Involvement of endogenous transforming growth factor-α in signal transduction pathway for interleukin-1β-induced hepatocyte proliferation

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Abstract. We studied the effects of interleukin (IL)-1β on DNA synthesis and cell proliferation in primary cultures of adult rat hepatocytes in order to elucidate the mechanisms of its action. Hepatocyte parenchymal cells maintained in a serum-free, defined medium synthesized DNA and proliferated in the presence of IL-1β (3-30 ng/ml), but not IL-1α (0.1-30 ng/ml) in a time- and dose-dependent manner. Specific inhibitors of growth-related signal transducers, such as AG1478, LY294002, PD98059, and rapamycin, completely abolished IL-1β-stimulated hepatocyte DNA synthesis and proliferation. Western blot analysis showed that IL-1β significantly stimulated mitogen-activated protein (MAP) kinase activation within 10 min. Addition of a monoclonal antibody against transforming growth factor (TGF)-α, but not a monoclonal antibody against insulin-like growth factor-I, to the culture dose-dependently inhibited IL-1β-induced hepatocyte mitogenesis. Culture medium TGF-α levels increased significantly within 3 min in response to IL-1β from baseline levels. Peak TGF-α levels (33 pg/ml) were reached at 10 min after IL-1β stimulation. These results indicate that the proliferative mechanism of action of IL-1β is mediated through an increase in autocrine secretion of TGF-α from primary cultured hepatocytes. Secreted TGF-α, in turn, acts as a complete mitogen to induce hepatocyte mitogenesis through the receptor tyrosine kinase/phosphatidylinositol 3-kinase/MAP kinase/mammalian target of rapamycin pathway.

1. Introduction

Adult rat hepatocytes rarely divide under normal physiological conditions. However, they remain capable of proliferation, and after hepatic resection, there is a prompt regenerative response to recruit mature hepatocytes into the cell cycle *in vivo* (Diehl and Rai, 1996; Galun and Axelrod, 2002). A variety of growth factors have been proposed to be involved in hepatocyte proliferation *in vivo* (Michalopoulos and DeFrances, 1997; Fausto et al., 2006). On the other hand, adult rat hepatocytes in primary culture can proliferate when cultured at low cell density in the presence of appropriate growth factors or cytokines such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), insulin, transforming growth factor-α (TGF-α) and tumor necrosis factor (TNF)-α (Kimura and Ogihara, 1997a, 1997b, 1997c, 1999; Okamoto et al., 2009). Therefore, primary cultures of adult rat hepatocytes are an excellent model system in which to study the mechanisms that control parenchymal cell proliferation.

The interleukin (IL)-1 family includes the structurally related proteins IL-1α, IL-1β and IL-1 receptor antagonist (IL-1ra), which bind to cell surface receptors. IL-1α and IL-1β are cytokines that have a wide array of effects on inflammatory and immune activity (Arend, 1991; Dripps et al., 1991; Li and Qin, 2005). There are conflicting data regarding the direct effects of IL-1s on cell proliferation. It has been reported that IL-1β can act as a growth factor for various cells, such as fibroblasts, airway smooth

muscle cells (Zhai et al., 2004), human astrocytoma cells (Lachman et al., 1987) and gastric epithelial cells (Beales, 2002). In contrast, cell growth inhibition by IL-1β has been observed in cultured melanoma and breast carcinoma cells (Dinarello, 1996). These results suggest that IL-1β is a bifunctional growth regulator, depending on cell type and experimental conditions.

In the liver, IL-1α and IL-1β are predominantly produced by Kupffer cells in response to infectious and inflammatory stimuli and play a significant role in the regulation of hepatocyte mitogenesis (Diehl and Rai, 1996). There have been few studies on the effects of IL-1s on hepatocyte DNA synthesis and cell proliferation in primary cultures of adult rat hepatocytes (Boulton et al., 1997; Nakamura et al., 1988; Wang et al., 1998). Nakamura et al. first reported that IL-1β strongly inhibited hepatocyte DNA synthesis induced by insulin plus EGF in primary cultures of adult rat hepatocytes. Although IL-1β inhibits EGF- and insulin-induced hepatocyte DNA synthesis, the mechanism of the inhibitory effects is yet to be established. On the other hand, the direct effects of IL-1s on hepatocyte mitogenesis have not been extensively studied. The main purpose of the present study is to investigate how the proliferative mechanisms of IL-1β are mediated.

Our results demonstrate that IL-1β, but not IL-1α, is a potent stimulator of DNA synthesis and cell proliferation in primary cultures of adult rat hepatocytes. The proliferative mechanisms of IL-1β are mediated by

autocrine secretion of the primary mitogen TGF-α from primary cultured hepatocytes, which, in turn, induces hepatocyte mitogenesis via the receptor tyrosine kinase/MAP kinase (extracellular signal-regulated kinase: ERK) signaling pathway.

2. Materials and Methods

2.1. Animals

Male Wistar rats weighing 200 - 220 g were obtained from Tokyo Experimental Animal Co. (Tokyo, Japan). Adaptation to a light-, humidity- and temperature-controlled room occurred over a minimum 3-day period prior to the start of experiments. Rats were fed a standard diet and given tap water *ad libitum*. Animals used in this study were handled in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society.

2.2. Hepatocyte isolation, culture and treatment

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (45 mg/kg). Hepatocytes were isolated from normal livers by the two-step *in situ* collagenase perfusion technique devised by Seglen to facilitate disaggregation of the adult rat liver (Seglen, 1975). Viability as tested by Trypan blue exclusion was more than 97%. Unless otherwise

indicated, freshly isolated hepatocytes were plated onto collagen-coated plastic culture dishes (35 mm diameter; Iwaki Glass Co., Tokyo, Japan) at a density of 3.3 × 10^4 cells/cm^2, and allowed to attach for 3 h on collagen-coated dishes in Williams' medium E containing 5% newborn calf serum, 0.1 nM dexamethasone, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.10 μg/ml aprotinin under 5% CO_2 in air at 37°C. Medium was then replaced by aspiration, and cells were further cultured in serum- and dexamethasone-free Williams' medium E supplemented with IL-1α or IL-1β. When appropriate, following agents were added; IL-1α or IL-1β with or without U-73122, U-73343, GF109203X, H-89, 2, 4-dideoxyadenosine, verapamil, somatostatin and inhibitors of growth-related signal transducers (i.e., AG1478, LY294002, PD98059 and rapamycin).

AG1478, LY294002, PD98059, U-73122, U-73343, GF109203X, and rapamycin were dissolved in 5% DMSO. All other agents were dissolved in phosphate-buffered saline (PBS; pH 7.4).

Hepatocyte DNA synthesis was assessed by measuring [^{3}H]-thymidine incorporation into acid-precipitable materials (Morley and Kingdon, 1972). After an initial attachment period of 3 h, hepatocytes were washed twice with serum-free Williams' medium E, followed by culture in medium containing IL-1α or IL-1β for a further 4 h. Cells were pulse-stimulated for 2 h with [^{3}H]-thymidine (1.0 μCi/well) at 2 h following the addition of IL-1α or IL-1β. Incorporation of [^{3}H]thymidine into DNA was determined as

described previously (Kimura and Ogihara, 1997a). Hepatocyte protein content was determined using a modified Lowry procedure (Lee and Paxman, 1972) with bovine serum albumin as a standard. Data are expressed as dpm/(h・mg cellular protein).

The number of nuclei rather than the number of cells was counted, as described previously (Nakamura et al., 1983), but with minor modifications (Kimura and Ogihara, 1997a).

2.3. Analysis of cell cycle by flow- and laser scanning-cytometry

Cell cycle progression by IL-1β was determined by using Muse™ cell cycle kit and Muse™ cell analyzer, in accordance with the manufacturer's instructions (Millipore, Darmstadt, Germany). The assay system allows for the quantitative measurement of percentage of cells in the G_0/G_1, S, and G_2/M phases of the cell cycle. The basic methodology of this assay system has been described elsewhere (Darzynkiewicz et al., 2010). Briefly, hepatocytes were cultured as described in Section 2.1. At each time point, hepatocytes were washed twice with ice-cold PBS, and were then solubilized with 0.1 M citrate-0.1% Triton X-100 solution and nuclei were isolated as described elsewhere (Kimura and Ogihara, 1997a). They were subjected to flow cytometry. Isolated nuclei rather than whole cells were analyzed because primary cultured hepatocytes are firmly attached to collagen-coated plastic dishes. The accuracy of DNA content measurement

for DNA ploidy or cell cycle phase estimation is much greater when isolated nuclei are analyzed rather than whole cells (Darzynkiewicz et al., 2010). However, this method may not detect mitotic cell nuclei, as the lysis of plasma membranes of mitotic cells lacking a nuclear envelope leads to dispersion of individual chromosomes.

2.4. Determination of receptor tyrosine kinase and extracellular signal-regulated kinase (ERK 1/2) activation

A 175-kDa protein was identified as the EGF/TGF-α receptor by immunoblotting with anti-phosphoreceptor tyrosine kinase antibody in accordance to the manufacturer's instructions, as described previously (Okamoto et al., 2009). Briefly, hepatocytes were freshly isolated and cultured as described in Section 2.2. Cultured hepatocytes were washed once with ice-cold phosphate-buffered saline (pH 7.4), and 1.2 ml of lysis buffer was added. Cell lysates were obtained by scraping cells in lysis buffer followed by sonication for 3 min. Cell lysates were centrifuged (3,000 × g for 3 min at 4°C) to remove cell debris, and were then denatured in boiling water for 5 min. For immunoblotting analysis, samples of supernatant (30 μg/lane) were resolved by SDS-polyacrylamide gel electrophoresis using a 7.5% polyacrylamide resolving gel (Laemmli, 1970), transferred to PVDF membrane and immunoblotted with the anti-phosphotyrosine antibody PY 20 (Towbin et al., 1979). Blots were

developed by enhanced chemiluminescence following incubation with HRP-conjugated secondary antibodies (Kimura et al., 2001). Proteins were quantified by densitometry after the membrane was developed with enhanced chemiluminescence reagent and exposure to Hyperfilm (Kodak, Tokyo). Densitometric analysis was performed using the NIH image program (ver. 1.6 for Macintosh). The tyrosine kinase activity of the phosphorylated p175-kDa protein (P-p175 kDa) was normalized against that of total p175-kDa protein. Supernatant protein concentration was determined using the modified Lowry method (Lee and Paxman, 1972).

Cell lysis and Western blotting procedure were carried out as described above, except that 20 μg of supernatant was applied to each lane of a 10% polyacrylamide resolving gel. Phosphorylated ERK isoforms (P-ERK1, P-p44 MAPK; and P-ERK2, P-p42 MAPK) were identified by Western blot analysis using anti-phospho-ERK1/2 monoclonal antibody, as described previously (Towbin et al., 1979). Phosphorylated ERK (P-ERK) activity was normalized against total ERK activity. Data were calculated in terms of arbitrary units and are expressed as means ± S.E.M. The autodiagram is a representation of three experiments using different cell preparations.

