Competence Effect of Basic Fibroblast Growth Factor on Cell Cycle in Subcultured Endothelial Cells of Rat Aorta by Flow Cytometry

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ABSTRACT—With flow cytometry, we investigated the effect of basic fibroblast growth factor (bFGF)-induced competence in subcultured endothelial cells (EC) (4–9-passage) of rat thoracic aorta. The cell population in each phase of the cell cycle was determined by a double staining technique with fluorescein isothiocyanate-conjugated mouse monoclonal antibody against the proliferation-associated nucleus antigen Ki-67 and propidium iodide for total DNA content. EC were cultured in medium containing 5% fetal bovine serum (FBS) for 6 days. After serum-starvation for 2 days, the treatment with bFGF (3–10 ng/ml) for 12 hr promoted the entry of cells into the G₁ phase from the G₀ phase concentration-dependently. bFGF (10 ng/ml) increased the cell population in the G₁ phase by 5% of the total EC, compared with the control culture without bFGF. A further 12–15-hr culture with 1% FBS after bFGF treatment promoted the entry of the cell into the S phase. Thus flow cytometric analysis demonstrates that bFGF stimulates the entry of EC into the G₁ phase from the G₀ phase.

Keywords: Basic fibroblast growth factor, Flow cytometry, Rat aortic endothelial cell, Ki-67 antigen, Propidium iodide

Abnormal proliferation of vascular endothelial cells (EC) causes many diseases such as diabetic retinopathy (1) and atherosclerosis (2). Development of these angiogenic diseases are reported to be induced several growth factors such as basic fibroblast growth factor (bFGF), platelet-derived growth factor and interleukin-8 (3–5). Growth factors are classified into two types, a competence factor and a progression factor, based on their modes of enhancement of cell proliferation (6). The competence factors advance the cell cycle from the G₀ to G₁ phase, whereas the progression factors promote entrance to the S phase from the G₁ phase (7). bFGF, a competence factor not a progression factor, can induce DNA synthesis only in combination with a progression factor-containing platelet-poor plasma in Balb/3T3 fibroblast (7). bFGF is also a potent mitogen for EC proliferation since the bFGF effect is enhanced in an autocrine manner in vitro (8). In the previous study, we demonstrated that bFGF hastens the starting time of [³H]-thymidine incorporation into subcultured EC of rat aorta, suggesting the competence effect of bFGF (9). However, it remains to be determined if bFGF is a competence factor in EC as well as in fibroblasts.

A proliferation-associated nucleus antigen, Ki-67, is a useful tool for detecting the transition from the G₀ to G₁ phase, because this antigen is expressed in actively growing cells but not in G₀-arrested (quiescent) cells (10, 11). In the present study using flow cytometry with double staining of Ki-67 antigen and total DNA, we provided evidence that bFGF stimulates the competence in quiescent EC of rat thoracic aorta.

MATERIALS AND METHODS

Cell culture

Rat aortic EC in primary culture were prepared by the method reported by Schwartz (12) and Nagaura et al. (9) as follows: The thoracic aorta of male Wistar rats (9-week-old, 243–302 g; SLC, Tokyo) was isolated, and the periadventitial connective tissue was carefully removed. The vessel was placed into a culture dish (100-mm diameter) filled with 10 ml Hanks' balanced salt solution (136.8 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 1.3 mM CaCl₂, 0.3 mM Na₂HPO₄, 5.6 mM glucose and 4.2 mM NaHCO₃; pH 7.3) and cannulated with a needle. The needle was fastened with a thread, and the other end of

the vessel lumen was closed with a vascular clamp. Hanks' balanced salt solution containing 2 mg/ml collagenase (Wako, Osaka) was injected into the vessel from the needle and incubated for 30 min at 37°C. Then the collagenase solution was washed out by 5–10 ml Dulbecco's modified Eagle medium (DMEM; Nissui, Tokyo) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Summit, Fort Collins, CO, USA), 160 U/ml penicillin G potassium (Banyu Seiyaku, Tokyo) and 100 µg/ml streptomycin sulfate (Meiji Seika, Tokyo) and 4.2 mM NaHCO₃. The cell-suspended medium was collected and added onto a Type I collagen (Koken, Tokyo)-coated, 24-well plate (Corning 258201; Iwaki Glass, Tokyo) at a density of 100–200 cells/0.5 ml/16-mm well. The cells were cultured in a humidified atmosphere of 5% CO₂ in air at 37°C. The non-endothelial cells such as smooth

muscle cells or fibroblasts were removed with a rubber cleaner (Ikemoto Scientific Technology, Tokyo) under an optical microscope. The cloned EC were treated with Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS) containing 0.5 mM EDTA and subcultured at a split ratio of 1:4. Subcultured EC were used at the 4th to 9th passage in each test. EC were identified by their typical cobblestone-like morphology and their ability to form tube-like structures in vitro (9, 13).

