Role of Interleukin-6 in the Control of DNA Synthesis of Hepatocytes: Involvement of PKC, p44/42 MAPKs, and PPAR δ

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Key Words

Interleukin-6 • PKC • p44/42 MAPKs • PPAR δ • Hepatocytes proliferation

Abstract

Interleukin-6 (IL-6) is a pleiotropic cytokine with a pivotal role in normal hepatic growth and liver regeneration. Therefore, in the present study, we examined the effect of IL-6 on cell proliferation and the related signaling pathways in primary cultured chicken hepatocytes. IL-6 increased the level of [³H]thymidine incorporation in a time (≥ 6 hr)- and a dose (≥ 0.1 ng/ml)-dependent manner. Indeed, IL-6 increased the number of BrdU-positive cells and the total number of cells. IL-6 (10 ng/ml) increased the level of IL-6R α and glycoprotein (gp) 130 (IL-6R β) protein expression, Janus Kinase (JAK) 2, signal transducer and activator of transcription (STAT) 3, PKC, p44/42 MAPKs phosphorylation, and PPAR δ protein expression. Inhibition of each pathways blocked IL-6-induced [³H]thymidine incorporation increase. IL-6 increased c-fos, c-jun, and c-myc proto-oncogene mRNA levels and the percentage of cells in the S phase according to fluorescence-activated cell sorter (FACS) analysis. IL-6-induced G1/S phase

progression was inhibited by AG 490 (2×10^{-5} M, JAK2 inhibitor), a STAT3 inhibitor peptide (10^{-5} M), bisindolylmaleimide I (10^{-6} M, PKC inhibitor), PD 98059 (10^{-5} M, p44/42 MAPKs blocker), or PPAR δ -specific small interfering RNAs (siRNAs). In conclusion, IL-6 stimulates the proliferation of primary cultured chicken hepatocytes through PKC, p44/42 MAPKs, and PPAR δ pathways.

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Introduction

IL-6, originally identified as hepatocyte-stimulatory factor, is a typical pleiotropic cytokine that modulates a variety of physiological events such as cell proliferation and differentiation and regulates specific gene expression, particularly the expression of the acute phase proteins (APP) in liver cells [1]. Recently, Zimmers et al. [2] reported that systemic IL-6 administration in nude mice results in massive hepatomegaly and hepatocyte hyperplasia in the absence of hepatic injury. This is the evidence of IL-6 acting as a complete mitogen in the liver rather than in its established role as a mediator of priming and cytoprotective functions during the initiation of liver regeneration. In vitro and in vivo data pertaining

to the effects of IL-6 on hepatocyte proliferation are controversial [2]. Kuma et al. [3] and Ren et al. [4] reported that in vitro treatment with IL-6 stimulated primary mouse hepatocyte proliferation, while other studies reported that IL-6 failed to affect primary hepatocyte proliferation [5] or inhibited proliferation of primary mouse [6, 7] and rat [8] hepatocytes. In vivo, injection of recombinant IL-6 protein alone in mice in the absence of hepatectomy has not been demonstrated to produce hepatocyte proliferation [9], although administration of IL-6 followed by NH₄Cl and glucagons has been observed to increase hepatocyte mitoses in intact rat livers [10]. IL-6 is involved in mediating cell cycle progression directly as well as activation of signaling pathways [11]. Recently, many studies are focusing on the signaling pathways that allow hepatocytes to maintain most of their homeostatic functions and proliferate at the same time. STAT3 as well as AP1 and HNF1 interact adaptively to liver injury by amplifying hepatic genes to maintain metabolic homeostasis. However, despite multiple studies of liver proliferation, many aspects of this phenomenon remain to be further understood and the role of IL-6 in liver biology is still an emerging area of investigation. Thus, in the present study, we will focus on IL-6 signaling pathways, which we believe are central for future development of new therapeutic reagents and strategies to maintain liver function and enhance the natural capacity for regeneration of the failing liver.