Cytosolic protein in hepatocytes was quantified using a modification of the Lowry procedure with bovine serum albumin as a standard (Lee and Paxman, 1972).

2.5. Neutralization of endogenous growth factors and assay of TGF-α levels in culture medium

In experiments employing neutralizing antibodies, serum-free primary cultured hepatocytes were treated with 10 ng/ml IL-1β in the presence or absence of varying concentrations of human IgG, monoclonal antibodies against IGF-I or TGF-α (12.5, 25, 37.5, 50, 62.5 or 80 ng/ml).

As some ingredients of Williams' medium E interfere with the enzyme-linked immunosorbent assay (ELISA) system, TGF-α secretion into a phosphate-buffered conditioned medium after addition of 10 ng/ml IL-1β with or without test substances was measured as described previously (Kimura et al., 2001). Briefly, after an attachment period of 3 h, cultured hepatocytes were washed three times with phosphate-buffered saline (PBS; pH 7.4) and culture medium was replaced with PBS containing 1.0 mM $CaCl_2$, 5.5 mM glucose and 0.10 μg/ml aprotinin (pH 7.4). Hepatocytes were preincubated in conditioned medium for 5 min in humidified 5% CO_2-95% air at 37°C, and were then stimulated with 10 ng/ml IL-1β in the absence or presence of test substances. At each time point, conditioned medium (50 μl) was collected and medium samples were tested with an ELISA kit for TGF-α according to the manufacturer's protocols (Calbiochem, San Diego, CA). Absorbance was determined at 490-nm using a micro-plate reader (Tecan Japan, Kanagawa, Japan). A standard curve was obtained in a linear range of 15.6 - 800 pmol/ml, with a minimum detectable limit of

about 7.8 pmol/ml.

2.6. Materials

The following reagents were obtained from Sigma Chemical Co. (St. Louis, MO); human recombinant IL-1α, IL-1β, human IgG, aphidicolin, dexamethasone, somatostatin, verapamil hydrochloride and aprotinin. Monoclonal antibodies against IGF-I were obtained from Oncogene Research Products (San Diego, CA). IL-1ra was obtained from R & D systems (Minneapolis, MN). Monoclonal antibody against TGF-α and BAPTA/AM [1,2-Bis(2-aminophenoxy)ethane-*N, N, N', N'*-tetraacetic acid tetrakis(acetoxymethyl ester)] was obtained from Santa Cruz Biotechnology (Dallas, TX). U-73122 (1-[6-[17β-3-methoxyestra-1, 3, 5(10)-trien-17-yl]-amino] hexyl]-1H-pyrrol-2, 5-dione), U-73343 (1-[6-[17β-3-methoxyestra-1, 3, 5(10)-trien-17-yl]-amino] hexyl]-2, 5-pyrrolidine-dione), GF109203X (2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide), 2,4-dideoxyadenosine, H-89 (N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide dihydrochloride), AG1478 (2-[4-morpholinyl]-8-phenyl-1(4H)-benzopyran-4-one), LY294002 (N-[3-chlorophenyl]-6,7-dimethoxy-4-quinazolinamine) and rapamycin were obtained from Enzo Life Sciences, Inc. (Farmingdale, NY). PD98059 (2'-amino-3'-methoxyflavone) was obtained from Calbiochem-Behring (La

Jolla, CA). Williams' medium E and newborn calf serum were purchased from Flow Laboratories (Irvine, Scotland). Collagenase (type II) was obtained from Worthington Biochemical Co. (Freehold, NJ). [Methyl-^{3}H] thymidine (20 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA). ELISA kit for TGF-α was obtained from Calbiochem (San Diego, CA). Muse™ cell cycle kit was obtained from Millipore (Darmstadt, Germany). All other reagents were of analytical grade.

2.7. Statistical analysis

Data are expressed as means ± S.E.M. Group comparisons were made by analysis of variance (ANOVA) for unpaired data, followed by post-hoc analysis using Dunnett's multiple comparison test. Differences of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Time course of induced stimulation of hepatocyte DNA synthesis and proliferation by IL-1β

We examined the effects of IL-1α and IL-1β on DNA synthesis and proliferation in primary cultures of adult rat hepatocytes in the absence of exogenously added primary mitogens such as EGF and insulin. IL-1α (30 ng/ml) and IL-1β (10 ng/ml) were added 3 h after plating when the change to serum-free culture medium was made (Fig. 1). When maintained for a short time in culture medium, hepatic parenchymal cells underwent time-dependent DNA synthesis and proliferation (i.e., an increase in the number of nuclei) in the presence of IL-1β (10 ng/ml), but not IL-1α (30 ng/ml). The onset of DNA synthesis was first observed at about 2.0 h and peaked at about 3.5 h after addition of IL-1β (Fig. 1A), while significant mitotic activity was observed at 3 h and peaked at about 4.0 h (Fig. 1B). Maximal stimulation of hepatocyte DNA synthesis and proliferation by 10 ng/ml IL-1β was about 8.0-fold and 1.2-fold, respectively. DNA synthesis and proliferative effects of 10 ng/ml IL-1β on primary cultured hepatocytes were almost completely inhibited by 20 ng/ml IL-1ra.

3.2 Effects of IL-1α and IL-1β on cell cycle progression in primary cultures of adult rat hepatocytes

We then examined effects of IL-1α (30 ng/ml) and IL-1β (10 ng/ml) on cell cycle progression in primary cultures of adult rat hepatocytes. Fig.2A shows that percent of total hepatocyte nuclei for G_0/G_1 phase of the cell cycle decreased significantly during the 3-h attachment period. The percent of total hepatocyte nuclei for the G_0/G_1 phase of the cell cycle decreased significantly during further 4-h culture in medium-treated control cells. Percentages further decreased significantly when IL-1β (10 ng/ml) or EGF (20 ng/ml) was added to the culture when compared with the respective control (medium alone) at 2 h and 3 h after addition of primary mitogen growth factor.

Fig. 2B shows that percent of total hepatocyte nuclei for S phase of the cell cycle did not change significantly during 3-h attachment period. The percent of total hepatocyte nuclei for S phase of the cell cycle did not increase significantly during further 4-h culture in medium-treated control or IL-α (30 ng/ml)-treated cells. In contrast, percentages were found to be significantly higher when IL-1β (10 ng/ml) or EGF (20 ng/ml) was added to culture when compared with the respective control (medium alone) at 1, 2, 3 and 4 h after addition of primary mitogens.

3.3. Dose-response effects of IL-1β on hepatocyte DNA synthesis and proliferation, and its blockade by IL-1ra

We then examined the dose-response relationship between IL-1β and

hepatocyte mitogenesis. IL-1β-induced [^{3}H]thymidine incorporation (i.e., DNA synthetic activity) was dose-dependent and plateaued at 10 ng/ml, with a half-maximal effective concentration (ED_{50}) value of 2.2 ng/ml (Fig. 3A). The proliferative effects of the IL-1β on cultured hepatocytes were very similar to those effects on DNA synthesis. In contrast, the effects of IL-1α on hepatocyte DNA synthesis and proliferation were negligible, being in the concentration range of 0.1 to 30 ng/ml (Fig. 3A).

As shown in Fig. 3B, DNA synthesis and proliferative effects of 10 ng/ml IL-1β on primary cultured hepatocytes were almost completely inhibited by 30 ng/ml IL-1ra with a half-maximal inhibitory concentration (IC_{50}) value of 7.2 and 7.1 ng/ml.

3.4. Effects of specific inhibitors of growth-related signal transducers on hepatocyte DNA synthesis and proliferation induced by IL-1β

We next investigated whether the mitogenic responses of hepatocytes to IL-1β were mediated by signal transducers, such as receptor tyrosine kinase, phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein (MAP) kinase kinase and mammalian target of rapamycin (mTOR), an upstream element of ribosomal protein p70 S6 kinase using the corresponding specific inhibitors of the signal transducers, AG1487 (10^{-6} M), a specific inhibitor of receptor tyrosine kinase, LY294002 (3×10^{-7} M), a specific inhibitor of PI3K, PD98059 (10^{-6} M), a specific inhibitor of ERK,

and rapamycin (10 ng/ml), a specific inhibitor of mTOR. When combined, AG1487 (10^{-6} M), LY294002 (3×10^{-7} M), PD98059 (10^{-6} M) and rapamycin (10 ng/ml) almost completely inhibited the IL-1β-induced hepatocyte DNA synthesis and proliferation during the early (Fig. 4A and B) and late phases of culture (data not shown), thus suggesting involvement of receptor tyrosine kinase, PI3K, ERK and mTOR. In addition, possible involvement of phospholipase C (PLC) in the IL-1β-induced hepatocyte DNA synthesis and proliferation was examined using U-73122. U-73122 (10^{-6} M) produced attenuation of IL-1β-induced hepatocyte DNA synthesis and proliferation, but U-73343, an inactive structural analogue, did not. GF109203X (10^{-6} M), a specific protein kinase C inhibitor did not inhibit IL-1β-induced hepatocyte DNA synthesis and proliferation. Furthermore, IL-1ra (20 ng/ml) inhibited IL-1β-induced hepatocyte DNA synthesis and proliferation. These specific inhibitors alone had no effect on hepatocyte DNA synthesis and proliferation.

3.5. Time-course and patterns of IL-1β stimulation of ERK isoform phosphorylation

We then examined the time-course and patterns of IL-1β stimulation of ERK isoform phosphorylation in primary cultured hepatocytes, as detected by Western blot analysis. The phosphorylated ERK2 band (P-ERK2) was induced after only 3 min, and peaked (about 3-fold increase) between 5 and

10 min after addition of 10 ng/ml IL-1β (Figs. 5A, B). ERK1 phosphorylation was not significantly affected by either medium alone (control) or by 10 ng/ml IL-1β treatment (Figs. 5A, C).

3.6. Effects of specific inhibitors of growth-related signal transducers on ERK phosphorylation induced by IL-1β

In order to obtain further support for ERK mediation of hepatocyte mitogenesis induced by IL-1β, we examined the effects of specific inhibitors of growth-related signal transducers on ERK2 phosphorylation induced by 10 ng/ml IL-1β for 10 min. Fig. 6A shows that IL-1β caused an increase in the phosphorylation of ERK2, but not ERK1. The phosphorylation of ERK 2 was completely inhibited by the IL-1β receptor antagonist IL-1ra at 20 ng/ml. When IL-1β was added in combination with PD98059 (10^{-6} M), the agent completely abolished IL-1β-induced ERK2 phosphorylation (Fig. 6A). Moreover, ERK2 phosphorylation induced by IL-1β was abolished by receptor tyrosine kinase inhibitor AG1478 (10^{-6} M) or PI3K inhibitor LY294002 (3×10^{-7} M), but not by rapamycin (10 ng/ml) (Fig. 6A). These results suggest that the mitogenic effects of IL-1β were mediated through activation of the receptor tyrosine kinase/PI3K/ERK 2 pathway. As U-73122 (10^{-6} M), verapamil (10^{-6} M) and somatostatin (10^{-7} M) inhibited IL-1β-induced ERK2 phosphorylation, IL-1β-mediated responses were modulated by PLC and extracellular Ca^{2+}, which is closely

associated with cell proliferation in primary cultures of adult rat hepatocytes (Fig. 6B). On the other hand, GF109203X (10^{-6} M), dideoxyadenosine and H-89 did not affect IL-1β-induced ERK2 phosphorylation, suggesting that protein kinase C, adenylate cyclase and protein kinase A are not likely to be involved in IL-1β-induced ERK2 phosphorylation (Fig. 6B).

