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NF-κB Is Induced in the Nuclei of Cultured Rat Aortic Smooth Muscle Cells by Stimulation of Various Growth Factors

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We investigated whether induction of transcription factor NF-κB is involved in the proliferation of cultured rat aortic smooth muscle cell using electrophoretic mobility shift assay and immunocytochemistry. NF-κB was induced in the nucleus in a dose-dependent manner when the smooth muscle cells were stimulated by various growth factors such as PDGF-BB, bFGF, EGF and IGF-1, but not growth inhibitors such as TGF-β and IFN-γ. Among growth factors, PDGF-BB and bFGF, more potent growth stimulators, induced higher κB binding activity than EGF or IGF-1. These evidences were also supported by the results obtained with immunocytochemistry. Immunocytochemistry also showed that the induced NF-κB contained p50 and p65. These results suggest that NF-κB induction may be involved in the proliferation of vascular smooth muscle cell.

Angioplasty is now recognized as a beneficial procedure to open clogged arteries. However, restenosis following angioplasty remains a crucial problem to be resolved and we in fact do not have any useful devices or agents to prevent restenosis. Proliferation of vascular smooth muscle cell has been shown to play an important role in the restenosis (1) and a lot of factors have been reported to stimulate the proliferation of smooth muscle cell in vivo and in vitro (2). However little is known on intracellular signaling pathway after these stimulation, especially on transcription factors influencing final events, growth related gene expression in smooth muscle cells.

NF-κB was originally identified as a transcription factor bound on the enhancer element of the κ light chain gene of B lymphocytes. Recently NF-κB has been found to be present in other cells and implicated in control of transcription of several genes involved in immune and inflammatory responses, cell growth and adhesion, including IL-2, IL-6, IL-8, GM-CSF, TNF-α, IFN-β and γ, VCAM-1, ICAM-1, ELAM-1, NOS, tissue factor and c-myc genes. In addition, it has been demonstrated that NF-κB activity comprised of several proteins such as p50, p52, p65, c-rel and Rel B, with differential regulation of their induction (3). Although NF-κB has been extensively investigated in hematopoietic cells, its activity and function in vascular smooth muscle cells have been incompletely elucidated.

These evidences led us to investigate whether NF-κB induction is involved in the proliferation of vascular smooth muscle cell. We assessed induction of NF-κB in smooth muscle cells stimulated by several growth factors, known as potent growth stimulators for smooth muscle cells, using electrophoretic mobility shift assay and immunocytochemistry.

MATERIALS AND METHODS

Chemicals. As mitogens for vascular smooth muscle cells, we employed platelet derived growth factor-BB (PDGF-BB; Becton Dickinson, Bedford, MA), basic fibroblast growth factor (bFGF; kindly provided by Takeda pharmaceutical

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company), epidermal growth factor (EGF; Sigma, St. Louis, MO), and insulin like growth factor-1 (IGF-1; Mallinckrodt Specialty Chemicals Co., Paris, KY). For growth inhibitors we used transforming growth factor-β (TGF-β; King Brewing, Kanagawa, Japan) and interferon-γ (IFN-γ; Chemicon, Temecula, CA).

Cell culture. Smooth muscle cells were explanted from a medial layer of rat aorta as previously described (4). They were cultured in M-199 medium (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) supplemented with 10% FBS (Gibco, Grand Island, NY), penicillin (100 U/ml), and streptomycin (100 μg/ml). Smooth muscle cells from the third to ninth passages were used for experiments. They were first starved with 0.1% FBS for 72 hours and then treated with several agents for experiments.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay was carried out with the method as described previously (5). In brief, nuclear protein extracts were prepared according to the method of Stein et al. (6). DNA probes included synthetic 27-bp oligonucleotides containing the κB enhancer in the flanking regions of the human interleukin-2 receptor α (IL-2Rα) gene (5′-CAACGGCAGGGGAATCTCCCTCTCCTT-3′). Double-stranded radiolabeled oligonucleotide probes were prepared by primer extension in the presence of ^{32}P-labeled (dATP and dCTP) using 8-bp primers and the Klenow fragment of DNA polymerase I.

Immunocytochemistry. Immunocytochemistry was performed according to the method as described previously (4). Briefly, smooth muscle cells were seeded in chamber slides and treated with various agents. They were then washed with PBS and fixed with 1% paraformaldehyde and 0.1% Triton X-100 in PBS for 30 min. Mouse monoclonal antibodies against p50 and p65 (Santa Cruz Biotechnology, Santa Cruz, CA) were used. Immunoreactivity was visualized with the indirect immunoperoxidase ABC method.

