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Stimulation of Cell Growth by Erythropoietin in RAW264.7 Cells: Association with AP-1 Activation

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Erythropoietin (EPO), a hematopoietic factor, is required for normal erythrocyte developments, but it has been demonstrated to have many other functions, and its receptor is localized in other tissues. In the present study, we investigated whether EPO can promote other cell proliferation and possible molecular mechanisms. EPO restored the inhibition of the RAW264.7 and PC12 cell growth by fetal bovine serum (FBS) withdrawal in a dose dependent manner, but not that of other cell types tested. The restoring effect of EPO was completed when the RAW264.7 cells were cultured in the medium containing as low as 3% of FBS, and 10 U/mL EPO could replace FBS. The restoring effect of EPO in the RAW264.7 cells was associated with the increased of c-Fos and c-Jun expression as well as AP-1 activation. These data demonstrate that EPO can stimulate RAW264. 7 cell as well as PC12 cell growth even when the cells were cultured without FBS or in the presence of small amounts of FBS in the medium, and this stimulating effect is associated with the activation of AP-1 transcription factor.

Key words: EPO, Cell growth, AP-1, Erythropoietin

INTRODUCTION

In the human, erythropoietin (EPO) is a glycoprotein hormone that is produced by peritubular cells in the kidneys of the adult and in hepatocytes in the fetus (Fisher, 2003; Jelkmann, 2004). The primary function of the EPO is to promote red cell production from erythrocyte progenitors in hemopoietic tissues (Liboi *et al.*, 1993; Sasaki *et al.*, 2000). EPO binds to an erythroid progenitor cell surface receptor called EPO receptor. Activation of EPO receptor leads to tyrosine phosphorylation of the EPO receptor and several proteins such as c-Jun and c-Fos, which is involved with cell proliferation (Adunyah *et al.*, 1996; Damen, *et al.*, 1995; Farrel *et al.*, 2004). However, recent advances in analytical techniques have led to the demonstration of EPO-R mRNA, EPO-R protein, EPO binding to EPO-R, and intracellular signaling in a variety of nonhemopoietic cells and organs, including the brain, cardiovascular tissues (endothelium, vascular smooth muscle and cardiomyocytes), the liver, gastrointestinal tissues, pancreatic islands, the kidney, the testis, and the female reproductive organs. Thus, EPO is a more pleiotropic survival and growth factor than initially thought (Adunyah *et al.*, 1996; Bergelson *et al.*, 1998; Patel and Sytkowski, 1995).

Similar to the finding by Sugawa *et al.* (2002), we previously also found that recombinant human EPO markedly enhanced the proliferation and differentiation of astrocytes (Lee *et al.*, 2004). Sigounas *et al.*, also reported that EPO increase endothelial cell proliferation, and promoted IL-3 induced cell proliferation in a synergistic manner (1997). Proliferation of microglial cell by EPO was also observed by Vairano *et al.* (2002). Therefore, it is possible that EPO can facilitate cell proliferation of RAW264.7 cells which belongs to the macrophage lineage.

A plethora of physiological and pathological stimuli such as growth factors induce and activate a group of DNA binding proteins that form AP-1 dimers (Hess *et al.*, 2004; Shaulian and Karin, 2001). These proteins include the Jun and Fos of transcription factors. Recent studies using cells and mice deficient in individual AP-1 proteins have begun to shed light on their physiological functions in the control of cell proliferation (Shaulian and Karin, 2001). Bergelson found that EPO activates AP-1 transcription factor

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through activation of tyrosine kinase (1998). Furthermore in the study of cell proliferation by EPO using murine erythroid cell line HCD57 as a model cell system for the study of erythroid proliferation, it was reported that AP-1 DNA-binding activity was increased during promotion of cell proliferation by EPO (Jacobs-Helber *et al.*, 1997, 1998). EPO also induces the expression of early response genes c-jun and c-fos, and induces activation of AP-1 during cell growth of hyman erythroleukemia K562 (Adunyah *et al.*, 1996) as well as in both transformed and normal erythroid cells (Patel and Sytkowski, 1995). In this experiment, we designed to study whether EPO may act as growth factor or have promoting effect on cell growth, and what the possible mechanisms are involved in several cells.