3.7. Effects of monoclonal antibodies against TGF-α or IGF-I on hepatocyte DNA synthesis and proliferation induced by IL-1β

Figs. 7A and 7B show that the addition of a neutralizing monoclonal antibody against TGF-α (1- 80 ng/ml) dose-dependently inhibited the growth-promoting effects of IL-1β (10 ng/ml) on hepatocyte DNA synthesis and proliferation, suggesting involvement of endogenous TGF-α secretion. IC_{50} values for these DNA synthetic and proliferative effects after 4 h of culture were 34 ng/ml and 38 ng/ml, respectively. In contrast, DNA synthetic and proliferative effects of IL-1β (10 ng/ml) were not affected significantly by treatment of hepatocytes with various concentrations of monoclonal antibody against IGF-I (1 - 80 ng/ml). Addition of human IgG protein (1- 80 ng/ml) to cultured hepatocytes had no significant effect on hepatocyte DNA synthesis or proliferation induced by 10 ng/ml IL-β (data not shown). These monoclonal antibodies alone or human IgG protein did not significantly influence hepatocyte DNA synthesis or proliferation

during 4 h of culture (data not shown).

3.8. Effects of specific inhibitors of growth-related signal transducers on phosphorylation of receptor tyrosine kinase induced by IL-1β

In order to confirm the notion that IL-1β induces hepatocyte DNA synthesis and proliferation through TGF-α receptor tyrosine kinase/ERK signaling pathway, we investigated whether IL-1β can actually stimulate phosphorylation of TGF-α receptor tyrosine kinase (p175 kDa). Fig. 8 shows that 10 ng/ml IL-1β caused an increase in the phosphorylation of TGF-α receptor tyrosine kinase (p175 kDa), peaking at about 2.4-fold (compared with controls) at 3 min after addition. IL-1ra (20 ng/ml) completely inhibited the IL-1β-induced phosphorylation of TGF-α receptor tyrosine kinase. AG1478 (10^{-6} M) significantly inhibited IL-1β-induced phosphorylation of TGF-α receptor tyrosine kinase. The PLC inhibitor U-73122 completely inhibited the IL-1β-induced phosphorylation of TGF-α receptor tyrosine kinase. In contrast, LY294002, PD98059 and rapamycin did not affect the IL-1β-induced phosphorylation of TGF-α receptor tyrosine kinase. These inhibitors alone did not affect the IL-1β-induced phosphorylation of TGF-α receptor tyrosine kinase.

3.9. IL-1β-induced increases in TGF-α in culture: time course study and dose-response relationship

In order to verify the model proposed above, it was important to demonstrate that treatment of primary cultured hepatocytes with IL-1β does in fact lead to increased secretion of TGF-α. We thus determined the culture medium TGF-α levels. A time course study showed that a detectable increase was seen within 3 min after 10 ng/ml IL-1β treatment, as compared with controls (Fig. 9A). Maximal levels (33 pg/ml) were reached at 10 min after IL-1β stimulation. When combined, IL-1ra (20 ng/ml) completely inhibited the IL-1β-induced secretion of TGF-α into culture medium. TGF-α levels remained significantly elevated for the duration of the experiment. The addition of IL-1α (30 ng/ml) alone had no significant effect (0 - 30 min) on TGF-α levels in culture medium.

As shown in Fig. 9B, IL-1β induced dose-dependent increases in the culture medium TGF-α levels during 10-min culture, with ED_{50} values of 2.5 ng/ml. The addition of IL-1α (0.1 - 30 ng/ml) to cultured hepatocytes had no significant effect on TGF-α levels in culture medium.

3.10. Regulation of IL-1β-induced increase in culture medium TGF-α levels: effects of inhibitors of the IL-1β receptor/phospholipase C pathway

In order to investigate the signal transducing mechanisms that mediate the IL-1β-induced increases in culture medium TGF-α levels by primary cultured hepatocytes, we examined the effects of PLC inhibitor U-73122, PKC inhibitor GF109203X, membrane-permeable chelator of Ca^{2+}

BAPTA/AM somatostatin, and L-type Ca^{2+} channel blocker verapamil. As shown in Fig. 10, the increase in TGF-α levels by 10 ng/ml IL-1β treatment was completely inhibited by IL-1ra (20 ng/ml). Addition of U-73122 (10^{-6} M) almost completely inhibited the 10 ng/ml IL-1β-induced increase in culture medium TGF-α levels, thus suggesting that PLC is closely involved in TGF-α secretion by cultured hepatocytes. Next, we evaluated the effects of somatostatin, which inhibits the release of certain gastrointestinal and pancreatic hormones (presumably by affecting decreasing cytosolic Ca^{2+} levels or attenuating cAMP levels) on the increase in culture medium TGF-α levels by 10 ng/ml IL-1β stimulation. Treatment of cultured hepatocytes for 30 min with somatostatin (10^{-7} M) caused marked inhibition of TGF-α release. In addition, the IL-1β-induced increase in TGF-α levels was also inhibited by verapamil (10^{-6} M) and BAPTA/AM (10^{-7} M). Moreover, this increase in TGF-α levels was not affected by GF109203X (10^{-6} M) (Fig. 10). In addition, the growth-related signal transducer inhibitors AG1478, LY294002, PD98059 and rapamycin had no significant effects on the IL-1β-induced increase in TGF-α secretion into culture medium. These inhibitors alone did not affect basal TGF-α levels.

4. Discussion

It has been reported that IL-1β strongly inhibits hepatocyte DNA synthesis induced by EGF and insulin in primary cultures of adult rat hepatocytes (Nakamura et al., 1988). They identified a unique high-affinity IL-1 receptor (i.e., type I receptor), which specifically binds the IL-1β, but not IL-1α, present on adult rat hepatocytes. Cloning of type I IL-1 receptor showed that it did not possess intrinsic tyrosine kinase activity, and the same is true for the low-affinity IL-1 receptor (i.e., type II receptor) (Kohira et al., 1993). However, why IL-1β inhibited hepatocyte DNA synthesis in the presence of EGF and insulin remains to be clarified (Nakamura et al., 1988; Wang et al., 1998).

On the other hand, we demonstrated that IL-1β, but not IL-1α, significantly induced time- and dose-dependent increases in DNA synthesis and proliferation in primary cultures of adult rat hepatocytes in the absence of exogenous primary growth factors or cytokines such as HGF, EGF, insulin and TGF-α (Figs. 1 and 3). As shown in Figs. 1 and 3, we found that the growth-promoting effects of IL-1β are completely inhibited by an antagonist of IL-1β receptor, IL-1ra (Dripps et al., 1991; Wang, et al., 1998), confirming that IL-1β apparently acts as complete mitogen via the IL-1β receptor in primary cultures of adult rat hepatocytes. However, the results in Fig. 1 show that the doubling time of primary cultured hepatocytes is about 3 - 4 h after IL-1β treatment. This appears to be very

fast. Therefore, we performed cell cycle analysis during primary culture. Under our culture conditions (i.e., low cell density and low dexamethasone), hepatocytes were able to progress from G_0 to G_1 phase in the absence of primary mitogens during the 3-h attachment period. These hepatocytes were arrested in the G_1 phase, and further progression to S phase was found to be dependent on IL-1β or EGF addition (Figs. 2A and 2B). It has also been reported that collagenase perfusion of the liver triggers the G_0/G_1 transition of quiescent normal rat hepatocytes (Loyer et al., 1996). Progression in G_1 is essential for making hepatocytes competent with growth factor (Collin de l'Heuter et al., 2012).

We then investigated the post-receptor mechanisms responsible for the proliferative action of IL-1β, as well as the intracellular signal transduction mechanisms. Specific inhibitors of growth-related signal transducers, such as U-73122 (Thompson et al., 1991), AG1478 (Levitzki and Gazit, 1995), LY294002 (Vlahos et al., 1994), PD98059 (Alessi et al., 1995) and rapamycin (Chung al., 1992) almost completely attenuated IL-1β-stimulated hepatocyte DNA synthesis and proliferation (Fig. 4). Inhibitor study suggests that mitogenic signaling through the IL-1β receptor pathway requires activation of PLC, receptor tyrosine kinase, PI3K, ERK (Davis, 1993) and mTOR, respectively (Fig. 4). As described above, however, IL-1β receptor does not possess intrinsic tyrosine kinase activity (Kohira et al., 1993). Therefore, it remains to be elucidated how

these factors are associated with one another. On the other hand, it is unlikely that IL-1β receptor/(Gq)/PLC/protein kinase C, or adenylate cyclase/protein kinase A pathway contributes to IL-1β-induced hepatocyte mitogenesis, as the specific inhibitor of protein kinase C GF109203X (Toullec et al., 1991), the direct inhibitor of adenylate cyclase 2,4-dideoxyadenosine (Holgate et al., 1980), nor the inhibitor of protein kinase A H-89 (Zusick et al., 1994) affected DNA synthesis and proliferation (Fig. 4).

In order to further support receptor tyrosine kinase/MAP kinase mediation of the IL-1β action, we first examined the effects of IL-1β on ERK activation. As shown in Figs. 5 and 6, IL-1β induced phosphorylation of ERK, and this was inhibited specifically by PD98059. These results are consistent with those reported in other cell types (Beales, 2002; Tominaga et al., 2000; Yang et al., 2000; Zhai et al., 2004). In addition, IL-1β-induced phosphorylation of ERK was completely inhibited by IL-1ra and AG1478 (Fig. 6A). We then investigated the mechanisms by which IL-1β-stimulated hepatocyte mitogenesis and receptor tyrosine kinase/ERK-mediated pathways are associated with one another. As shown in Fig. 5, we found that IL-1β stimulates ERK2 phosphorylation, but not ERK1 phosphorylation. Carcamo-Orive et al. demonstrated that continued expression of cyclin-D1 in the G_0/G_1 phase is dependent on ERK2 expression by PDGF, and that the ablation of ERK1 influences neither the

proliferation capacity of human mesenchymal stem cells, nor the expression pattern of cyclin-D1. In agreement with a recent report, our results indicated that ERK2 play a key role in hepatocyte proliferation. The involvement of ERK2 in the IL-1β-induced hepatocyte proliferation was almost completely blocked by the MEK inhibitor PD98059 (Fig. 6A).