Cell proliferation assay. To examine the proliferation of smooth muscle cell, 3 μCi/ml of [^{3}H]-thymidine (NEN Research Products, Boston, MA) was added to wells after treatment with any agents for 24 hours (7), employed the exact protocol used for electrophoretic mobility shift assay and immunocytochemistry. After cells were incubated for an additional 4 hours, They were fixed with ice-cold 10% TCA for 20 minutes and then rinsed twice with 0.5 ml of 10% TCA. They were then solubilized in 0.5 ml of 0.1N NaOH and TCA-insoluble fractions of cell lysates were counted in a liquid scintillation counter (TRI-CARB 4430, Packard Instrument, Downers Grove, IL).

RESULTS

Electrophoretic Mobility Shift Assay

To determine whether growth factors stimulate κB binding activity in smooth muscle cells, cultures starved with 0.1% FBS for 72 hours were treated with several growth stimulators or inhibitors for varying times. We have previously reported that two bands, termed B1 and B2, were found in phorbol ester-activated Jurkat T cells with electrophoretic mobility shift assay, and that the more rapidly migrating B2 complex was composed of the P50 and partial degraded products of P50, in contrast the more slowly migrating B1 complex contained not only these smaller proteins but also p65 and c-rel (5). In a preliminary study, we confirmed that two bands obtained in smooth muscle cells stimulated with 10% FBS were in the same position as those in phorbol ester-activated Jurkat T cells (data not shown). Electrophoretic mobility shift assay with the nuclear extract from smooth muscle cells starved with 0.1% FBS for 72 hours showed a faint B1 and B2 bands (Fig. 1). When cultures were stimulated by 10% FBS, κB binding activity was increased 30 min after stimulation (B1 and B2 complexes; Fig. 1). Growth factors such as PDGF-BB, bFGF, EGF and IGF-1 induced κB binding activity at 1 to 3 hours after stimulation, however growth inhibitors such as TGF-β and IFN-γ did not Fig. 1). These growth factors increased κB binding activity in a dose-dependent fashion (Fig. 2) and the increased κB binding activities were transient, with the peak at approximately 3 hours after stimulation. Among these 4 growth factors, PDGF-BB induced the highest κB binding activity and IGF-1 the lowest at a same dose (Fig. 1, 2). We verified that the formation of both the B1 and B2 complexes was blocked by the addition of a 100 fold excess of unlabeled wild type, but not mutant, IL-2Rα κB oligonucleotides, indicating the sequence-specific nature of these DNA-protein interactions (data not shown).

Immunocytochemistry

To clarify which component of rel family members contributes to the complexes, we performed immunocytochemistry with antibodies for p50 and p65. Starved smooth muscle cells

