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Overexpression of Regucalcin Suppresses Cell Death and Apoptosis in Cloned Rat Hepatoma H4-II-E Cells Induced by Lipopolysaccharide, PD 98059, Dibucaine, or Bay K 8644

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Abstract The effect of regucalcin, a regulatory protein in intracellular signaling pathway, on cell death was investigated by using the cloned rat hepatoma H4-II-E cells overexpressing regucalcin. The hepatoma cells (wild-type) and stable regucalcin (RC)/pCXN2 transfectants were cultured for 72 h in medium containing 10% fetal bovine serum (FBS) to obtain subconfluent monolayers. After culture for 72 h, cells were further cultured for 12–72 h in medium without FBS containing either vehicle or lipopolysaccharide (LPS; 0.1 or 1.0 μg/ml). The number of wild-type cells was significantly decreased by culture for 24 or 48 h in the presence of LPS (0.1 or 1.0 μg/ml). The effect of LPS (0.1 or 1.0 μg/ml) in decreasing the number of hepatoma cells was significantly prevented in transfectants overexpressing regucalcin. However, the culture with LPS (0.1 or 1.0 μg/ml) for 72 h caused a significant decrease in cell number of transfectants. Ca^{2+}/calmodulin-dependent nitric oxide (NO) synthase activity was significantly decreased by culture with LPS (1.0 μg/ml) for 24–72 h of wild-type cells. This decrease was significantly prevented in transfectants. LPS (0.1 or 1.0 μg/ml)-induced decrease in the number of wild-type cells was significantly prevented by culture with caspase-3 inhibitor (10^{-8} M). Moreover, the number of wild-type cells was significantly decreased by culture with PD 98059 (10^{-6} M), dibucaine (10^{-6} M), or staurosporine (10^{-6} M), which is an inhibitor of various protein kinases. The effect of PD 98059 or dibucaine on the number of wild-type cells was not observed in transfectants, although the effect of staurosporine was seen in transfectants. Culture with Bay K 8644 (2.5×10^{-6} M), an agonist of Ca^{2+} entry in cells, caused a significant decrease in the number of wild-type cells. Such an effect was not seen in transfectants. The presence of LPS did not significantly decrease the number of wild-type cells in the presence of Bay K 8644. Agarose gel electrophoresis showed the presence of low-molecular-weight deoxyribonucleic acid (DNA) fragments of adherent wild-type cells cultured with Bay K 8644, and this DNA fragmentation was significantly prevented in transfectants. This study demonstrates that overexpression of regucalcin has a suppressive effect on cell death induced by LPS or various intracellular signaling-related factors. J. Cell. Biochem. 93: 598–608, 2004.

Key words: regucalcin; cell death; apoptosis; NO synthase; lipopolysaccharide; protein kinase inhibitor; Bay K 8644; hepatoma cells

Regucalcin was found as a novel Ca^{2+}-binding protein not including the EF-hand motif [Yamaguchi and Yamamoto, 1978; Shimokawa and Yamaguchi, 1993; Misawa and Yamaguchi, 2000]. The gene of regucalcin is highly conserved in vertebrate species [Shimokawa and Yamaguchi, 1993; Misawa and Yamaguchi, 2000]. The rat and human regucalcin genes are localized on chromosome X [Shimokawa et al., 1995; Bhattacharya et al., 2002]. Regucalcin is greatly expressed in liver and kidney cortex, and the expression is mediated through Ca^{2+}-signaling mechanism due to hormonal stimulation [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993; Murata and Yamaguchi, 1999]. AP-1 and NFI-A1 have been found to be transcriptional factors for the enhancement of regucalcin gene promoter

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activity [Murata and Yamaguchi, 1998; Misawa and Yamaguchi, 2002].

Regucalcin has been demonstrated to play a multifunctional role as a regulatory protein on intracellular signaling process in the cytoplasm and nucleus of cells [Yamaguchi, 2000a,b reviews]. Regucalcin plays a role in the maintenance of intracellular Ca^{2+} homeostasis and in the regulation of various Ca^{2+}-dependent protein kinases and tyrosine kinases, protein phosphatases, nitric oxide (NO) synthase, and the suppression of nuclear DNA and RNA syntheses [Yamaguchi, 2000a,b; Tsurusaki and Yamaguchi, 2002a,b; Izumi et al., 2003]. Regucalcin may play a pivotal role in the regulation of cell function.