In previous studies, we demonstrated that prostaglandin E_1, IP receptor agonist and TNF-α induced hepatocyte DNA synthesis and proliferation through autocrine secretion of complete mitogen TGF-α, which, in turn, stimulated hepatocyte mitogenesis through TGF-α receptor tyrosine kinase/ERK pathway (Kimura et al., 2001, Kimura et al., 2009, Okamoto et al., 2009). Therefore, we hypothesized that the IL-1β receptor/(Gq)/PLC/Ca^{2+} pathway stimulates secretion of a complete mitogen by primary cultured hepatocytes in an autocrine manner, inducing hepatocyte DNA synthesis and cell proliferation through stimulation of the downstream receptor tyrosine kinase/ERK pathway. Either TGF-α or IGF-I is a potential candidate as the primary growth factor, since hepatocytes express mRNA for both TGF-α and IGF-I, and the cells can synthesize and store these primary growth factors (Michalopoulos and DeFrances, 1997).

In order to investigate the possibility that TGF-α or IGF-I mediates IL-1β-induced hepatocyte DNA synthesis and proliferation in primary cultures, we examined the effects of neutralizing monoclonal antibodies against TGF-α and IGF-I on the hepatocyte mitogenesis induced by IL-1β.

As shown in Fig. 7, the results clearly indicated that IL-1β-induced hepatocyte DNA synthesis and proliferation were almost completely inhibited by a monoclonal antibody against TGF-α, but not by a monoclonal antibody against IGF-I. Therefore, the results suggest that cytokine TGF-α is stored within the parenchymal hepatocytes and is triggered to secrete to the culture medium through IL-1β stimulation. Moreover, with regard to the action of endogenous TGF-α, we showed that 10 ng/ml IL-1β stimulation caused a rapid increase in phosphorylation of TGF-α receptor tyrosine kinase (p175kDa). IL-1ra and AG1478 completely antagonized the IL-1β-induced phosphorylation of TGF-α receptor tyrosine kinase (Fig. 8).

In order to verify the model proposed above, it is important to demonstrate that treatment of primary cultured hepatocytes with IL-1β does in fact lead to increased secretion of TGF-α into culture. We obtained data showing that IL-1β produced significant time- and dose-dependent increases in TGF-α in the culture medium (Fig.9A and B). In addition, according to our previous study on the TGF-α signaling system in primary cultures of adult rat hepatocytes (Kimura and Ogihara, 1999), the growth-promoting signaling of TGF-α is mainly mediated through the receptor tyrosine kinase/PI3K/ERK/mTOR pathway. Thus, the TGF-α releasing action of IL-1β appears to be sufficient to explain the phenomenon of induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes.

The relationships between IL-1β-induced TGF-α secretion and hepatocyte mitogenesis were confirmed by investigating the regulatory mechanisms behind the rapid increase in culture medium TGF-α levels by IL-1β receptor stimulation. As shown in Fig. 4, IL-1β-induced hepatocyte mitogenesis was inhibited by the PLC inhibitor U-73122 (Thompson et al., 1991), but not by its inactive analog, U-73343. Therefore, the functional IL-1β receptors found in hepatocytes in primary cultures are likely to stimulate PLC and increase Ca^{2+} mobilization to secrete TGF-α as a primary mechanism of IL-1β-induced hepatocyte DNA synthesis and proliferation (Figs. 4 and 10). The importance of intracellular Ca^{2+} was further supported by the observation that the L-type Ca^{2+} channel blocker verapamil and BAPTA/AM (Szabo et al., 2008) inhibit hepatocyte DNA synthesis and proliferation induced by IL-1β (data not shown) by inhibiting TGF-α secretion (Fig. 10). In addition, somatostatin strongly inhibited IL-1β-induced ERK2 phosphorylation by inhibiting TGF-α secretion (e.g., Figs. 6B and 10). Taken together, these observations indicate the need for increases in intracellular Ca^{2+} during IL-1β-induced hepatocyte DNA synthesis and proliferation, which is mediated through autocrine secretion of TGF-α. In support of our findings, it has been reported that PLC mediates IL-1β receptor-stimulated mucin secretion in middle ear epithelial cells (Kerschner et al., 2006).

On the other hand, it is unlikely that IL-1β receptor/(Gq)/PLC/protein

kinase C, or the adenylate cyclase/protein kinase A pathway contributes to IL-1β-induced hepatocyte mitogenesis, as the inhibitor of protein kinase C GF109203X, the direct inhibitor of adenylate cyclase 2,4-dideoxyadenosine, and the inhibitor of protein kinase A H-89 had no effect on DNA synthesis and proliferation or ERK activation (Figs. 4 and 6B). In addition, the growth-related signal transducer inhibitors AG1478, LY294002, PD98059 and rapamycin had no significant effects on IL-1β-induced increases in culture medium TGF-α levels (Fig. 10). These agents only blocked IL-1β receptor signaling by inhibiting corresponding growth-related signal transducer activities induced by TGF-α, thereby blocking hepatocyte DNA synthesis and proliferation (Figs. 4A and B). Consequently, the suppression of IL-1β receptor signaling by these specific growth-related signal transducer inhibitors may occur downstream of TGF-α secretion. Taken together, our findings confirm that the ability of IL-1β to promote hepatocyte mitogenesis is due to an indirect effect of secreted TGF-α in an autocrine manner. This is a unique intracellular signal transduction mechanism when compared with classical growth factors such as EGF, HGF and insulin.

Based on the above biochemical and pharmacological analysis, we propose that IL-1β receptor is linked to a G-protein (possibly Gq) and stimulates the activity of PLC. This causes an increase in intracellular Ca^{2+} levels, which induces TGF-α secretion in an autocrine manner. The secreted

TGF-α can induce hepatocyte DNA synthesis and proliferation via the TGF-α receptor tyrosine kinase/PI3K/ERK/mTOR pathway. A diagram of the proposed mechanism for this IL-1β receptor-mediated signaling pathway for hepatocyte DNA synthesis and proliferation is shown in Fig. 11.

With regard to the physiological significance of the IL-1β effects, Higashitsuji et al. reported that there was a transient increase in expression of IL-1β mRNA at 0.5 ~ 1.0 h after partial hepatectomy *in vivo* (Higashitsuji et al., 1995). Therefore, IL-1β, as a pro-inflammatory factor, plays an important role in liver regeneration during early phase of liver injury. In addition, based on the present results, IL-1β may also induce hepatocyte proliferation during the very early phases of liver injury in an autocrine manner *in vivo*. Studies into such processes remain to be conducted.

In conclusion, we provide evidence that IL-1β, but not IL-1α, is a potent initiator of DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. The IL-1β receptor/(Gq)/PLC/Ca^{2+}-dependent autocrine secretion of TGF-α occurring in response to IL-1β receptor activation is an essential step in the stimulation of hepatocyte DNA synthesis and proliferation. The secreted TGF-α, in turn, acts as a primary mitogen to induce hepatocyte mitogenesis through the receptor tyrosine kinase/ERK pathway in primary cultured hepatocytes (Fig. 11). As IL-1β is the main

cytokine secreted by non-parenchymal hepatocytes, the regulation of hepatocyte proliferation is likely to involve locally produced IL-1β, generated in response to hepatic damage or injury, acting as an inducer during liver regeneration *in vivo*.

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Figure Legends

Fig. 1. Effects of IL-1α and IL-1β on hepatocyte DNA synthesis and cell proliferation in primary cultures of adult rat hepatocytes. Hepatocytes at a cell density of 3.3×10^4 cells/cm^2 were plated and cultured for 3 h as described in Section 2.2. After an attachment period of 3 h (time zero), the medium was rapidly replaced with serum-free Williams' Medium E and hepatocytes were cultured with IL-1α (30 ng/ml) or IL-1β (10 ng/ml) with or without IL-1ra (20 ng/ml) for various durations. DNA synthesis and cell proliferation were assessed as described in Section 2.3. Results are expressed as means ± S.E.M. of three separate experiments. *$P<0.05$, **$P<0.01$ compared with controls.

Fig. 2 Effects of IL-1α and IL-1β on cell cycle progression in primary cultures of adult rat hepatocytes. Hepatocytes at a cell density of 3.3×10^4 cells/cm^2 were plated and cultured for 3 h as described in Section 2.2. After an attachment period of 3 h (time zero), the medium was rapidly replaced with serum-free Williams' Medium E and hepatocytes were cultured with IL-1α (30 ng/ml), IL-1β (10 ng/ml), and EGF (20 ng/ml) for various durations. At each time point, isolated hepatocyte nuclei (10^5/ml) were subjected to cell cycle analysis (A); percent of total hepatocyte nuclei in G_0/G_1 phase, (B); percent of total hepatocyte nuclei in S phase of cell cycle. Arrows indicate time of cytokine addition. Results are expressed as means

± S.E.M. of three separate experiments. *P<0.05, **P<0.01 compared with control (hepatocyte nuclei before culture). #P<0.05, ##P<0.01 compared with respective control.

Fig. 3. Dose-response effects of IL-1β on hepatocyte DNA synthesis and cell proliferation, and its blockade by IL-1ra in primary cultures of adult rat hepatocytes. Hepatocytes at a cell density of 3.3×10^4 cells/cm^2 were plated and cultured for 3 h as described in the legend to Fig. 1. After medium change, cultured hepatocytes were treated with increasing concentrations of IL-1α (0.1-30 ng/ml) and IL-1β (0.1-30 ng/ml), without or with IL-1ra (0.1-30 ng/ml) for 4 h of culture. DNA synthesis and cell proliferation were assessed as described in Section 2.2. Results are expressed as means ± S.E.M. of three separate experiments. *P<0.05, **P<0.01 compared with control.

Fig. 4. Effects of specific inhibitors of growth-related signal transducers on hepatocyte DNA synthesis and cell proliferation induced by IL-1β. Hepatocytes at a cell density of 3.3×10^4 cells/cm^2 were plated and cultured for 3 h as described in the legend to Fig. 1. After an attachment period of 3 h (time zero), medium was rapidly replaced with serum-free Williams' Medium E, and hepatocytes were cultured with 10 ng/ml IL-1β in the presence or absence of specific inhibitors of growth-related signal

transducers for an additional 4 h. Concentrations were as follows: IL-1ra (20 ng/ml); U-73122 (10^{-6} M); U-73343 (10^{-6} M); GF109203X (10^{-6} M); AG1478 (10^{-6} M); LY294002 (3×10^{-7} M); PD98059 (10^{-6} M); and rapamycin (10 ng/ml). Hepatocyte DNA synthesis (A) and proliferation (B) were determined as described in Section 2.2. Data are expressed as means ± S.E.M. of three separate experiments. **P<0.01 compared with respective controls.