MATERIALS AND METHODS

Cell culture

RAW264.7, a mouse macrophage-like cell line was obtained from the American Type Culture Collection (Cryosite, Lane Cove, NSW, Australia). Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin, and fetal bovine serum were purchased from Gibco Life Technologies (Rockville, MD, U.S.A.). RAW264.7 cells were grown in DMEM with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in 5% CO_2 humidified air.

WST-8 assay

The growth of cells was assessed by WST-8 reduction assay kit (Dojindo Laboratories, Tokyo, Japan) according to the instructions of the manufacturer. WST-8 was added to the culture and incubated for 3 h at 37°C prior to the colorimetry. Percentage of WST-8 reduction activity was represented as the ratio of activity at different time points. Cell growth was obtained by scanning with a microplate reader at 450 nm.

Activation of AP-1

Gel mobility shift assay was done using a slight modification of a previously described method (Jung *et al.*, 2003). Briefly, the cultured cells were washed three times with ice-cold phosphate buffered saline (PBS, pH 7.6) and pelleted. The pellets were resuspended in 400 μL of cold buffer containing 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and then centrifuged at 11,000 × g for 4 min to remove everything except the nuclei. The pellets were resuspended in a second buffer containing 20 mM HEPES, 20% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.2 mM PMSF. After centrifugation at 11,000 × g for 6 min, the supernatant contained the nuclear proteins. The protein level was determined by a microplate modification of the Bradford method (Bio-Rad Bulletin 1177, Bio-Rad Lab., Richmond, CA). The DNA binding activity of transcription factors was assayed according to the manufacturer's instructions (Promega Co., Madison, WI). In brief, 10 μg of nuclear protein was incubated in 25 μL total volume of incubation buffer (10 mM Tris pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 4% glycerol, 0.08 mg/mL salmon sperm DNA) at 4°C for 15 min followed by another 20 min incubation with 100 μCi [γ-^{32}P] ATP-labeled oligonucleotide containing AP-1 binding site at room temperature. For the competition assay, 50x, 100x or 200x excess of unlabeled double-stranded oligonucleotide of the AP-1 binding site were used as specific competitors. 200x excess of labeled double-stranded oligonucleotide of the SP-1 binding site was used as a nonspecific competitor. Ten μg of antibodies to c-jun, c-fos were added to the binding reaction for the supershift assay of AP-1. The DNA-protein binding complex was run on a 6% non-denatured polyacrylamide gel at 150 volts for 2 h. Gels were dried and autoradiographed using Kodak MR film at -80°C overnight.

Western blotting

Nuclear extracts were obtained RAW264.7 cell for a Western blotting (c-Jun/c-Fos) as previously described (Jung *et al.*, 2003). Equal amounts of proteins (20 ig) were separated on a SDS-polyacrylamide gel (12%) and then transferred to a nitrocellulose membrane (Hybond ECL, Amersham .Pharmacia Biotech, Piscataway, NJ). Blots were blocked for 2 h at room temperature with 5% (w/v) non-fat dried milk in Tris-buffered saline [10 mM Tris.HCl (pH 8.0) and 150 mM NaCl] containing 0.05% Tween 20. The membrane was then incubated for 3 h at room temperature with specific antibodies. Rabbit polyclonal antibodies against c-Jun and c-Fos (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), were used in this study, at dilutions specified by the manufacturer. The blot was then incubated with the corresponding conjugated anti-rabbit immunoglobulin G-horseradish peroxidase (Santa Cruz Biotechnology). Immunoreactive proteins were detected with the ECL Western blotting detection system. The relative density of the protein bands was quantified by densitometry using Electrophoresis Documentation and Analysis System 120 (Eastman Kodak, Rochester, NY).

RESULTS

Effect of EPO on the cell proliferation of RAW264.7 cultured in FBS free medium

We investigated whether EPO may restore cell proliferation of RAW264.7 cells cultured in FBS free medium.