A primary culture of hepatocytes has been used in many biophysiological studies of liver function because it retains many liver-specific functions and responds to various hormones through the expression of the liver-specific functions [12, 13]. The primary chicken hepatocytes culture system used in this study also retains the in vitro differentiated phenotype that is typical of the liver, including albumin expression [14], P450 1A induction [14], tyrosine aminotransferase expression [15], and ascorbate recycling [16]. The fully differentiated hepatocytes continue to carry the burden of maintaining homeostasis for the entire body and at the same time, restoring liver mass. Therefore, we aimed to determine the effects of IL-6 on DNA synthesis of primary cultured chicken hepatocytes and its related signaling pathways.

Materials and Methods

Materials

Two-week-old male White Leghorn chickens were obtained from Dae Han Experimental Animal Co, Ltd. (Chungju, Korea). All animal management procedures were performed

according to the standard protocols at Seoul National University. Appropriate management of the experimental samples and quality control of the laboratory facility and equipment were maintained. A STAT3 inhibitor peptide and AG 490 were purchased from Calbiochem (La Jolla, CA, USA). Fetal bovine serum (FBS) was supplied by Gibco (Rockville, MD, USA). Interleukin-6, bisindolylmaleimide I, PD 98059, class IV collagenase, and monoclonal anti- β -actin antibody were obtained from Sigma Chemical Company (St. Louis, MO, USA). The cyclinD1, cyclinE, CDK4, CDK2, p21, p27, phospho-Rb (ser 780), IL-6R α , gp130, phospho-STAT3, STAT3, JAK2, PKC-pan, - α , - δ , - θ , - ζ , phospho-p44/42 MAPKs, p44/42 MAPKs, PPAR δ , lamin, and goat anti-rabbit IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The phospho-JAK2 and phospho-pan PKC antibodies were obtained from Cell Signaling (Beverly, MA, USA). Liquiscint was obtained from National Diagnostics (Parsippany, NY, USA). All other reagents were of the highest purity commercially available.

Primary culture of chicken hepatocytes

The chicken hepatocytes were prepared and maintained as a monolayer culture, as described elsewhere [14]. Briefly, the chicken hepatocytes were isolated from a liver that had been starved for 24 hr by perfusion with 0.05% collagenase. Hepatocytes with > 90% viability, as verified by a trypan blue exclusion test, were used for subsequent plating. The hepatocytes were plated (5.0×10^5 cells/ 60-mm collagen-coated dish) in an incubation medium (William's medium) containing 75 U/ml penicillin and 75 U/ml streptomycin, 1 μ g/ml insulin, 10^{-12} M dexamethasone, 5 μ g/ml transferrin, 10^{-8} M T₃, and 5% FBS, and incubated for 4 hr at 37°C in 5% CO₂. The medium was changed one day after plating to achieve the monolayer culture and every two days thereafter. To confirm purity of hepatocytes, we measured the hepatocyte specific markers such as albumin, α -fetoprotein, and α -antitrypsin. Before the experiments, cells were incubated with fresh serum-free William's medium including all supplements for 24 hr to synchronize and then the experiments were carried out.

[³H]Thymidine incorporation

[³H]thymidine incorporation experiments were carried out as described by Brett et al. [17] and Zhang et al. [18]. The cells were cultured in a single well until they reached 50% confluence; the cells were then washed twice with PBS and maintained in serum-free William's medium including all supplements. After 24 hr incubation, the cells were washed twice with PBS, and incubated with fresh serum-free William's medium including all supplements and indicated agents. After the indicated incubation period, 1 μ Ci of [methyl-³H]thymidine (specific activity: 74 GBq/mmol, 2.0 Ci/mmol; Amersham Biosciences, Buckinghamshire, UK) was added to the cultures. The cells were incubated with [³H]thymidine for 1 hr at 37°C. The cells were then washed twice with PBS, fixed in 10% trichloroacetic acid (TCA) at 23°C for 15 min, and then washed twice with 5% TCA. The acid-insoluble material was dissolved in 0.2 N NaOH for 12 hr at 23°C. Aliquots were removed so that the level of radioactivity could be determined using a liquid scintillation

counter (LS 6500, Beckman Instruments, Fullerton, CA). All values are reported as the means ($\pm$ S.E.) of triplicate experiments. The values were converted from absolute counts to a percentage of the control in order to allow comparison between experiments.