Fig. 5. Effects of IL-1β on ERK phosphorylation in primary cultured hepatocytes. Hepatocytes at a cell density of 3.3×10^4 cells/cm^2 were plated and cultured for 3 h as described in the legend to Fig. 1. After an attachment period of 3 h (time zero), medium was rapidly replaced with serum-free Williams' Medium E, and hepatocytes were cultured with 10 ng/ml IL-1β for various durations. Phosphorylation of ERK 1/2 by IL-1β was assessed as described in Section 2.4. A typical Western blot image is shown on the top of the figure. Results are expressed as a percentage of the respective control value (means ± S.E.M. of three separate experiments). *P<0.05, **P<0.01 compared with controls.

Fig.6. Effects of specific inhibitors of growth-related signal transducers on ERK phosphorylation induced by IL-1β. Hepatocytes at a cell density of 3.3 $\times 10^4$ cells/cm^2 were plated and cultured for 3 h as described in the legend

to Fig. 1. After an attachment period of 3 h (time zero), medium was rapidly replaced with serum-free Williams' Medium E, and hepatocytes were cultured with IL-1β with or without specific inhibitors of growth-related signal transducers for 10 min. Phosphorylation of ERK 1/2 stimulated by IL-1β was assessed as described in Section 2.4. A typical Western blot image is shown on the top of the figure. Concentrations were as follows: (A), IL-1ra (20 ng/ml); AG1478 (10^{-6} M); LY294002 (3×10^{-7} M); PD98059 (10^{-6} M); and rapamycin (10 ng/ml); (B), U-73122 (10^{-6} M); U-73343 (10^{-6} M); GF109203X (10^{-6} M); verapamil (10^{-6} M); dideoxyadenosine (10^{-6} M); H-89 (10^{-6} M); and somatostatin (10^{-7} M). Results are expressed as a percentage of respective control values (means ± S.E.M. of three separate experiments). **$P<0.01$ compared with respective controls.

Fig. 7. Effects of monoclonal antibodies against IGF-I and TGF-α on IL-1β-induced hepatocyte DNA synthesis and proliferation. Hepatocytes at a cell density of 3.3×10^4 cells/cm^2 were plated and cultured for 3 h as described in the legend to Fig. 1. After an attachment period of 3 h (time zero), medium was rapidly replaced with serum-free Williams' Medium E, and hepatocytes were cultured with various concentrations of monoclonal antibodies against TGF-α, and IGF-I in the presence of 10 ng/ml IL-1β for an additional 4 h. Hepatocyte DNA synthesis (A) and proliferation (B) were

determined as described in Section 2.2. and 2.5. Data are expressed as means ± S.E.M. of three separate experiments. *$P<0.05$, **$P<0.01$ compared with respective controls.

Fig. 8. Effects of specific inhibitors of growth-related signal transducers on phosphorylation of TGF-α receptor tyrosine kinase induced by IL-1β. Hepatocytes at a cell density of 3.3×10^4 cells/cm^2 were plated and cultured for 3 h as described in the legend to Fig. 1. After an attachment period of 3 h (time zero), medium was rapidly replaced with serum-free Williams' Medium E, and hepatocytes were cultured with 10 ng/ml IL-1β with or without inhibitors of growth-related signal transducers for 3 min. Phosphorylation of TGF-α receptor tyrosine kinase (p175 kDa) induced by IL-1β was assessed as described in Section 2.4. A typical Western blot image is shown on the top of the figure. Concentrations were as follows: IL-1ra (20 ng/ml); AG1478 (10^{-6} M); LY294002 (3×10^{-7} M); PD98059 (10^{-6} M); rapamycin (10 ng/ml); and U-73122 (10^{-6} M). Results are expressed as a percentage of respective control values (means ± S.E.M. of three separate experiments). **$P<0.01$ compared with respective controls.

Fig. 9. IL-1β-induced secretion of TGF-α by primary cultured hepatocytes: time-course study (A) and dose-response relationship (B). Hepatocytes were isolated and cultured as described in Section 2.2. At each time point, the

conditioned medium (50 μL) was collected and medium samples were tested by ELISA for TGF-α according to the manufacturer's instructions, as described in Section 2.5. Concentrations of ILs were as follows: IL-1α (30 ng/ml); IL-1β (10 ng/ml); and IL-1ra (20 ng/ml). Results are expressed as means ± S.E.M. of three separate experiments. *P<0.05, **P<0.01 compared with respective controls.

Fig.10. IL-1β-induced secretion of TGF-α by primary cultured hepatocytes; effects of specific inhibitors of various signal transducers. Hepatocytes were isolated and cultured as described in Section 2.2. After 10-min incubation of hepatocytes with 10 ng/ml IL-1β in the presence or absence of specific inhibitors, conditioned medium (50 μl) was collected and medium samples were tested by ELISA for TGF-α in accordance with the manufacturer's instructions, as described in Section 2.5. Concentrations of specific inhibitors of signal transducers were as follows: IL-1ra (20 ng/ml), U-73122 (10^{-6} M); GF109203X (10^{-6} M); BAPTA/AM (10^{-7} M); verapamil (10^{-6} M); somatostatin (10^{-7} M); AG1478 (10^{-6} M); LY294002 (3×10^{-7} M); PD98059 (10^{-6} M); and rapamycin (10 ng/ml). Results are expressed as means ± S.E.M. of three separate experiments. **P<0.01 compared with respective controls.

Fig. 11. Signal transduction pathway for IL-1β-induced DNA synthesis and proliferation in primary cultures of adult rat hepatocytes. Abbreviations; RTK: receptor tyrosine kinase; PLC: phospholipase C; PIP_2: phosphatidylinositol 4, 5-bisphosphate; DG: diacylglycerol; IP_3: inositol 1,4, 5-trisphosphate; PI3K: phosphoinisitide 3-kinase; ERK2: extracellular signal-regulated kinase 2; and mTOR: mammalian target of rapamycin.

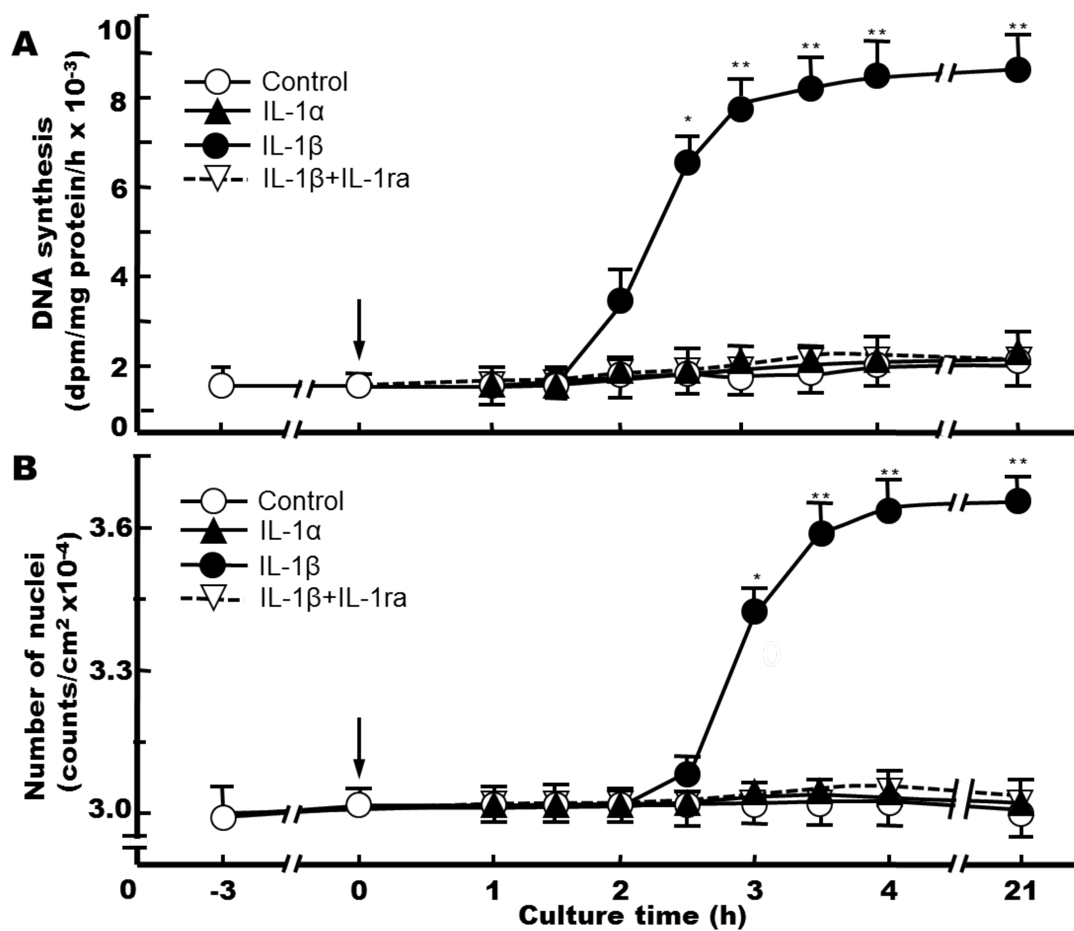

Fig.1

Kimura M. et al.

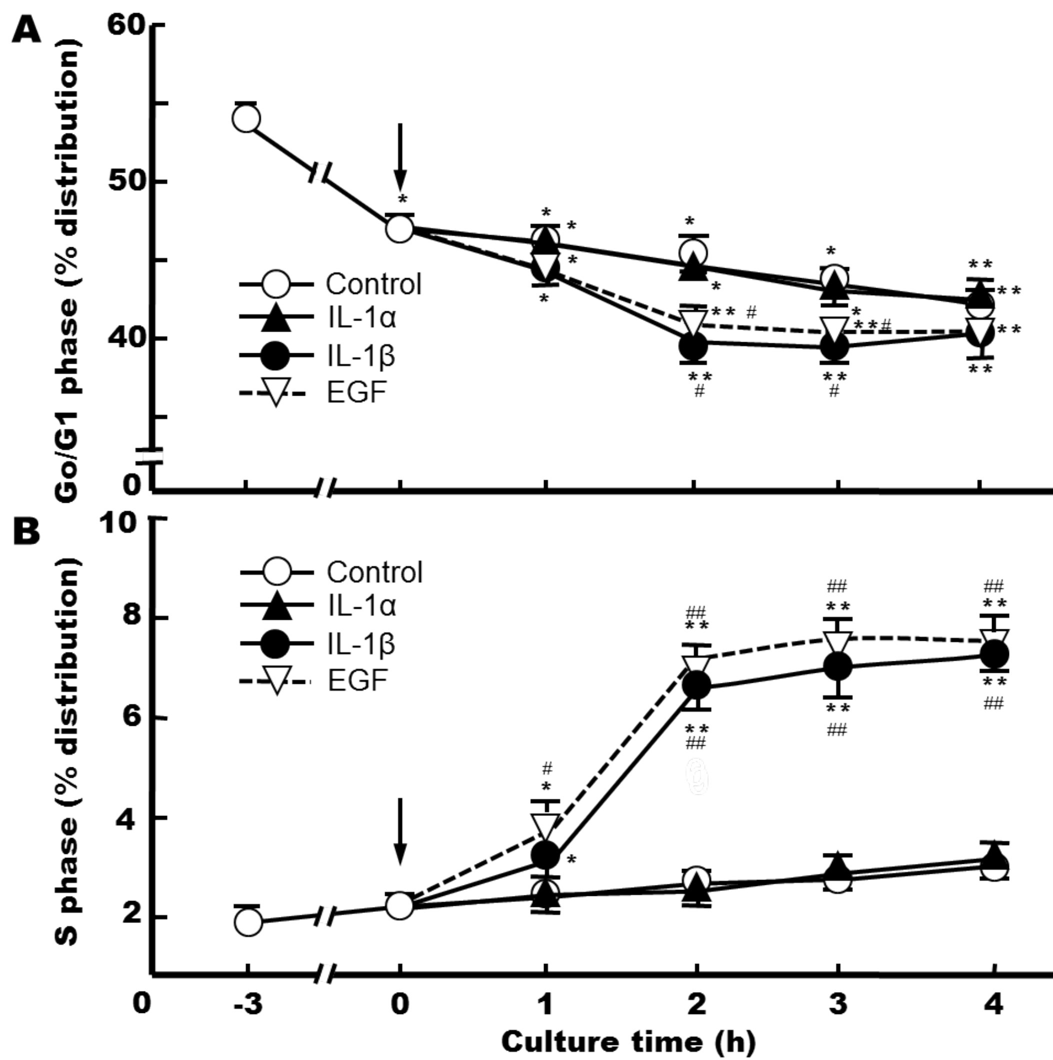

Fig.2

Kimura M. et al.