We conducted this experiment in different time periods and concentrations of EPO of cell culture. Cells were plated at a density of 10^3 cells/well in 96-well plates and cultured in DMEM with 10% FBS at 37°C for 24 h. To study effect of EPO on cell proliferation in serum free medium, the cells were then further cultured on medium with or without FBS for 24, 48 and 72 h, respectively. Twenty-four hours before cell viability assay, the cells were treated with several dose of EPO (1-20 U/mL) or vehicle. Finally cell proliferation was determined by a manufactured cell counting kit as well as morphological observation. In this study, we discovered that EPO treatment on FBS free medium restored cell proliferation of RAW264.7 cells in dose-dependent (Fig. 1 and Fig. 3 A). We also investigated this EPO effect can be occurred in other cells. Since PC12 cells could be grown in the medium containing 5% FBS as well as 10% Horse Serum (HS), we investigated whether EPO can stimulate PC12 cell growth cultured either in FBS and HS free medium. As seen in Fig. 2 and Fig. 3B, EPO restored cell growth of PC12 cells cultured FBS free medium, but EPO could not restore cell growth culture HS free medium. This stimulating effect of cell growth, however, was not achieved in other cells such as THP-1, a monocyte derived cells and SK-N-SH, neuroblastoma derived cells (Fig. 3C and D).

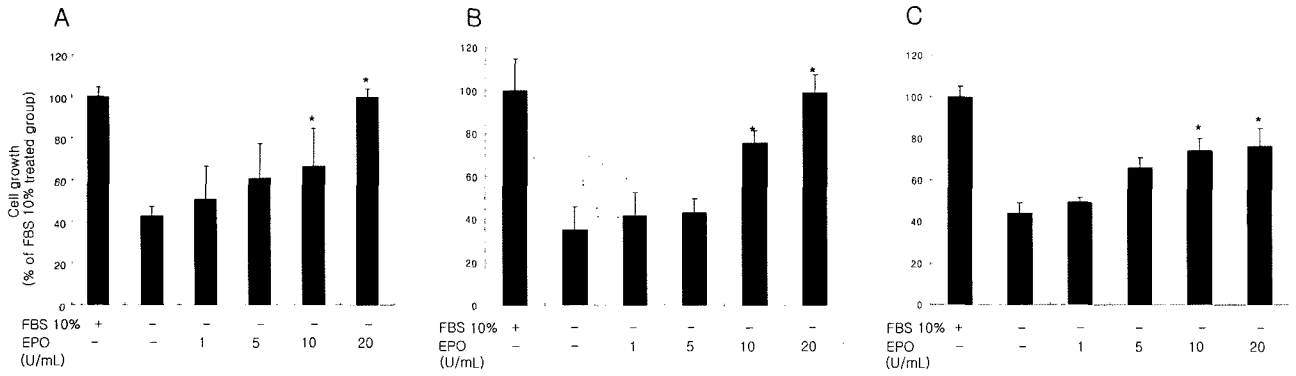

Fig. 1. Cell growth and morphological changes of RAW264.7 cells. RAW264.7 cells were seeded at a density of 10^3 cells/well in 96-well plates and cultured in DMEM with 10% FBS for 24 h. Medium was replaced with or without FBS, and then cells were treated with several dose of EPO (1-20 U/mL) or vehicle for 24 h (A), 48 h (B) and 72 h (C), respectively. Cell growth was determined by WST-8 assay as described under Materials and Methods. The values are mean ± S.D. of three experiments, with triplicate of each experiment. *P<0.05 indicate significantly different from the FBS withdrawal cells without EPO.