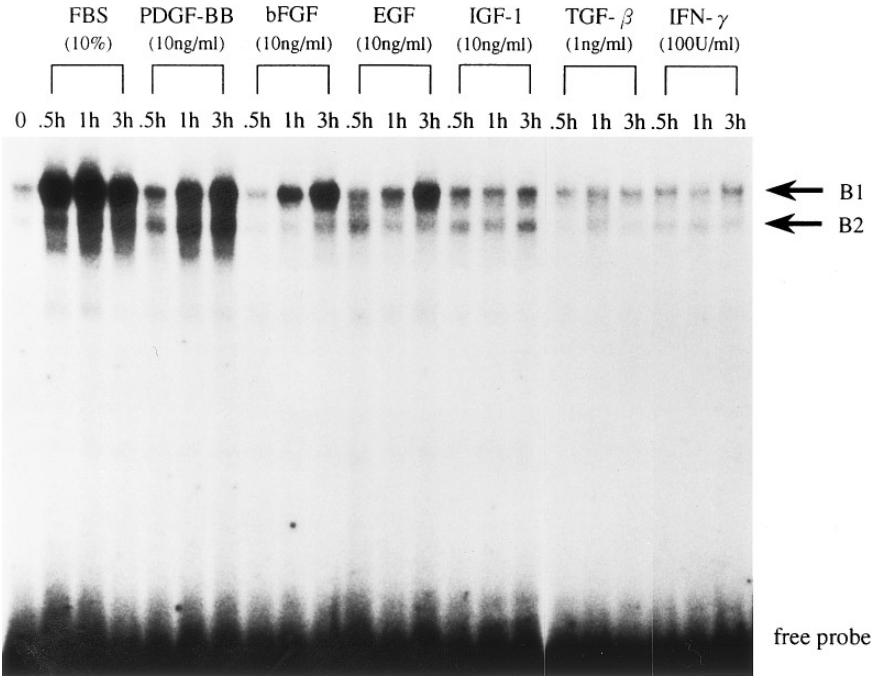

FIG. 1. Electrophoretic mobility shift assay showing the induction of κB binding activity after treatment with various agents. Cultured smooth muscle cells were starved with 0.1% FBS for 72 hours. The cells were then treated with various growth factors or cytokines. Nuclear protein extracts were obtained at varying time and electrophoretic mobility shift assay was performed. B1 and B2 denote the principle inducible κB specific DNA–protein complexes.

had a little immunoreactivity for p50 in the nucleus, but not p65 (Fig. 3). On the other hand, in the cytoplasm, they showed moderate amount of immunoreactivity for p50 and p65. Once they were stimulated by 10% FBS, PDGF-BB, bFGF, EGF or IGF-1, nuclear staining was prominent (Fig. 3). However, significant changes of immunoreactivity in the cytoplasm before and after stimulation were not detected. Specificity for immunoreactivity was verified using non-immune serum IgG.

Cell Proliferation Assay

We carried out [^{3}H]-thymidine incorporation assay, with the exact protocol used for electrophoretic mobility shift assay, to investigate whether degrees of NF-κB induction are related to growth stimulative activity. As shown in Fig. 4, PDGF-BB stimulated DNA synthesis about 4 times more than that in control, bFGF about 3 times, EGF about 2 times and IGF-1 about 1.2 times. As expected, TGF-β and IFN-γ did not increase [^{3}H]-thymidine incorporation into the cells.

DISCUSSION

In the present study, we demonstrated that NF-κB was induced in nuclei of smooth muscle cells stimulated by various mitogens such as PDGF-BB, bFGF, EGF and IGF-1, but not by inhibitory agents such as TGF-β and IFN-γ. Furthermore, we clarified that induced NF-κB in the nuclei of smooth muscle cells consisted of p50 and p65 with differential expression pattern. When smooth muscle cells were starved with 0.1% FBS for 72 hours, immunocytochemistry revealed that p50 was slightly but significantly detected in the nucleus, but p65 was

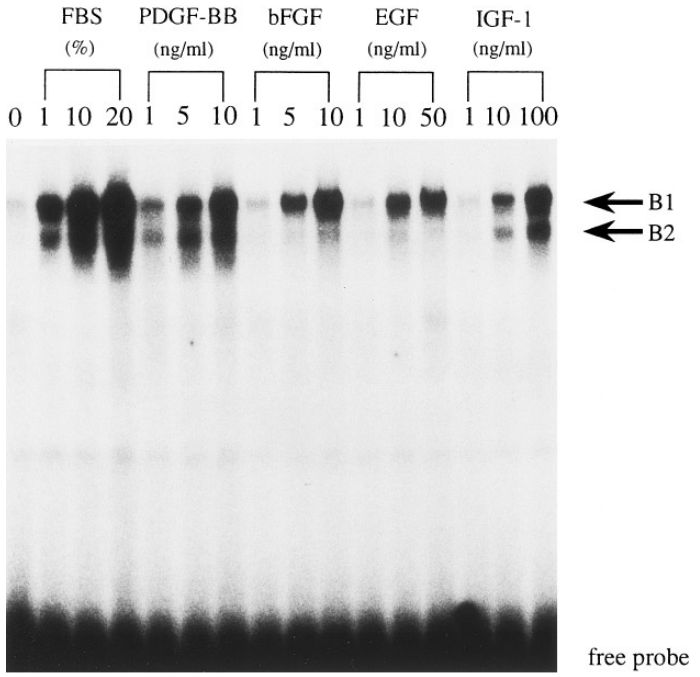

FIG. 2. Dose-dependent induction of κB binding activity by various growth factors. Cultured smooth muscle cells were stimulated by various growth factors for 3 hours. Nuclear extracts were prepared and electrophoretic mobility shift assay was performed. Various growth factors induced κB binding activities in a dose-dependent manner.