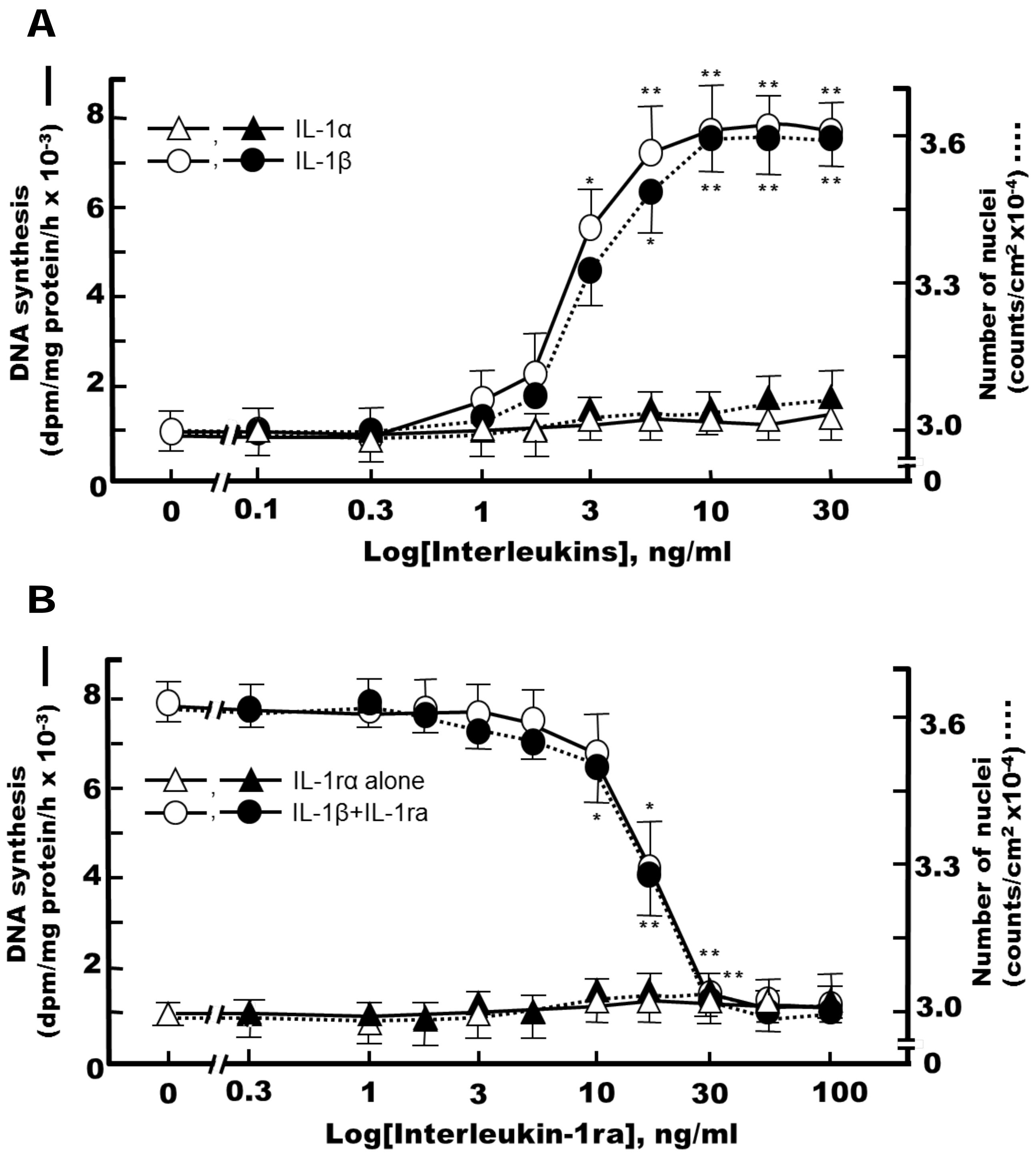

Fig.3

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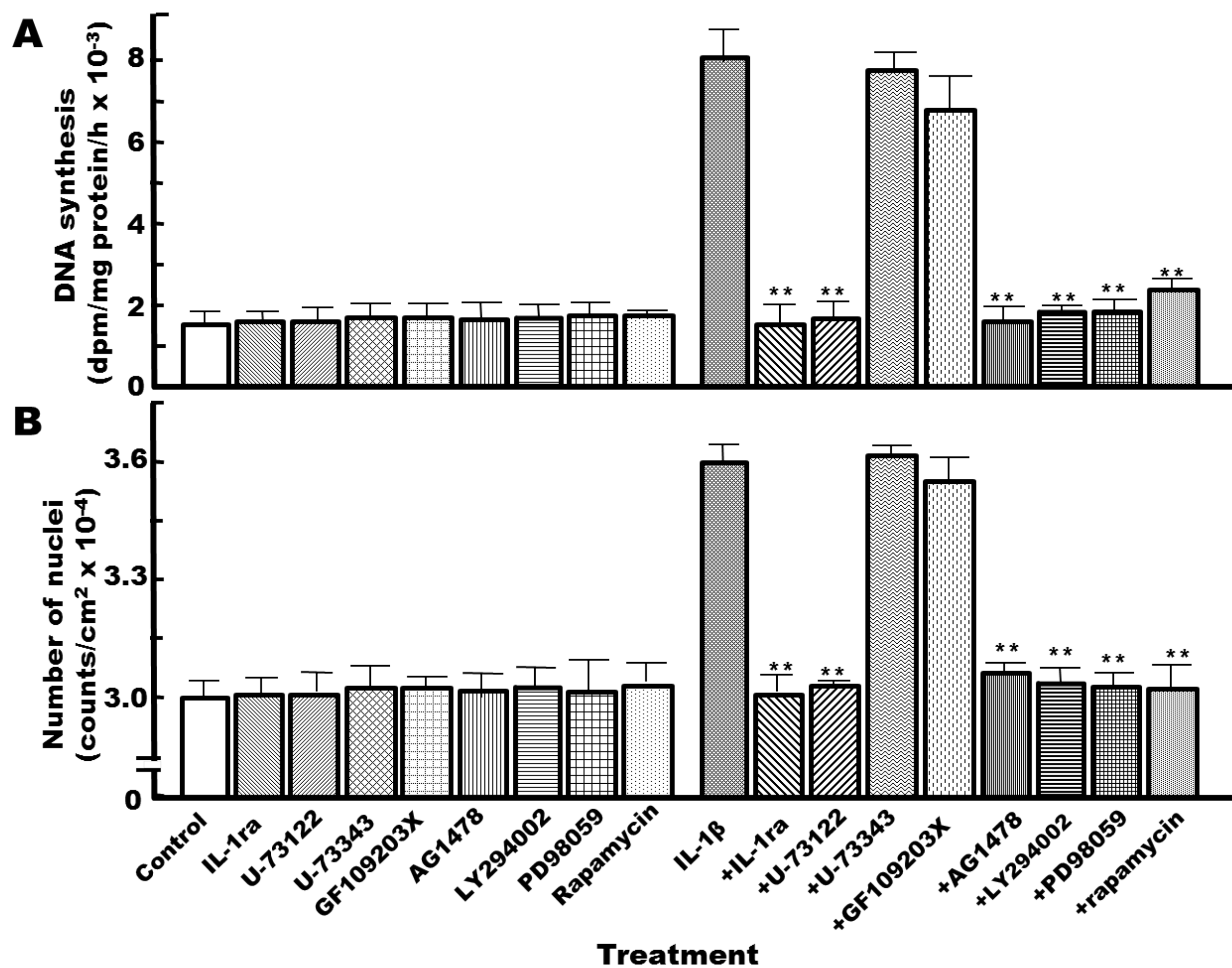