To determine the number of viable cells, the cells were washed twice with PBS and trypsinized from the culture dish. The cell suspension was mixed with 0.4% (wt/vol) trypan blue solution, and the number of live cells was determined using a hemocytometer. Cells failing to exclude the dye were considered nonviable.

PPAR δ small interfering ribonucleic acid transfection

PPAR δ small interfering ribonucleic acid transfection was carried out as described by Gardmo et al. [19]. Cells were grown in each dish until they reached 75% confluence. They were then transfected for 24 hr with either a SMART pool of the small interfering RNAs specific to PPAR δ (200 pmole) or a non-targeting small interfering RNA (as negative control; 200 pmole; Dharmacon, Inc., Lafayette, CO) using Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. The PPAR δ -specific siRNA sequences are CCG CAU GAA GCU CGA GUA U; GUA UAC AGA CUG ACG GAA C; GGA GCA UCC UCA CCG GCAA; CAG UGG AGA CAG UCC GAG A.

Bromodeoxyuridine incorporation

The level of DNA synthesis was determined by measuring the level of 5-bromo-2'-deoxyuridine (BrdU) (a thymidine analog) incorporation. The cells were then treated with IL-6 for 12 hr. Fifteen μ M BrdU was added, and incubation was continued for an additional 1 hr. After several washes with PBS, the cells were fixed with methanol [10% (vol/vol) for 10 min at 4°C], followed by incubation in 1 N HCl for 30 min at room temperature. The cells were then washed and incubated with 0.1 M sodium tetraborate for 15 min. Alexa Fluor 488-conjugated mouse anti-BrdU mAb (diluted 1:200; Molecular Probes, Eugene, OR, USA) in 2% BSA-PBS was incubated with the cells overnight at 4°C. After a PBS wash, the coverslips were mounted onto glass slides with Dako Fluorescent mounting medium using gelvatol, and the slides were examined by optical microscopy (Fluoview 300, Olympus, Tokyo, Japan).

Fluorescence-activated cell sorter (FACS) analysis

The cells were incubated with IL-6 for 12 hr. The cells were then dissociated in trypsin/EDTA, pelleted by centrifugation, and resuspended at approximately 10^5 cells/ml in PBS containing 0.1% BSA. Subsequently, the cells were fixed in 70% ice-cold ethanol, followed by incubation in freshly prepared nuclei staining buffer [250 μ g/ml propidium iodide (PI) and 100 μ g/ml RNase] for 30 min at 37°C. Cell cycle histograms were generated after analyzing the PI-stained cells by FACS (Beckman Coulter, CA). At least 3×10^4 events were recorded for each sample. The samples were analyzed using CXP software (Beckman Coulter, Fullerton, CA, USA).

RNA isolation and real-time RT-PCR

Total RNA was extracted using STAT-60, a monophasic

solution of phenol and guanidine isothiocyanate from Tel-Test, Inc. (Friendwood, TX, USA). The primers were 5-CGT TGC AGA CTG AGA TTG CC-3 (sense) and 5-ACC GGA CAG GTC CAC ATC TG-3 (antisense) for c-fos, 5-AAC TCG GAC CTT CTC ACG TCG-3 (sense) and 5-TGC TGAGGT TGG CGT AGA CC-3 (antisense) for c-jun, and 5-TCC ATT CCG AGG CCA CAG CAA G-3 (sense) and 5-TCA GCT CGT TCC TCC TCT GAC G-3 (antisense) for c-myc.

Real-time quantification of the RNA targets was performed using a Rotor-Gene 6500 real-time thermal cycling system (Corbett Research, NSW, Australia) and the QuantiTect SYBR Green RT-PCR kit (QIAGEN, CA, USA). The reaction mixture (20 μ l) contained 200 ng of total RNA, 0.5 μ M of each primer, the appropriate amounts of enzymes, and fluorescent dyes, as recommended by the supplier. The Rotor-Gene 6500 cyclor was programmed as follows: 30 min at 50°C for reverse transcription; 15 min at 95°C for DNA polymerase activation; 15 sec at 95°C for denature; 45 cycles of 15 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C. Data collection was carried out during the extension step (30 sec at 72°C). The PCR reaction was followed by melting curve analysis to verify the specificity and identity of the RT-PCR products; this analysis can distinguish specific PCR products from any non-specific PCR product resulting from primer-dimer formation. The temperature of the PCR products was increased from 65°C to 99°C at a rate of 1°C/5 sec, and the resulting data was analyzed using the software provided by the manufacturer.