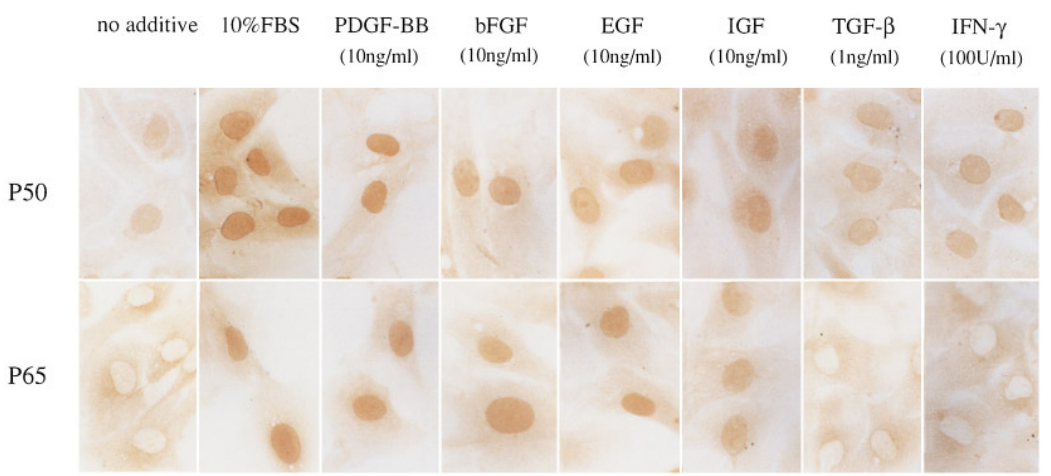

FIG. 3. Immunocytochemistry showing nuclear staining for κB binding proteins, p50 and p65, after treatment with several agents. Cultured smooth muscle cells were seeded in chamber slides, starved and treated with several agents for 3 hours. They were then fixed and stained with antibodies against p50 or p65. Nuclear staining was prominent for both p50 and p65 after stimulation with various growth factors.

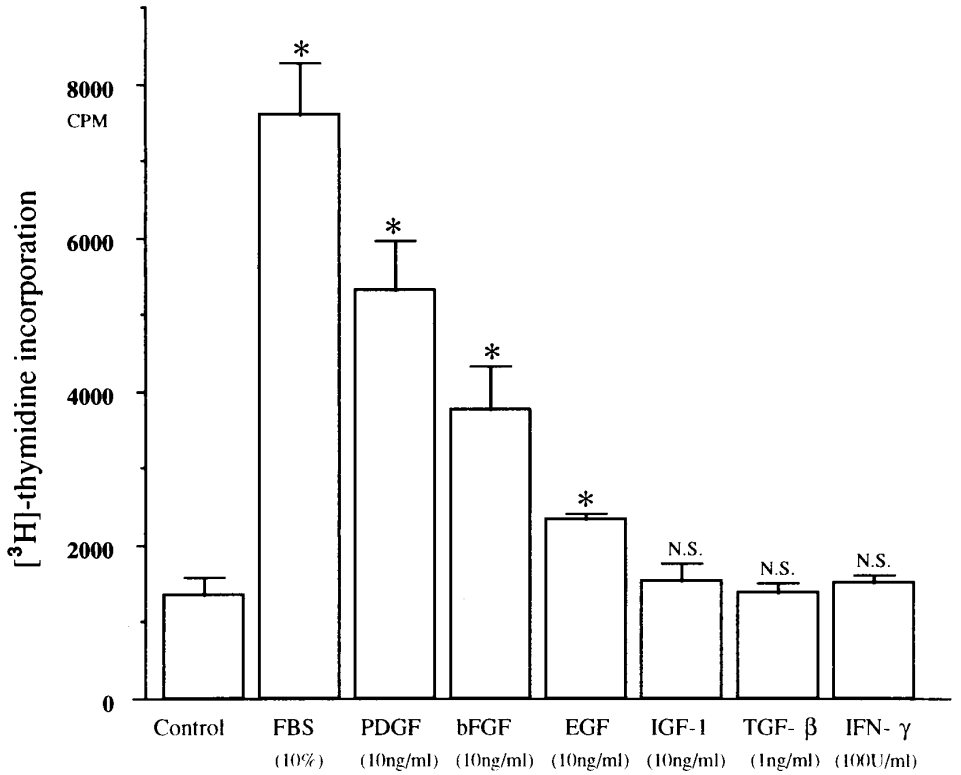

FIG. 4. [^{3}H]-thymidine incorporation after treatment with several agents. Cultured smooth muscle cells were starved and treated with several agents for 24 hours. They were then incubated with [^{3}H]-thymidine for 4 hours. [^{3}H]-thymidine incorporation of the cells was counted using a liquid scintillation counter.