Experimenta protocol

Subcultured EC were seeded at a density of 1.5×10^5 cells/1.5 ml/collagen-coated 35-mm dish (Falcon 3001; Nippon Becton Dickinson, Tokyo) and incubated with 5% FBS-DMEM 6 days. In the case of flow cytometric analysis of cells during the exponential phase, EC at day 3

Fig. 1. Diagram for analysis of competence stimulation by basic fibroblast growth factor (bFGF) with [³H]-thymidine incorporation (above) and flow cytometry (below) in subcultured endothelial cells (EC) of rat aorta. Subcultured EC were seeded at a density of 1.5×10^5 cells/1.5 ml/collagen-coated 35-mm dish and incubated in 5% FBS-DMEM for 6 days. At the indicated time points, a–h, the EC were double-stained with fluorescein isothiocyanate (FITC)-conjugated Ki-67 monoclonal antibody and propidium iodide and then analyzed by flow cytometry. After a 6-day culture with 5% FBS and 2-day serum starvation, EC were treated for 12 hr with (A) or without bFGF (B), and then cultured with 1% FBS. For investigating the effect of 10% FBS, EC were first treated for 12 hr with 10% FBS, subjected to serum starvation, and then cultured for a further 12–15 hr with 10% FBS (C).

were used (Fig. 1, A-a). The subconfluent cells (6-day culture) were synchronized by serum starvation for 2 days (Fig. 1, A-b), and then the medium was exchanged with fresh serum-free DMEM containing bFGF (Fig. 1, A-c: 3 and 10 ng/ml, Fig. 1, B-e: 0 ng/ml) and applied to EC for 12 hr. Then the medium was washed out and replaced with 1% FBS-DMEM to continue the culture further for 12–15 hr (Fig. 1, A-d and B-f).

Flow cytometric analysis

In the previous paper (9), the competence effects of bFGF were investigated by analyzing the time course (day and hr) of [3 H]-thymidine incorporation. Under the same experimental conditions, the EC were analyzed by flow cytometry. At the time points of -12 (before), 0 and 12–15 (after bFGF pretreatment for 12 hr) hr, the cells were harvested by treating them with PBS containing 0.5 mg/ml trypsin and 0.54 mM EDTA and then suspended in 10% FBS-DMEM. After washing them twice with PBS, 10^6 EC were fixed at -10°C to -8°C with 1 ml of periodate-lysine-paraformaldehyde solution for 15 min (14). The EC were washed twice with PBS, and they were centrifuged at $1800 \times g$ for 5 min. The cell pellet was first stained at 4°C for 1 hr with $10 \mu\text{l}$ fluorescein isothiocyanate (FITC)-conjugated mouse anti-Ki-67 monoclonal antibody (mAb) (1:1 dilution, DAKO-Ki-67; Dako, Kyoto) for the proliferation-associated nucleus antigen. After washing out the antibody twice with PBS, the EC were secondly stained at 4°C for 30 min with 1 ml PBS containing $50 \mu\text{g/ml}$ propidium iodide (PI; Molecular Probes, Eugene, OR, USA) and ribonuclease A (100

U/ml; Sigma Chemical, St. Louis, MO, USA) for total DNA content. FITC-conjugated mouse IgG₁ (Dako) was used as a negative control.

The doubly stained EC were analyzed by a Epics Elite flow cytometer (Coulter, Hialeah, FL, USA) equipped with an argon laser (the excitation wavelength at 488 nm). Green FITC fluorescence (520–530 nm) was measured using a 525-nm band pass filter, and red PI fluorescence ($>625 \text{ nm}$) was detected by a photomultiplier tube. Cytograms of 6.5×10^3 to 10^4 cells showing the green and the red fluorescence, and the side and the forward angle light scatter were analyzed by a computer workstation, where cell-clumps, dead cells and cell debris were excluded by gating the light scatter signal. Autofluorescence belonging to Ki-67 negative cells was adjusted within the region of 0.1 to 3 fluorescence intensity using the cells treated with FITC-conjugated IgG₁ (Dako) as a negative control antibody.

Materials

Recombinant human bFGF (154 amino-acid form, molecular weight of 17 kilodaltons, expressed in *Escherichia coli* and purified) was kindly supplied by Kaken Seiyaku (Kyoto).