Fig.4

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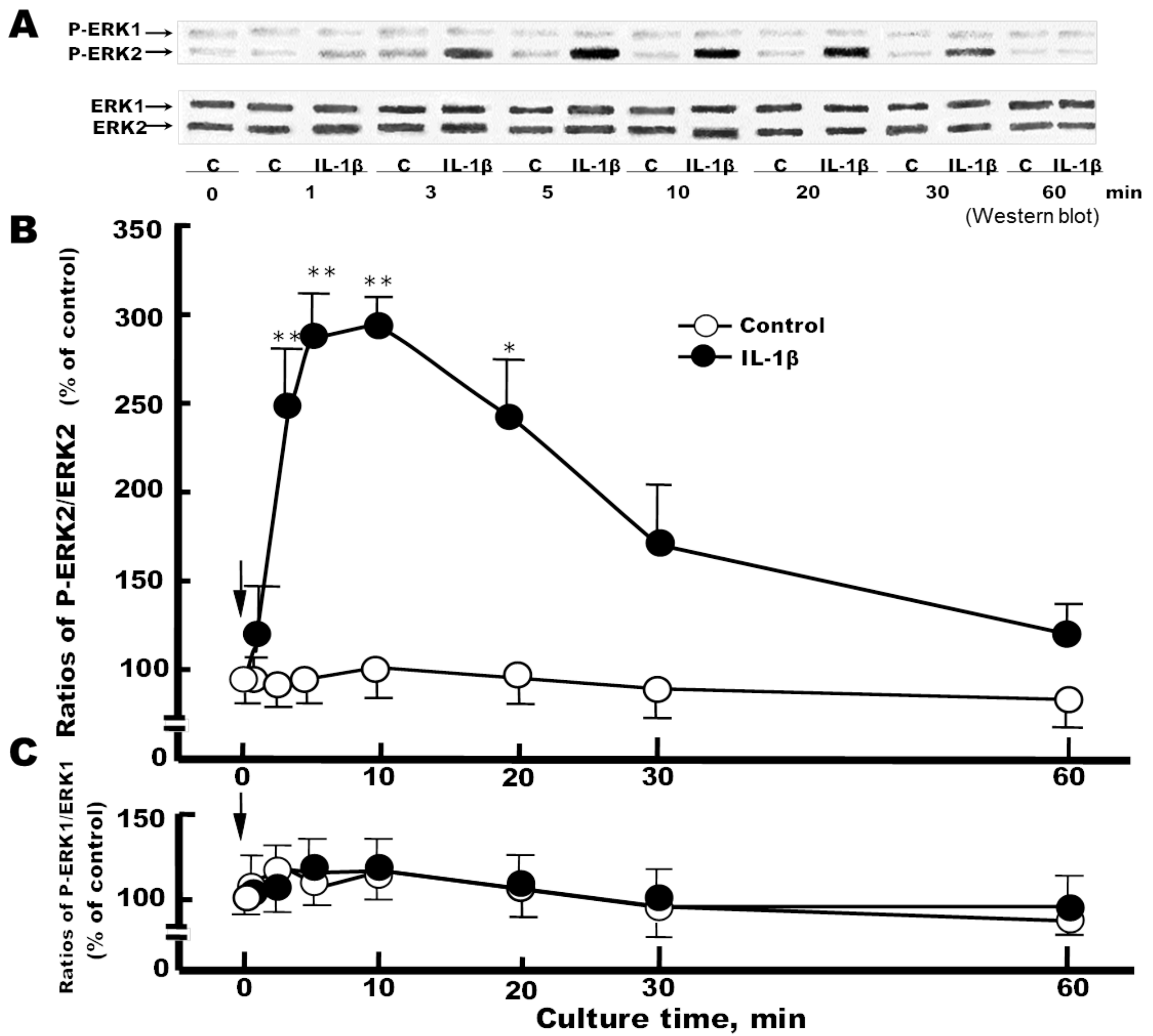

Fig.5

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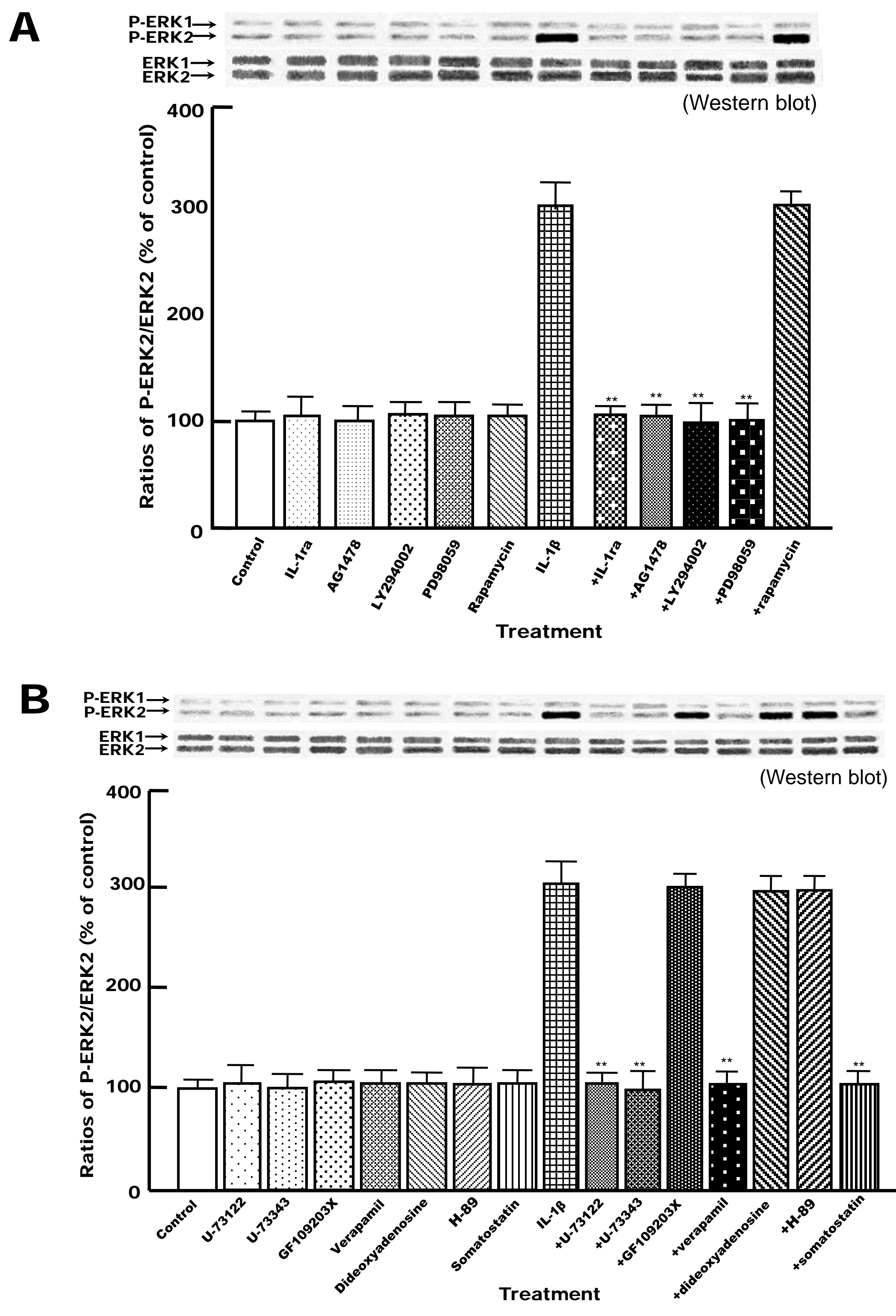

Fig.6

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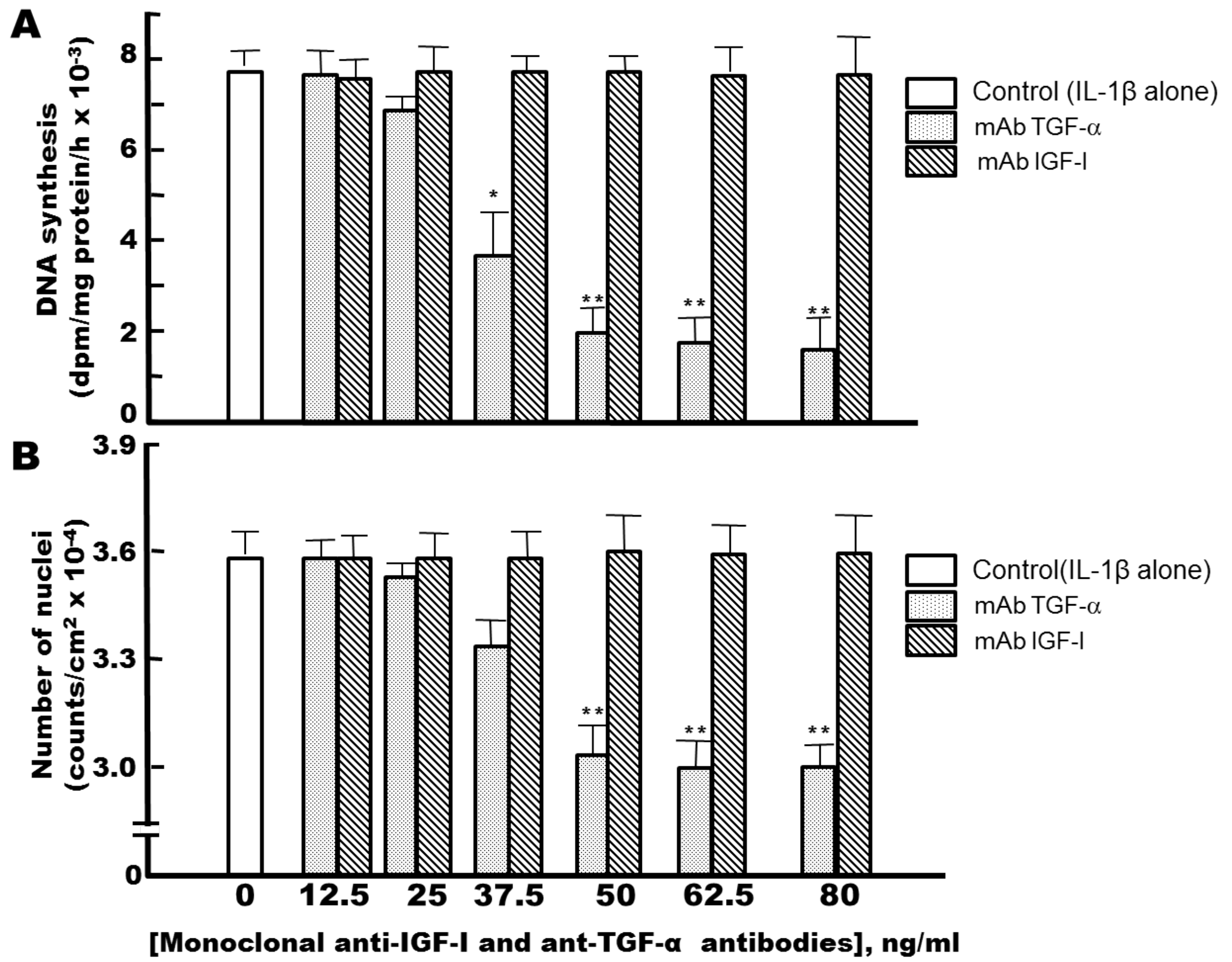

Fig.7

Kimura M. et al.