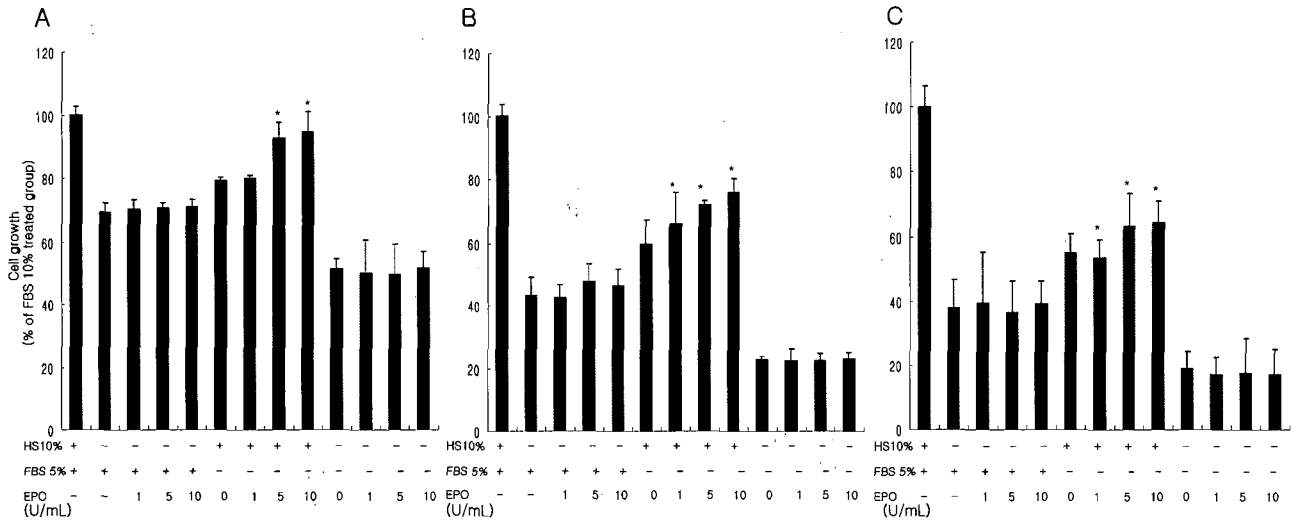

Fig. 2. Cell growth and morphological changes of PC12 cell. PC12 cells were seeded at a density of 10^3 cells/well in 96-well plates and cultured in DMEM with 10% HS and 5% FBS for 24 h. Medium was replaced with or without HS and FBS or both, and then cells were treated with several dose of EPO (1-20 U/mL) or vehicle for 24 h (A), 48 h (B) and 72 h (C), respectively. Cell growth was determined by WST-8 assay as described under Materials and Methods. The values are mean ± S.D. of three experiments, with triplicate of each experiment. *P<0.05 indicate significantly different from the FBS withdrawal cells without EPO.