RESULTS

Flow cytometric distribution pattern of rat aortic EC population in the cell cycle

After a 3 day-culture with 5% FBS-DMEM, rat aortic EC were distributed at the G₀, G₁, S and G₂/M phases of

Fig. 2. Typical cytograms of subcultured EC (rat aorta) treated with 5% FBS for 3 days. EC were stained with FITC-conjugated mouse monoclonal antibody against Ki-67 (a) or FITC-conjugated mouse IgG₁ as a negative control. Logarithmic fluorescence intensity of FITC and linear intensity of propidium iodide (PI) are displayed with 67-channel resolution. The debris and clumps of cells were excluded by taking advantage of the PI peak and PI integral parameters. The 10^4 EC were used in each cytogram. The 5% FBS-stimulated EC were composed of $69.2 \pm 1.9\%$ at the G₀ phase, $9.8 \pm 2.3\%$ at the G₁ phase, $17.3 \pm 1.1\%$ at the S phase and $9.3 \pm 0.8\%$ at the G₂/M phase ($n=5$). For further explanations, see the legend for Fig. 1.

Fig. 3.

Fig. 4. Effect of 10% FBS on the cell cycle of subcultured EC of rat aorta. Typical cytograms before the treatment with 10% FBS (b), 12 hr after the treatment with 10% FBS (g) of EC (6.5×10^3 – 10^4 cells) doubly stained with FITC-conjugated monoclonal antibody against Ki-67 and PI for total DNA content. The 10% FBS was applied after a 6-day culture followed by 2-day serum starvation. The 10% FBS was then replaced with 10% FBS (h) for 12–15 hr. For further explanations, see legends for Figs. 1 and 2.

the cell cycle by gating analysis for red fluorescence of propidium iodide bound to DNA and green fluorescence of FITC bound to Ki-67 (Fig. 2). The fluorescence of cells treated with FITC-conjugated Ki-67 mAb was within the region of 3 to 100 intensity of fluorescence (Fig. 2a), whereas autofluorescence of cells treated with FITC-conjugated mouse IgG₁ was within the region of 0.1 to 3 intensity of fluorescence (negative control in Fig. 2). The percentages of cells at each phase relative to the total number of EC were estimated to be $69.2 \pm 1.9\%$ at the G₀ phase, $9.8 \pm 2.3\%$ at the G₁ phase, $17.3 \pm 1.1\%$ at the S phase and $9.3 \pm 0.8\%$ at the G₂/M phase ($n=5$). Most of the EC belonging to the S and G₂/M phases were Ki-67-negative.

Competence effect of bFGF on rat aortic EC proliferation

After a 6-day culture with 5% FBS-DMEM, rat aortic EC were further cultured under serum-starvation for 2 days (Fig. 3b). The percentages were $70.7 \pm 7.1\%$ at the G₀ phase, $20.9 \pm 7.4\%$ at the G₁ phase, $7.9 \pm 7.4\%$ at the S phase, $4.4 \pm 1.2\%$ at the G₂/M phase ($n=3$). Then, the EC were stimulated for 12 hr without bFGF as a control culture (Fig. 3e) or with bFGF (3 ng/ml: Fig. 3c₁, 10 ng/ml: Fig. 3c₂). At 0 hr (12 hr after the treatment without bFGF), the percentages were $82.9 \pm 1.7\%$ at the G₀ phase, $10.8 \pm 2.2\%$ at the G₁ phase, $6.9 \pm 0.6\%$ at the S phase and $3.1 \pm 0.4\%$ at the G₂/M phase (Fig. 3e, con-

trol culture, $n=4$). The treatment with bFGF (10 ng/ml) for 12 hr slightly decreased the cells in the G₀ phase to $78.2 \pm 2.3\%$ and significantly increased those in the G₁ phase to $15.6 \pm 2.5\%$ (Fig. 3c₂ and A-c, $n=4$). Few effects were observed at the S and G₂/M phases. Similar effects were obtained by treatment with 3 ng/ml bFGF, although these effects were not significant.

After a further 12- to 15-hr culture with 1% FBS after removing the above medium, the percentages were $80.9 \pm 1.2\%$ at the G₀ phase, $11.0 \pm 0.8\%$ at the G₁ phase, $8.4 \pm 1.1\%$ at the S phase and $3.1 \pm 0.4\%$ at the G₂/M phase (Fig. 3f, $n=4$). Treatment with 10 ng/ml bFGF significantly increased the S phase cells to $9.0 \pm 1.6\%$ and G₂/M phase cells to $3.3 \pm 0.7\%$ (Fig. 3d₂, $n=5$). The percentages in EC precultured with 3 ng/ml bFGF were $76.6 \pm 2.4\%$ at the G₀ phase, $14.4 \pm 1.2\%$ at the G₁ phase, $8.5 \pm 1.6\%$ at the S phase and $3.5 \pm 1.1\%$ at the G₂/M phase (Fig. 3d₁, $n=3$). These results demonstrate that bFGF treatment stimulates cells to enter the G₁ phase from the G₀ phase by 5%. The transition from the G₀ to G₁, S and G₂/M phases was advanced more rapidly by 10 ng/ml bFGF than by 3 ng/ml bFGF.