Recent study has demonstrated that regucalcin has a suppressive effect on cell proliferation. Regucalcin has shown to inhibit nuclear function in regenerating rat liver with proliferative cells [Katsumata and Yamaguchi, 1998; Omura and Yamaguchi, 1999; Tsurusaki and Yamaguchi, 2002a,b], suggesting that the protein has a suppressive effect on proliferation of liver cells. Regucalcin has been shown to translocate to the nucleus of rat liver, and the nuclear localization of regucalcin is enhanced in regenerating rat liver [Tsurusaki et al., 2000; Tsurusaki and Yamaguchi, 2002b]. Moreover, regucalcin has a suppressive effect on cell proliferation and DNA synthesis in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin [Misawa et al., 2002; Tsurusaki and Yamaguchi, 2003]. Regucalcin may have a role as suppressor in the regulation of proliferation of liver cells.

The effect of regucalcin on cell death and apoptosis, however, has not been fully clarified. More recently, it has been shown that regucalcin has a suppressive effect on cell death and apoptosis induced by stimulation of tumor necrosis factor-α (TNF-α) or thapsigargin, an inhibitor of Ca^{2+}-ATPase in the endoplasmic reticulum (Ca^{2+} store) in cells [Izumi and Yamaguchi, 2004], suggesting a role of regucalcin in the regulation of cell death and apoptosis.

The present study, moreover, was undertaken to determine whether regucalcin has a suppressive effect on cell death and apoptosis, which is induced by LPS or various intracellular signaling-related factors. We found that overexpression of regucalcin has a preventive effect on cell death and apoptosis induced by LPS, protein kinase inhibitors (PD 98059 or dibucaine), or an agonist (Bay K 8644) of cellular Ca^{2+} entry in the cloned rat hepatoma H4-II-E cells.

MATERIALS AND METHODS

Chemicals

α-Minimal essential medium (α-MEM) and penicillin-streptomycin solution (5,000 U/ml of penicillin and 5,000 μg/ml of streptomycin) were obtained from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS), lipopolysaccharide (LPS), PD 98059, dibucaine, staurosporine, Bay K 8644, arginine, β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), citrulline, calmodulin (52,000 U/mg protein from bovine brain), phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, MO). Calcium chloride, caspase 3/CPP 32 inhibitor W-1 (caspase inhibitor), and other chemicals were purchased from Wako Pure Chemical Co. Ltd. (Osaka, Japan). Reagents used were dissolved in distilled water, and some reagents were passed through ion-exchange resin to remove metal ions.

Regucalcin Transfectants

Regucalcin transfectants, which are overexpressing regucalcin in the cloned rat hepatoma H4-II-E cells, were used in this experiments as reported previously [Misawa et al., 2002]. The cDNA encoding rat regucalcin was isolated and cloned into the pBluscript vector [Shimokawa and Yamaguchi, 1993]. The regucalcin cDNA contains *P*st I site downstream of the translational stop codon, and a *P*st I site and an *Eco*RI upstream of the regucalcin cDNA. The *Eco*RI fragment (containing the complete coding cDNA) was cloned into the *Eco*RI site of the pCXN2 expression vector [Niwa et al., 1991]. The resultant plasmid was designated as regucalcin (RC)/pCXN2 [Misawa et al., 2002].

For transient transfection assay, the H4-II-E cells were grown on 35-mm dishes to approximately 70% confluence. Each of RC/pCXN2 and pCXN2 vector alone was transfected into H4-II-E cells using the synthetic cationic lipid components, a Tfx-20 reagent, according to the manufacturer's instructions (Promega, Madison WI) [Misawa et al., 2002]. At 48 h after transfection, cells were harvested and used for subsequent experiments. H4-II-E cells were transfected with RC/pCXN2 vector alone using a Tfx-20 reagent. After 24 h, neomycin

(1.0 mg/ml Geneticin G418, Sigma) was added to cultures for selection and cells were plated at limiting dilution. Multiple surviving clones were isolated, transferred to 35-mm dishes, and grown in the medium without neomycin. Regucalcin was stably expressed in the transfectants [Misawa et al., 2002]. In experiments, transfectants were cultured for 72 h in α-MEM containing 10% FBS.