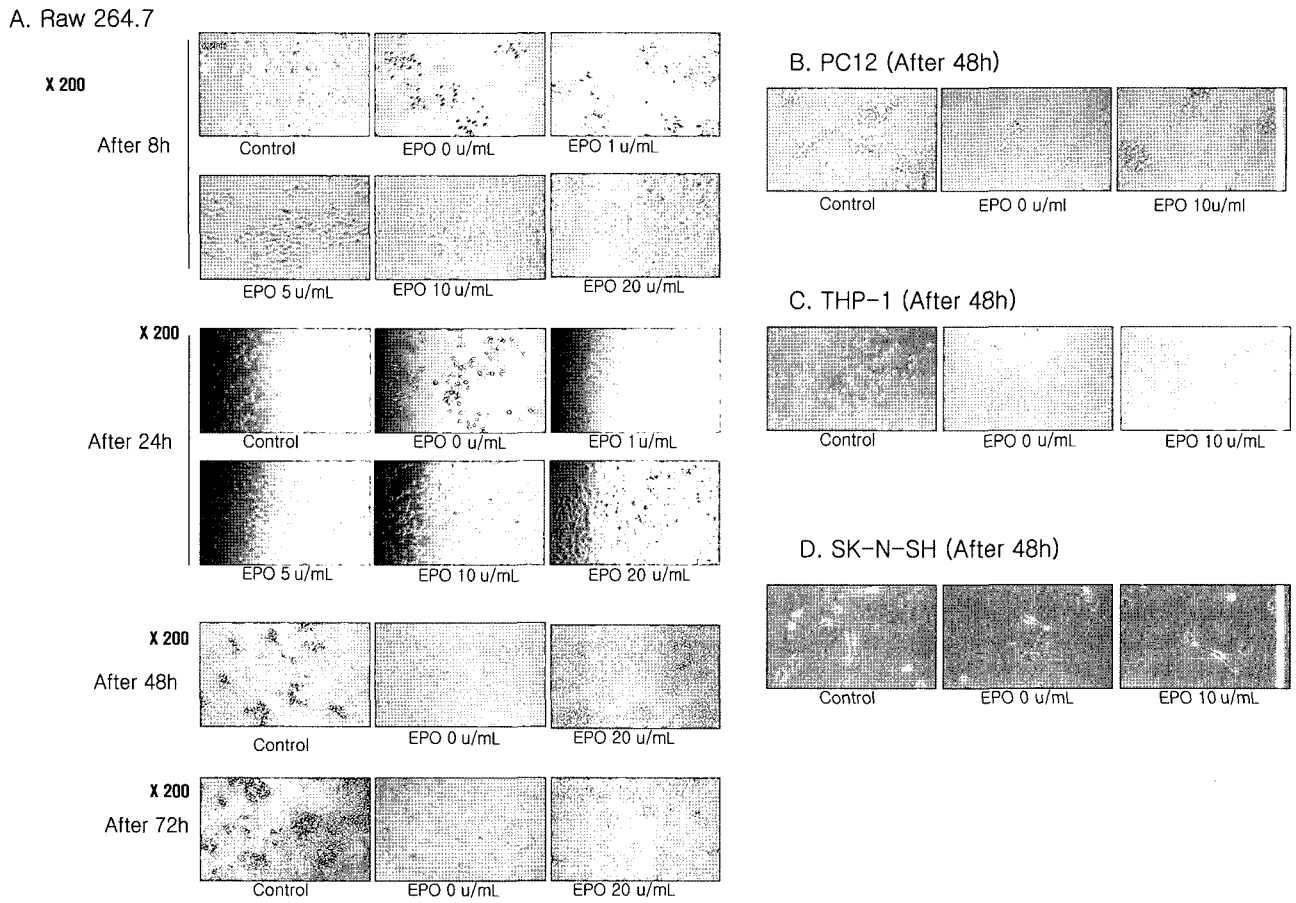

Fig. 3. Cell growth and morphological changes by EPO. Cells were seeded at a density of 10^3 cells/ well in 96-well plates and cultured in DMEM with 10% FBS (10% HS and 5% FBS in the PC12 cells) for 24 h. Medium was replaced with or without FBS (with HS in the PC12 cells), and then cells were treated with several dose of EPO (1-20 U/mL) or vehicle for 8-72 h in RAW264.7 cells (A), or 48 h in other cells (B-D), respectively. Cell growth was observed and morphological changes were pictured under microscope (magnification, 200X) between 8-72 h or 48 h.

Replacement of FBS with EPO on RAW264.7 cells proliferation

We next designed the culture of RAW264.7 cells treated with FBS at varied concentrations (between 1 and 10% of FBS in the total medium) of FBS, and further cultured on medium with or without EPO 10 U/mL for 48 h. As shown Fig. 4, RAW264.7 cells treated by EPO with 2-3% FBS on 48 h culture showed as much cell growth as on medium with 10% FBS without EPO, suggesting that EPO can replace the effect of FBS in cell growth of RAW264.7 cells.

Induction of AP-1 activation

In the present study, to investigate a possible mechanism of RAW264.7 cell proliferation by EPO, we focused on the potential role of AP-1 (c-Jun/c-Fos) in mediating the cell proliferation by EPO. Nuclear extracts were obtained in order to observe translocation of AP-1 (c-Jun/c-Fos) into nucleus by EPO. An electrophoretic mobility shift assay (EMSA) and Western blotting are used for AP-1 (c-Jun/c-Fos) detection. In a preliminary experiment, we demonstrated the most significant activation of AP-1 (c-Jun/c-Fos) at 2 h after the EPO (10 U/mL) treatment in RAW264.7 cell. Thus we treated several concentration of EPO (1-10 U/mL) on RAW264.7 cells cultured in medium without FBS for 2 h, and then the AP-1 activity was determined. As shown in Fig. 5A, it represented that AP-1 in RAW264.7 cells cultured in medium with FBS was activated, and dramatically down regulated when the cells were cultured in FBS free medium as related with the cell growth inhibition. However, once cells were replaced with EPO, the AP-1 activity was increased. The change in the activity of AP-1 was correlated with the expression of c-Jun and c-Fos (Fig. 5B). These observations strongly suggest that AP-1 plays as an important intracellular signal in the EPO-mediated cell proliferation in RAW264.7 cells.