Quiescent (serum-starvated) EC (Fig. 4b) were cultured with 10% FBS for 12 hr (Fig. 4g, 0 time point) and for an additional 12–15 hr (Fig. 4h) as a positive control for the above effect of bFGF. The percentages were $82.3 \pm 3.9\%$ at the G₀ phase, 10.9 ± 3.4 at the G₁ phase, $7.0 \pm 1.2\%$ at

Fig. 3. Competence effect of bFGF on subcultured EC of rat aorta. Typical cytograms (above) before the treatment with bFGF (b) and 12 hr after the treatment with bFGF (c: 0 ng/ml, c₁: 3 ng/ml, c₂: 10 ng/ml) of EC (6.5×10^3 – 10^4 cells) doubly stained with FITC-conjugated monoclonal antibody against Ki-67 and PI for total DNA content. bFGF was applied after a 6-day culture followed by 2-day serum starvation. The respective concentrations of bFGF were replaced and then the cells were treated with 1% FBS (f, d₁, d₂) for 12–15 hr. Percent changes (below) of G₀, G₁, S and G₂/M phases in EC cultured for 0 hr (A-c) and 12–15 hr (A-d) with 1% FBS after treatment with 3 ng/ml (hatched column) and 10 ng/ml (shaded column) bFGF. The values are means $\pm$ S.E.M. ($n=3-5$). * $P < 0.05$ and ** $P < 0.01$: significantly different, determined by the paired *t*-test. For further explanations, see legends for Figs. 1 and 2.

the S phase and $3.0 \pm 0.4\%$ at the G_2/M phase at 0-hr time point after 12-hr culture with 10% FBS (Fig. 4g, $n=3$). After the additional 12- to 15-hr treatment with 10% FBS, the percentages were $75.8 \pm 4.1\%$ at the G_0 phase, $13.4 \pm 2.4\%$ at the G_1 phase, $10.7 \pm 1.5\%$ at the S phase and $4.6 \pm 0.9\%$ at the G_2/M phase (Fig. 4h, $n=4$). These results suggest that the quiescent EC were advanced to the G_1 , S, G_2/M phases by 10% FBS. Thus, only a part of the EC-population was transferred by 10% FBS, as well as 3–10 ng/ml bFGF.

DISCUSSION

bFGF is localized in the nuclear chromatin, nucleoli, cytosol and cytoplasmic vesicles in subconfluent EC (15), and it may stimulate EC growth in an autocrine manner. The concept of the competence phenotype and the progression phenotype in the cell cycle has been proposed by Pledger et al. (6). It is very important to investigate the cell cycle in abnormally proliferating cells, which are detected in angiogenic diseases. EC composed of a vascular lumen are normally in a quiescent state. However, angiogenic stimuli can induce EC to sprout from a blood vessel and proliferate (16). In the previous study, we have devised a convenient competence assay to evaluate the effects of growth factors on the competence phase of cell proliferation with two indices, the starting time of [3 H]-thymidine incorporation and its incorporation rate; Our study indicated that the treatment with bFGF (1, 10 and 30 ng/ml) for 12 hr promotes initiation of [3 H]-thymidine incorporation into aortic EC (17, 18). Furthermore, the present flow cytometric analysis demonstrated that the treatment with bFGF increased the population at the G_1 phase, compared with the control without bFGF. These results provide evidence that bFGF is a competence factor in rat aortic EC. It is considered that bFGF-induced competence may cause the abnormal proliferation of EC in angiogenic diseases.

In the previous flow cytometric analysis of smooth muscle cells from rat aorta, high levels of Ki-67 antigen expression are observed at the G_0 , $G_{1, \text{early}}$, S and G_2/M phases, whereas there was only a low level at the $G_{1, \text{late}}$ phase (19). In contrast, the Ki-67 expression in EC of rat aorta was negative level and hardly detected at G_0 , S and G_2/M phases, despite high level at only G_1 phase. The same phenomena in bovine EC were reported by Antoine et al. (20). These results suggest that the expression of other proteins in addition to Ki-67 proteins may also be involved in EC proliferation.

In conclusion, we provide evidence that bFGF stimulates the entry of EC into the G_1 phase from the G_0 phase, as a competence factor.

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