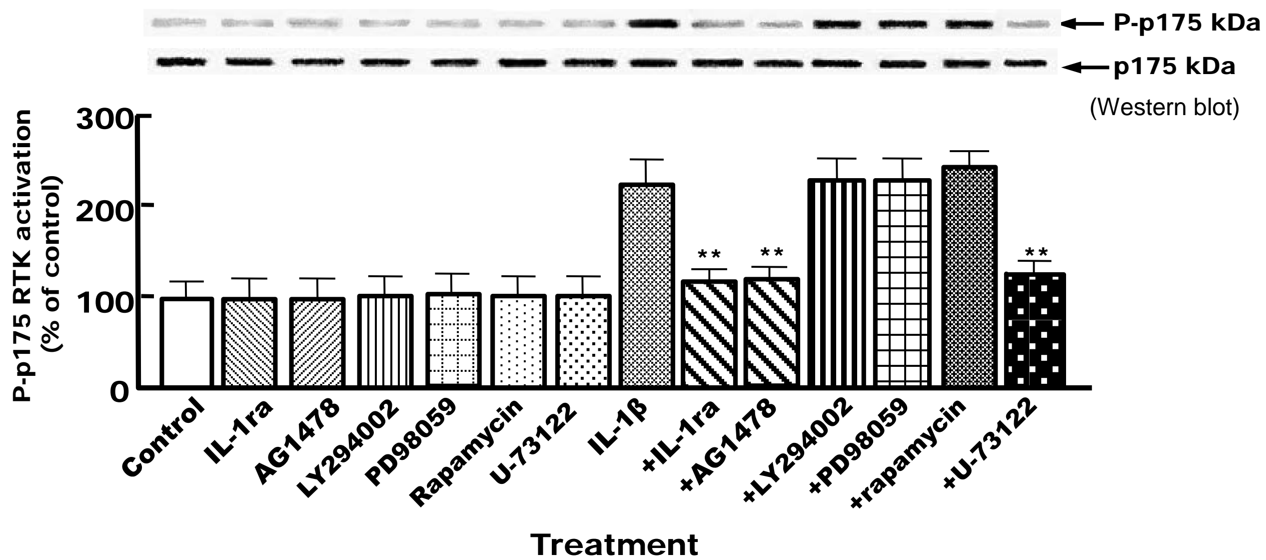

Fig.8

Kimura M. et al.

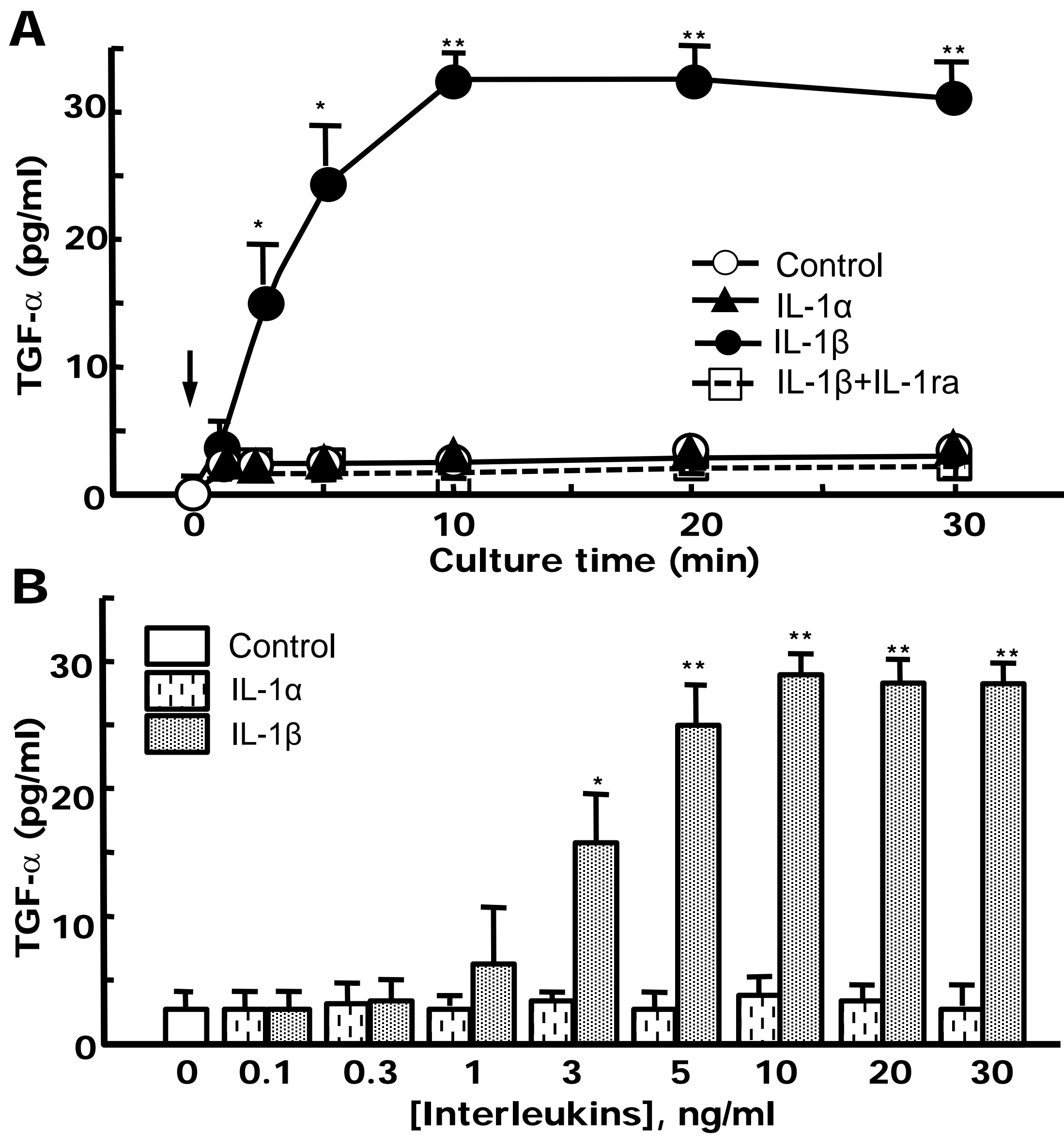

Fig.9

Kimura M. et al.

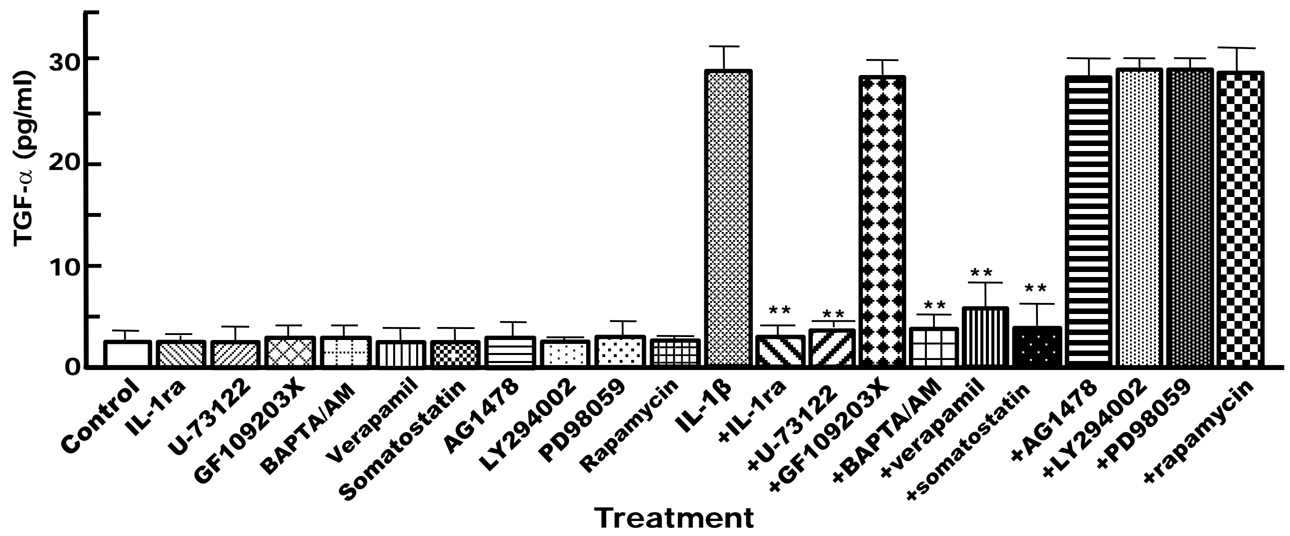

Fig.10

Kimura M. et al.

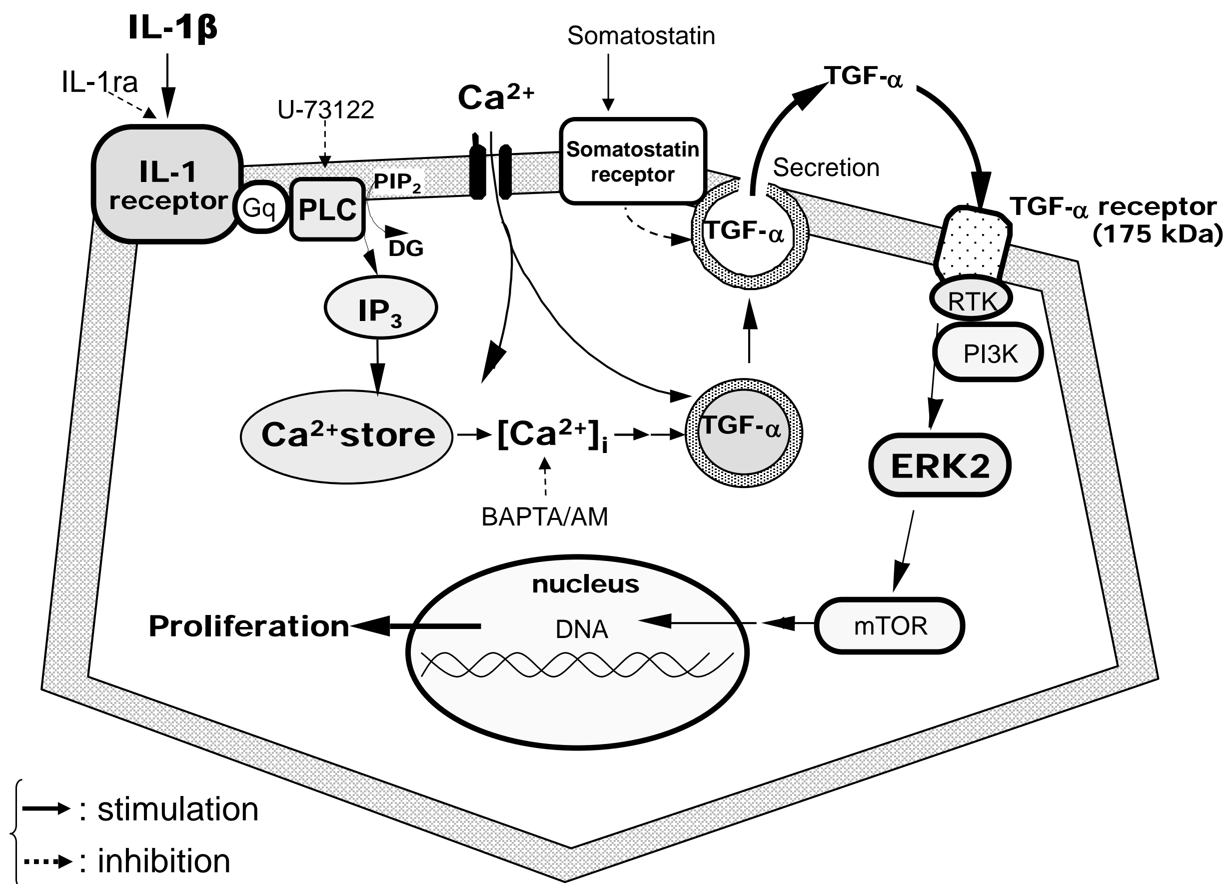

Fig.11

Kimura M. et al.