DISCUSSION

EPO is firstly regarded having an effect to inhibit

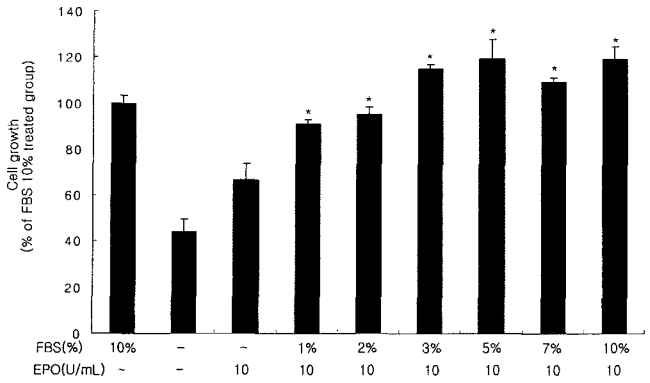

Fig. 4. The replacement of FBS with EPO on RAW264.7 cell proliferation. RAW264.7 Cells were seeded at a density of 10^3 cells/well in 96-well plates and cultured in DMEM with 10% FBS at for 24 h. RAW264.7 cells were then cultured with FBS at various concentrations between 1 and 10% of total medium and further cultured in medium with or without EPO 10 U/mL 48 h. Cell growth was determined by WST-8 assay as described under Materials and Methods. The values are mean ± S.D. of three experiments, with triplicate of each experiment. *P<0.05 indicate significantly different from the FBS withdrawal cells without EPO.

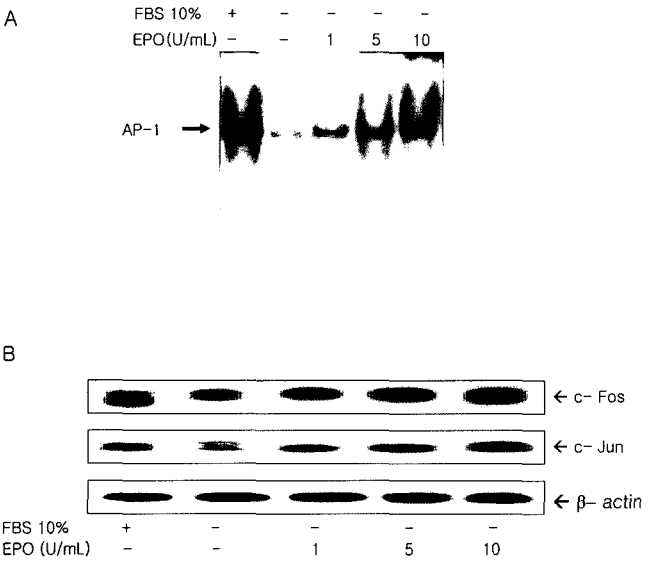

Fig. 5. Effect of EPO on the DNA binding activity of AP-1 and related gene expression in RAW264.7 cells. Nuclear extracts were prepared from RAW264.7 cells, which were incubated with several dose of EPO for 1 h. Gel mobility shift assay was done with nuclear protein extracted from 1 h exposed cells (A). Equal amounts of whole nucleus lysates were subjected to electrophoresis and analyzed by western blot for cell proliferation regulatory molecules (c-Jun, c-Fos). Levels of proteins were determined by using specific primary antibody followed by peroxidase-conjugated secondary antibody and visualization by enhanced chemiluminescence detection system (B). Figure is representative of three experiments.

