tyrosine kinases are inactivated, PDGF induces $[Ca^{2+}]_i$ elevations mediated by a Ca^{2+} influx but cannot stimulate cell cycle progression (Kobayashi et al., 1994). In accordance with this result, the finding of the present study that endothelin-1 and angiotensin II induced typical $[Ca^{2+}]_i$ transients in G_0 cells, but were not able to cause progression of the cell cycle in G_0 cells, is also compatible with the notion that $[Ca^{2+}]_i$ elevations, per se, may not be a major factor for the G_0/G_1 transition, as induced by either vasoconstrictors or growth factors. We therefore propose that $[Ca^{2+}]_i$ elevations may have little importance in competence proliferation, which is a conversion from the G_0 to the G_1 phase in vascular smooth muscle cells.

The proliferation of vascular smooth muscle cells in atherosclerosis and in restenosis after vascular injury is thought to be due to the synergistic proliferative effects of various kinds of growth factors released at the site of both atherosclerotic lesions and vascular injury (Ross, 1986; Owens, 1989). It has been reported that the plasma and tissue levels of endothelin-1 and angiotensin II are elevated in patients with atherosclerosis and hypertension (Alderman et al., 1991; Bobik and Campbell, 1993). In addition, an in vivo study showed that the infusion of angiotensin II results in an increase in DNA synthesis and cell proliferation in vascular smooth muscle cells (Daemaen et al., 1991). These findings suggest the possible participation of endothelin-1 and angiotensin II, together with other growth factors, in the progression of cell proliferation of vascular smooth muscle cells in atherosclerotic lesions. The findings in the present study indicate that the proliferative effects of endothelin-1 and angiotensin II on vascular smooth muscle cells depend on the competent state of vascular smooth muscle cells, and therefore, endothelin-1 and angiotensin II require the exposure of vascular smooth muscle cells to competent growth factors in advance to exert their proliferative effects. These results are compatible with the notion that endothelin-1 and angiotensin II may play an important role in the pathogenesis of atherosclerosis as a progression factor, but not as an initiator of atherosclerosis. This notion is also supported by the previous observation that vasoconstrictors including angiotensin II stimulate proliferation of vascular smooth muscle cells in hypertensive rats, but much less in normotensive rats (Bunkenberg et al., 1992), since it is well known that vascular smooth muscle cells from hypertensive rats are more competent than those from normotensive rats (Berk et al., 1989).

In summary, our novel finding is that vasoconstrictors, endothelin-1 and angiotensin II, are not competence growth factors (to stimulate progression from the G_0 to the G_1 phase), but progression growth factors (to stimulate progression from the G_1 to the S and M phases) for vascular smooth muscle cells, which thus indicates that endothelin-1 and angiotensin II both play an important role in atherosclerosis as promoters, but not as initiators. Comparative studies with primary cultured and passaged vascular smooth muscle cells suggest that variation in the competent state of cultured cells even after serum deprivation may contribute to the discrepancies in the previous studies concerning the effects of endothelin-1 and angiotensin II on cell growth in serum-deprived cells. We also propose that the $[Ca^{2+}]_i$ elevation induced by endothelin-1 and angiotensin II, per se, may not be a major factor for a competence transition from the G_0 to the G_1 phase in vascular smooth muscle cells.

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