Cell Culture

The cloned rat hepatoma H4-II-E cells and the transfectant of H4-II-E cells (1.0×10^5) were maintained for 72 h in α-MEM supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin in humidified 5% CO_2/95% air at 37°C to obtain subconfluent monolayers [Misawa et al., 2002]. After culture, cells were washed three times with phosphatate-buffered saline (PBS), and the cells were incubated for 12–72 h in α-MEM without 10% FBS in the absence or presence of LPS (0.1 or 1.0 μg/ml of culture medium). Cells were washed three times with PBS after culture, and the number of cells was counted. The polled cells were scraped into 0.5 ml of ice-cold 0.25 M sucrose solution, and disrupted for 30 s with an ultrasonic device. Scrapped cells were also homogenized in Potter-Elvehjem homogenizer with a Teflon pestle. The homogenates were spun at 5,500*g* in a refrigerated centrifuge for 5 min to remove nuclei and cell debris. The 5,500*g* supernatant was pooled to assay NO synthase activity [Izumi et al., 2003]. Protein concentration in the 5,500*g* supernatant of cell homogenate was determined by the method of Lowry et al. [1951].

Cell Counting

After trypsinization of each of the culture dishes using 0.2% trypsin plus 0.02% EDTA in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) for 2 min at 37°C, cells were collected and centrifuged in a PBS at 100*g* for 5 min. The cells were resuspended on PBS solution and stained with eosin. Cell numbers were counted under a microscope using a Hemacytometer plate. For each dish, we took the average of two countings.

Assay of NO Synthase

NO synthase activity in the 5,500*g* supernatant of the cloned rat hepatoma cell homogenate was estimated by the procedure of Lee and Stull [1998] with a minor modification. The enzyme activity was measured for 60 min at 37°C in a reaction mixture (1.0 ml) containing 100 mM HEPES, pH 7.2, 4 mM β-NADPH, 2 mM L-arginine, and the cell protein (50–80 μg/ml) in the absence or presence of both calcium chloride (10 μM) and calmodulin (2.5 μg/ml). The enzyme reaction was terminated by the addition of 1.0 ml of cold 10% trichloroacetic acid and centrifuged to precipitate protein. Produced citrullin in the supernatant was quantified by the method of Boyde and Rahmatullah [1980]. Results were expressed as nanomoles of cirtrullin produced per minute (min) per milligram (mg) of cell protein. Protein concentration was determined by the method of Lowry et al. [1951] using bovine serum albumin as the standard.

Analysis of DNA Fragmentation

The cloned rat hepatoma H4-II-E cells and the transfectant of H4-II-E cells (1×10^5) were cultured for 72 h in α-MEM without 10% FBS in the absence or presence of LPS (0.1, 1, or 10 μg/ml) or Bay K 8644 (2.5×10^{-6} M). The culture supernatant was removed and adherent cells were then lysed in 10 mM Tris-HCl, pH 7.4, 10 mM EDTA (neutralized), and 0.5% Triton X-100. Low-molecular-weight DNA fragments were separated by electrophoresis in 1.5% agarose gel [Preaux et al., 2002]. Gels were visualized by ethidium bromide staining with a UV transilluminator (Funakoshi Co. Ltd., Tokyo, Japan).

Statistical Analysis

Data were expressed as the mean ± SEM. The significance of difference between the values was estimated by Student's *t*-test or by analysis of variance (ANOVA) for comparing multiple groups. A *P*-value of <0.05 was considered to indicate statistically significant difference.

RESULTS

Effect of LPS on Hepatoma Cells Overexpressing Regucalcin

The hepatoma cells (wild-type), pCXN2 transfected cells (mock-type), or stable regucalcin (RC)/pCXN2 transfectants were cultured for 72 h in medium containing 10% FBS to obtain subconfluent monolayers. The proliferation of the cells was significantly suppressed in transfectants cultured for 72 h, as shown previously

[Tsurusaki and Yamaguchi, 2003]. After culture for 72 h, cells were changed to medium not containing FBS in the absence or presence of LPS (0.1 or 1.0 μg/ml of medium), and further cultured for 12–72 h. The expression of regucalcin in the cells was remarkable in transfectants (data not shown), as shown previously [Misawa et al., 2002; Izumi et al., 2003].