not, suggesting that p50 is constitutively expressed in the nuclei of smooth muscle cells. When they were once activated, both p50 and p65 were strongly induced into the nuclei. These evidences were also supported by the results obtained with electrophoretic mobility shift assay. Interestingly, PDGF-BB and bFGF, more potent growth factors, as assessed by [^{3}H]-thymidine incorporation assay, exhibited higher κB binding activity than EGF or IGF-1 at a same concentration. These results strongly suggest that transcription factor NF-κB induction may be involved in the proliferation of vascular smooth muscle cells. Autieri et al. have reported that addition of p65 antisense oligonucleotides inhibited human vascular smooth muscle cell proliferation (8). Bellas et al. have reported that microinjection of purified IκB-α, specific inhibitors of NF-κB/Rel activity, or double-stranded oligonucleotides for NF-κB inhibited the proliferation of cultured bovine vascular smooth muscle cells (9). On the basis of our and their data, we conclude that transcription factor NF-κB induction is important in the proliferation of vascular smooth muscle cells.

Although NF-κB was first identified as an inducible B lymphocyte specific factor, it is now recognized a ubiquitously expressed factor present in most kinds of cells. Extensive studies on NF-κB have been performed using hematopoietic cells, however there have been only a few recent reports demonstrating that NF-κB played a role in some physiological processes in vascular smooth muscle cells. These include that fibronectin attachment activated NF-κB p50/p65 heterodimerization in smooth muscle cells (10), and the activation of NF-κB was involved in thrombin signaling (11) in addition to two reports described above. However, the

present study is the first to directly demonstrate the induction of NF-κB into the nucleus in vascular smooth muscle cells stimulated by several important growth factors.

NF-κB complexes are found mainly in the cytoplasm prior to stimulation, inactive form bound to a member of the IκB family of inhibitor proteins (IκB-α, β and γ, and Bcl-3 et al.). A wide range of stimuli, including viruses, antigens, lipopolysaccharide, or other stimuli, lead the NF-κB dimers to dissociate from the IκB molecules and translocate them to the nucleus, where they bind to κB sequence in active form (3). We clarified the induction of NF-κB into the nucleus of the smooth muscle cells after stimulation of growth factors with two methods, immunocytochemistry and electrophoretic mobility shift assay. In immunocytochemistry, we had expected to detect the change of immunoreactivity in the cytoplasm as well as in the nucleus before or after stimulation. We, however, could not observe the significant change in the cytoplasm. A possibility is that the amount of NF-κB translocated in the nucleus may be small compared with that stored in the cytoplasm.

In conclusion, transcription factor NF-κB is induced in vascular smooth muscle cells by stimulation of various growth factors, but not growth inhibitors and NF-κB induction may be involved in the proliferation of vascular smooth muscle cell.

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REFERENCES

1. Forrester, J. S., Fishbein, M., Helfant, R., and Fagin, J. (1991) *J. Am. Coll. Cardiol.* **17,** 758–769.
2. Ward Casscells (1991) *Prog. Growth Factor Res.* **3,** 177–206.
3. Thanos, D., and Maniatis, T. (1995) *Cell* **80,** 529–532.
4. Biro, S., Fu, Y. M., Yu, Z. X., and Epstein, S. E. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 654–658.
5. Arima, N., Molitor, J. A., Smith, M. R., Kim, J. H., Daitoku, Y., Greene, W. C. (1991) *J. Virol.* **65,** 6892–6899.
6. Stein, B., Rahmsdorf, H. J., Litfin, M., Herrlich, P. (1989) *Mol. Cell. Biol.* **9,** 5169–5181.
7. Miyata, M., Biro, S., Kaieda, H., and Tanaka, H. (1995) *FEBS lett* **377,** 493–496.
8. Autieri, M. V., Yue, T. L., Ferstein, G. Z., and Ohlstein, E. (1995) *Biochem. Biophys. Res. Commun.* **213,** 827–836.
9. Bellas, R. E., Lee, J. S., Sonenshein, G. E. (1995) *J. Clin. Invest.* **96,** 2521–2527.
10. Qwarnstrom, E. E., Ostberg, C. O., Turk, G. L., Richardson, C. A., Bomsztyk, K. (1994) *J. Biol. Chem.* **269,** 30765–30768.
11. Nakajima, T., Kitajima, I., Shin, H., Takasaki, I., Shigeta, K., Abeyama, K., Yamashita, Y., Tokioka, T., Soejima, Y., and Maruyama, I. (1994) *Biochem. Biophys. Res. Commun.* **204,** 950–955.