Preparation of cytosolic and total membrane fraction

The medium was removed, and the cells were washed twice with ice-cold PBS, scraped, harvested by microcentrifugation and resuspended in buffer A (137 mM NaCl, 8.1 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄, 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 10 μ g/ml leupeptin, pH 7.5). The resuspended cells were lysed mechanically on ice by trituration using a 21.1-gauge needle. The lysates were first centrifuged at 1,000 $\times$ g for 10 min at 4°C. The supernatants were further centrifuged at 100,000 $\times$ g for 1 hr at 4°C to prepare the cytosolic and total particulate fractions. The particulate fraction containing the membrane fraction was washed twice and resuspended in buffer A with 1% Triton X-100. The protein level in each fraction was quantified using the Bradford method [20].

Western blot analysis

Cell homogenates (40 μ g of protein) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF). The blots were then washed with TBST (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20). The membrane was blocked with 5% skim milk for 1 hr and incubated with the appropriate primary antibody at the dilutions recommended by the supplier. The membrane was then washed, and the primary antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit-IgG. The antibodies were incubated with the membranes at 4°C. The bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, England).

Fig. 1. Effect of IL-6 on DNA synthesis. (A) The primary cultured chicken hepatocytes were incubated for various times (0-24 hr) with or without IL-6 (10 ng/ml) and pulsed with 1 μ Ci of [^{3} H]thymidine for the final 1 hr. The values represent the mean $\pm$ S.E. of four independent experiments with triplicate dishes. * p < 0.05 vs. Control. (B) Cells were incubated for 12 hr with various IL-6 concentrations (0-20 ng/ml) and pulsed with 1 μ Ci of [^{3} H]thymidine for the final 1 hr. The values represent the mean $\pm$ S.E. of four independent experiments with triplicate dishes. * p < 0.05 vs. Control. (C) BrdU-positive cells were measured in response to various IL-6 concentrations (0-20 ng/ml) for 12 hr. The values represent four independent experiments. * p < 0.05 vs. Control. (D) Cells were treated with IL-6 (0-20 ng/ml) for 12 hr, and the cells were counted using a hemocytometer. The values represent three independent experiments. * p < 0.05 vs. Control.

Statistical analysis

Results are expressed as the mean $\pm$ the standard error (S.E.). All experiments were analyzed by ANOVA. In some experiments, a comparison of the treatment means was made with the control using a Bonferroni-Dunn test. A p -value < 0.05 was considered significant.

Results

Effect of IL-6 on DNA synthesis and IL-6 receptors

The most effective treatment time and concentration of IL-6 on [^{3} H]thymidine incorporation were determined by observing chicken hepatocytes at various times

(0-24 hr) and concentrations (0-20 ng/ml) of IL-6. As shown in Figures 1A and B, IL-6 increased the maximal level of [^{3} H]thymidine incorporation at 12 hr with 10 ng/ml IL-6. We confirmed the effect of IL-6 on cell proliferation conducting BrdU incorporation and counting total cell numbers. The level of BrdU incorporation and the total cell numbers reached a maximum in response to 10 ng/ml IL-6 for 12 hr (Fig. 1C, 1D). Therefore, 10 ng/ml IL-6 for 12 hr was used for further experiments.

IL-6 increased the level of IL-6 α and gp130 protein expression (Fig. 2A). IL-6 also increased the level of JAK2 and STAT3 phosphorylation (Fig. 2B). In order to determine the involvement of JAK2 and STAT3 in IL-6-induced hepatocytes proliferation, the level of