apoptosis of erythrocytic progenitors and stimulate young red blood cells to grow. Resent researches in analytical techniques, however, have demonstrated EPO-R mRNA, EPO-R protein, EPO binding to EPO-R, and intracellular signaling in diverse nonhemopoietic cells and organs (Jelkmann and Wagner, 2004). The present study demonstrated that EPO can stimulate RAW264.7 cells as well as PC12 cells, and this cell growth stimulating effect may replace the usage of FBS (at least, the amount of FBS can be reduced) in the cell culture medium. We previously found that EPO stimulated the proliferation/and differentiation of embryonic stem cells into astrocytes, a macrophage class cell in the brain (Lee *et al.*, 2004). This cell growth promoting effect was also found in PC12 cells when this cell was cultured in FBS free medium, but not HS free medium, suggesting that EPO can replace growth factors in FBS. However, we did not found the cell growth promoting effect in other cells such as THP-1 and SK-N-SH cells. We do not know why other cells would not respond to EPO in the present study, but it is possible that the cell growth stimulating effect of EPO may be cell type specific. Alternatively, EPO may have different functions depending on cell types. It is noteworthy to understand that EPO stimulates cell growth in PC12 cells but it did not stimulate cell differentiation (data not shown). However, EPO stimulates embryonic stem cell differentiation into astrocytes but not into neuron (unpublished data). We recently also found that EPO can produce nerve growth factor (NGF) in astrocytes which can stimulate proliferation of astrocytes, but stimulate neuronal cell differentiation (unpublished data). We did not determine some of soluble growth factor which may be produced in RAW264.7 cells as well as PC12 cells by EPO as seen in astrocytes, but we can not exclude this possibility. Therefore, three results suggest that EPO may stimulate cell growth and differentiation directly (autocrine effect) or may produce cell specific soluble growth factors which modulate cell growth and differentiation in a paracrine way. Even though, we did not directly demonstrate the critical growth factor derived by EPO in RAW264.7 cells and PC 12 cells, it is possible that EPO can stimulate cell growth in a cell type specific manner, and can replace FBS in the medium of some cell culture.

We also found that AP-1 was activated in the full grown RAW264.7 cells, but it was reduced by the withdrawal of FBS in accord with the change of cell growth. However, after treatment of EPO, AP-1 activation was restored with accompaniment of the cell growth. The change in AP-1 activation may be due to the change of the expression of c-Fos. This result is similar to the result reported by Bergelson showing that AP-1 activation was increased by EPO in HCD57 cells, a hematopoietic cell (1998). They demonstrated that the AP-1 DNA binding activity observed in HCD57 cells corresponded to a biological function of AP-1 in these cells. Inhibition of this activity was associated with cell growth inhibition demonstrated by using

dominant negative AP-1 mutant TAM-67 transfected HCD57 cells. The dramatic inhibitory effect on the rate of proliferation under the presence of the AP-1 inhibitor suggests that AP-1 may play a central role in the regulation of EPO-dependent growth and survival in these erythroleukemia cells. It has been reported that EPO drived differentiation of immature erythroid cells, colony-forming units-erythroid cells (CFU-Es), proerythroblasts, and a number of erythroid cell lines (Adachi and Saito, 1992; Sawyer and Penta, 1994; Wojchowski and He, 1993). There is a growing amount of evidence that Jun may play a role in the regulation of cell growth. c-Jun can homodimerize and heterodimerize with other AP-1 factors, and then strongly transforms rat embryo fibroblasts (Schutte *et al.*, 1989). We also demonstrated that stimulation of RAW264.7 cell proliferation by EPO is accompanied by an immediate increase in AP-1 activity. This result is consistent with a previously published report which showed an EPO-induced increase in AP-1 DNA binding activity in a cell line (Patel and Sytkowski, 1995). Therefore, increase of c-Jun and Fos protein expression may be important in the activation of AP-1, and this signal may be critically involved in EPO-induced cell proliferation. Collectively, EPO can promote cell growth in a cell type specific manner, and the promoting effect in cell growth is associated with AP-1 signal.

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