Fig. 2. Involvement of IL-6 receptors and JAK/STAT in IL-6-induced DNA synthesis. (A) Primary cultured chicken hepatocytes were incubated with IL-6 (10 ng/ml) for 12 hr. The plasma membrane protein was extracted and examined for IL-6R α , gp130, and β -actin by Western blotting. The lower panels denote the means $\pm$ S.E. of four experiments for each condition determined from densitometry relative to β -actin. * p < 0.05 vs. Control, # p < 0.05 vs. Control. (B) Cells were incubated with IL-6 (10 ng/ml) for various times (0-120 min). The total protein was extracted and examined for phospho-JAK2, phospho-STAT3, JAK2, and STAT3 by Western blotting. The lower panels denote the means $\pm$ S.E. of four experiments for each condition determined from densitometry relative to JAK2 or STAT3. * p < 0.05 vs. Control, # p < 0.05 vs. Control. (C) Cells were treated with AG 490, or a STAT3 inhibitor for 30 min before IL-6 treatment for 12 hr, and pulsed with 1 μ Ci of [³H]thymidine for the final 1 hr. The values represent the mean $\pm$ S.E. of four independent experiments with triplicate dishes. * p < 0.05 vs. Control, ** p < 0.05 vs. IL-6 alone.

Fig. 3. Involvement of PKCs in IL-6-induced DNA synthesis. (A) Primary cultured chicken hepatocytes were incubated with IL-6 (10 ng/ml) for various times (0-120 min). The total protein was extracted and examined for phospho-PKC and pan-PKC by Western blotting. The lower panels denote the means $\pm$ S.E. of four experiments for each condition determined from densitometry relative to pan-PKC. * p < 0.05 vs. Control. (B) PKC- α , - δ , - θ and - ζ isoforms present in either the cytosol or membrane compartments were detected by Western blotting as described in Materials and Methods. The bands are 80-90 kDa for PKC-pan, - α , - δ , - θ and - ζ and 41 kDa for β -actin. The example shown is representative of four experiments. (C) Cells were treated with bisindolylmaleimide I for 30 min before IL-6 treatment for 12 hr, and pulsed with 1 μ Ci of [³H]thymidine for the final 1 hr. The values represent the mean $\pm$ S.E. of four independent experiments with triplicate dishes. * p < 0.05 vs. Control, ** p < 0.05 vs. IL-6 alone.

[³H]thymidine incorporation was measured following treatment with AG 490 (JAK2 inhibitor), or a STAT3 inhibitor. Figure 2C shows that IL-6 increased the level of [³H]thymidine incorporation (38% increase vs.

control; p < 0.05); this increase was blocked by the AG 490 (38% decrease vs. IL-6 alone; p < 0.05) and a STAT3 inhibitor peptide (40% decrease vs. IL-6 alone; p < 0.05). This highlights the relevance of the

Fig. 4. Involvement of p44/42 MAPKs in IL-6-induced DNA synthesis. (A) Primary cultured chicken hepatocytes were incubated with IL-6 (10 ng/ml) for various times (0-120 min). The total protein was extracted and examined for phospho-p44/42 MAPKs and p44/42 MAPKs by Western blotting. The lower panels denote the means $\pm$ S.E. of three experiments for each condition as determined from densitometry relative to p44/42 MAPKs. * $p < 0.05$ vs. Control. (B) Cells were treated with PD 98059 for 30 min before IL-6 treatment for 12 hr and pulsed with 1 μ Ci of [³H]thymidine for the final 1 hr. The values represent the mean $\pm$ S.E. of four independent experiments with triplicate dishes. * $p < 0.05$ vs. Control, ** $p < 0.05$ vs. IL-6 alone. (C, D) Cells were treated with a STAT3 inhibitor or bisindolylmaleimide I for 30 min before treatment with IL-6 for 1 hr. The total protein was extracted and phospho-p44/42 MAPKs and p44/42 MAPKs were examined by Western blotting. The lower panels denote the means $\pm$ S.E. of four experiments for each condition as determined from densitometry relative to p44/42 MAPKs. * $p < 0.05$ vs. Control, ** $p < 0.05$ vs. IL-6 alone.

JAK2/STAT3 pathway to hepatocytes proliferation.

Involvement of PKC and p44/42 MAPKs in IL-6-induced DNA synthesis

IL-6 increased the level of PKC phosphorylation in a time-dependent manner (≥ 10 min) (Fig. 3A). In addition, IL-6 stimulated the translocation of PKC- α , - δ , - θ

and - ζ from the cytosol to the plasma membrane (Fig. 3B). The level of [³H]thymidine incorporation was measured following bisindolylmaleimide I (PKC inhibitor) treatment to determine the involvement of PKC in IL-6-induced hepatocytes proliferation. Figure 3C shows that the IL-6-induced increase in the level of [³H]thymidine incorporation (38% increase vs. control; $p < 0.05$) was

Fig. 5. Involvement of PPAR δ in IL-6-induced DNA synthesis. (A) Primary cultured chicken hepatocytes were incubated with IL-6 (10 ng/ml) for 12 hr. The nuclear membrane protein was extracted and examined for PPAR δ and lamin by Western blotting. The lower panels denote the means $\pm$ S.E. of three experiments for each condition as determined from densitometry relative to lamin. * $p < 0.05$ vs. Control. (B) Cells were transfected for 24 hr with either a SMART pool of PPAR siRNAs (200 pmole) or a nontargeting control siRNA (200 pmole) using Lipofectamine2000 before IL-6 treatment for 12 hr. The nuclear membrane protein was extracted and PPAR δ and lamin were examined by Western blotting. The lower panels denote the means $\pm$ S.E. of three experiments for each condition as determined from densitometry relative to lamin. * $p < 0.05$ vs. Control. (C) Cells were transfected for 24 hr with either a SMART pool of PPAR siRNAs (200 pmole) or a nontargeting control siRNA (200 pmole) using Lipofectamine2000 before IL-6 treatment for 12 hr and pulsed with 1 μ Ci of [³H]thymidine for the final 1 hr. The values represent the mean $\pm$ S.E. of four independent experiments with triplicate dishes. * $p < 0.05$ vs. Control, ** $p < 0.05$ vs. IL-6 alone. (D) Cells were treated with PD 98059 for 30 min before treatment with IL-6 for 12 hr. The nuclear membrane protein was extracted and PPAR δ and lamin were examined by Western blotting. The lower panels denote the means $\pm$ S.E. of three experiments for each condition as determined from densitometry relative to lamin. * $p < 0.05$ vs. Control, ** $p < 0.05$ vs. IL-6 alone.

blocked by PKC inhibitor (28% decrease vs. IL-6 alone; $p < 0.05$). These results show that PKC is involved in hepatocytes proliferation.

IL-6 increased the level of p44/42 MAPKs phosphorylation in a time-dependent manner (≥ 10 min) (Fig. 4A). In order to determine the involvement of

Fig. 6. Effect of IL-6 on proto-oncogenes expression levels and percentage of cells in S phase. (A) Primary cultured chicken hepatocytes were incubated with IL-6 for 1 hr and proto-oncogene (c-fos, c-jun and c-myc) mRNA levels were determined by real-time RT-PCR. Each example shown is representative of three experiments. * $p < 0.05$ vs. Control. (B) Effect of IL-6 on cell cycle progression. Representative FACS data (a) and corresponding histograms (b) for chicken hepatocytes incubated with IL-6 for 12 hr, washed with PBS, fixed, stained, and analyzed by flow cytometry. The gates were configured manually to determine the percentage of cells in the G1, S and G2 phases based on DNA content. The example shown is representative of three experiments.

p44/42 MAPKs in IL-6-induced hepatocytes proliferation, the level of [^{3} H]thymidine incorporation was measured following treatment with PD 98059 (p44/42 MAPKs inhibitor). Figure 4B shows that IL-6-induced increase in the level of [^{3} H]thymidine incorporation (39% increase vs. control; $p < 0.05$) was blocked by the p44/42 MAPKs inhibitor (38% decrease vs. IL-6 alone; $p < 0.05$). This shows that p44/42 MAPKs are related to hepatocytes proliferation. In experiments to examine the relationship between the signaling molecules in the cells, phosphorylation of p44/42 MAPKs was blocked by a STAT3 inhibitor and staurosporine (Fig. 4C, 4D). This suggests that STAT3 and PKC are upstream signal molecules of p44/42 MAPKs. The STAT3 inhibitor did not block the phosphorylation of PKC, and staurosporine

did not block the phosphorylation of STAT3 (data not shown), which suggests that STAT and PKC are parallel signals that converge at p44/42 MAPKs.

Involvement of PPAR δ in IL-6-induced DNA synthesis

IL-6 increased the level of PPAR δ protein expression on the nuclear membrane (Fig. 5A). In order to determine the involvement of PPAR δ in IL-6-induced hepatocytes proliferation, the level of [^{3} H]thymidine incorporation was measured following transfection with the PPAR δ -specific small interfering RNAs (siRNAs). The level of PPAR δ protein expression level was significantly blocked in PPAR δ -specific siRNA transfected cells (Fig. 5B). These results suggest that

Fig. 7. Effect of IL-6 on cell cycle regulator protein expression levels. (A, B, C) Primary cultured chicken hepatocytes were treated with AG 490, a STAT3 inhibitor, bisindolylmaleimide I, or PD 98059 for 30 min before IL-6 treatment for 12 hr and examined for cyclin D1, cyclin E, CDK4, CDK2, phospho-Rb, p21, p27 and β -actin by Western blotting. The example shown is representative of four experiments. (D) Cells were transfected for 24 hr with either a SMART pool of PPAR δ siRNAs (200 pmole) or a nontargeting control siRNA (200 pmole) using Lipofectamine2000 before IL-6 treatment for 12 hr and examined for cyclin D1, cyclin E, CDK4, CDK2, phospho-Rb, p21, p27 and β -actin by Western blotting.

PPAR δ -specific siRNA transfection is useful in primary cultured chicken hepatocytes. Figure 5C showed that the IL-6-induced increase in the level of [^{3} H]thymidine incorporation (36% increase vs. control; $p < 0.05$) was blocked when cells were transfected with a pool of PPAR δ -specific siRNAs (24% decrease vs. IL-6 alone; $p < 0.05$). Thus, PPAR δ is related to hepatocytes proliferation. The p44/42 MAPKs inhibitor blocked PPAR δ protein expression, which suggests that p44/42 MAPKs is an upstream signal molecule of PPAR δ (Fig. 5D). Each inhibitors itself did not affect [^{3} H]thymidine incorporation.

Effect of IL-6 on proto-oncogenes and cell cycle regulator proteins

IL-6 increased the c-fos, c-jun, and c-myc proto-oncogene mRNA levels (Fig. 6A). IL-6 also increased the percentage of the cell population in the S phase (control: 11.8%, IL-6: 68.8%) (Fig. 6B). The change in cell cycle regulator proteins in response to IL-6 was examined. IL-6 increased the levels of cyclin D1, cyclin E, CDK4, and CDK2 proteins as well as of Rb (ser780) phosphorylation, and decreased the level of p21^{WAF/Cip1} and p27^{kip1} expression. However, JAK2, STAT3, PKC,

and p44/42 MAPKs inhibitors and the PPAR δ -specific siRNAs blocked the IL-6-induced increase in cyclin D1, cyclin E, CDK4, and CDK2 proteins as well as of Rb (ser780) phosphorylation. In addition, JAK2, STAT3, PKC, and p44/42 MAPKs inhibitors and the PPAR δ -specific siRNAs blocked the IL-6-induced decrease in p21^{WAF/Cip1} and p27^{kip1} (Fig. 7A, 7B, 7C, 7D).

Discussion

The present study demonstrates that interleukin-6 (IL-6) stimulates the proliferation of primary cultured chicken hepatocytes through PKC, p44/42 MAPKs, and PPAR δ . IL-6 initiates its actions by binding to a specific receptor complex on the cell membrane. We found that IL-6 increased expression of IL-6 receptors [an 80-kDa binding protein (IL-6R α) and a 130-kDa transmembrane signal transducing component (gp130)]. This is biologically significant because the cells with high IL-6 receptor levels have significantly higher rates of proliferation [21]. Increased expression would be expected to increase the number or sensitivity of cells able to respond to the cytokine, suggesting a feed-forward

mechanism whereby IL-6 regulated responsiveness of liver cells. In many cases, there were both increased IL-6 and increased IL-6 receptor [22, 23], so that there seems to be two independent modes of arriving at increased levels of IL-6 signal transduction. The binding of IL-6 to IL-6R α triggers homodimerization of gp130 and activates the Janus kinases (JAK) [24]. Subsequently, gp130 is phosphorylated by JAK, and the phosphotyrosines recruit the signal transducer and activator of transcription 3 (STAT3) and protein tyrosine phosphatase 2 (SHP-2) [25, 26]. These results suggest that activation of tyrosine kinases such as JAK and STAT is essential for the action of IL-6 in primary chicken hepatocytes. Similarly, we found that IL-6 increased JAK2 and STAT3 phosphorylation as well as inhibition of JAK2 and STAT3 blocked the DNA synthesis. Dierssen et al. [27] supports our results, they reported that gp130-dependent STAT signaling via SOCS3 controls the timing of G1/S phase transition, thereby providing protective signals that are important to allow hepatocyte proliferation.

IL-6 increases the level of PKC activation, especially of Ca²⁺-independent PKCs [28, 29]. In the present study, we observed that IL-6 increased both Ca²⁺-independent and Ca²⁺-dependent PKC translocation to the plasma membrane, and the PKC inhibitor blocked hepatocyte proliferation. These results suggest that IL-6-induced hepatocytes proliferation is dependent on the PKC pathway fully. Also, El-Masri and Portier. [30] reported that PKC pathways can have a critical effect on the rate at which hepatocytes enter cell division. IL-6 stimulated a MAPK cascade in normal hepatic cells [31]. Moreover, activation of ERK2 results in gene expression to promote growth or mitosis [2]. In the present study, we also found that IL-6 increased the p44/42 MAPKs phosphorylation and the p44/42 MAPKs inhibitor blocked the proliferation of hepatocytes, which suggests that p44/42 MAPKs is involved in IL-6-induced hepatocyte proliferation. Recently, it was suggested that ligand activation of PPAR δ induces expression of p44/42 MAPKs [32], which could theoretically promote cell growth and inhibit apoptosis. PPAR β/δ is widely expressed in various tissues, including liver [33]. It has recently become clear that PPAR δ has a function in epithelial tissues, but due to inconsistent reports, its exact role remains controversial. Present study showed first time that IL-6-induced PPAR δ stimulated hepatocytes proliferation as a downstream signaling molecule of STAT3.

In the present study, IL-6 increased proto-oncogene expression, which suggests that IL-6 itself increases the cell cycle regulator proteins. Indeed, control of the cell

Fig. 8. Hypothetical model of the signaling pathways underlying IL-6-induced hepatocyte proliferation. IL-6 stimulates JAK/STAT and PKC phosphorylation via IL-6Rs, which stimulates p44/42 MAPKs phosphorylation and PPAR δ expression. Finally, these molecules induce cell cycle regulator proteins increase.

cycle regulator proteins (cyclin D1, cyclin E, CDK4 and CDK2) is dependent on the JAK2/STAT3, PKC, p44/42 MAPKs, and PPAR δ pathways. Previous studies reported that culture of fibroblasts with IL-6 induces c-Jun, JunB and c-Fos mRNA [34] and IL-6-dependent signals trigger nuclear events such as DNA binding of STAT3/APRF and nuclear cyclin A expression as a marker of the S-phase, which regulates hepatocytes proliferation [35]. In addition, the activation of p44/42 MAPKs decreased the expression of the cyclin-dependent protein kinase inhibitor, p21^{WAF1/Cip1}, leading to cell-cycle (G1 phase) progression and hepatocytes proliferation [10]. Consistent with the present results, overexpression of PPAR δ in vascular smooth muscle cells increased cell proliferation by increasing cyclin A and CDK2 and by decreasing p57^{Kip2} [36]. These results suggest that IL-6 alone is sufficient to induce cell cycle progression beyond the G₁ phase of the cell cycle. Based upon these results, we suggest hypothetical model depicting the signaling mechanisms for IL-6-induced hepatocyte proliferation (Fig. 8). We think that the

discovery of the role played by IL-6 in stimulating hepatocyte proliferation, together with the other results shown in this study, represent a significant advance in our knowledge of how hepatic growth is maintained by IL-6 and has application in the development of liver regeneration method. In conclusion, IL-6 induces the proliferation of primary cultured chicken hepatocytes through PKC, p44/42 MAPKs, and PPAR δ pathways.

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