Hepatoma cells with subconfluent monolayers were cultured for 12, 24, 48, or 72 h in medium without FBS containing either vehicle or LPS (0.1 or 1.0 ng/ml) (Fig. 1). The number of wild-type cells was significantly decreased by culture of 24–72 h in the presence of LPS (0.1 or 1.0 μg/ml). With culture of 12 h, the number of wild-type cells was significantly decreased in the presence of 0.1 ng/ml of LPS. The effect of LPS (0.1 or 1.0 ng/ml) in decreasing the number of hepatoma cells (wild-type) was not observed in stable RC/pCXN2-transfected cells cultured for 12, 24, or 48 h. With culture of 72 h, cell number of transfectants was significantly decreased in the presence of LPS (0.1 or 1.0 ng/ml).

Hepatoma cells with subconfluent monolayers with 10% FBS for 72 h were further cultured for 24, 48, or 72 h in medium without FBS containing either vehicle or LPS (1.0 μg/ml) (Fig. 2). The lysate of wild-type cells obtained with culture for 24, 48, or 72 h was used to assay NO synthase activity without or with calcium chloride (10 μM) plus calmodulin (2.5 μg/ml) addition in the enzyme reaction mixture. NO synthase activity was significantly increased by Ca^{2+}/calmodulin addition. Culture with LPS (1.0 μg/ml) for 24, 48, or 72 h of wild-type cells caused a significant decrease in NO synthase activity without or with Ca^{2+}/calmodulin addition. The effect of Ca^{2+}/calmodulin addition in increasing NO synthase activity was abolished in transfectants cultured for 24–72 h. Moreover, the suppression of Ca^{2+}/calmodulin-dependent NO synthase activity induced by culture with LPS for 24–72 h was not observed in transfectants.

Effect of Various Inhibitors on Hepatoma Cells Overexpression Regucalcin

Hepatoma cells (wild-type) and transfectants with subconfluent monolayers were cultured for 24 h in medium without FBS containing either vehicle or LPS (0.1 or 1.0 μg/ml) in the absence or presence of various inhibitors with an effec-

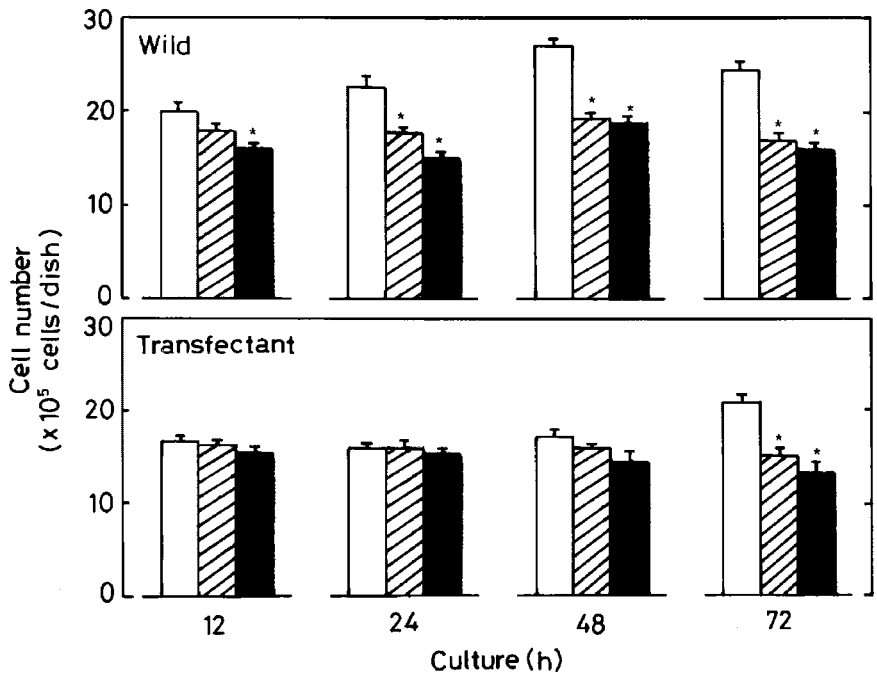

Fig. 1. Effect of lipopolysaccharide (LPS) on the number of the cloned rat hepatoma H4-II-E cells (wild-type) or RC/pCXN2-transfected cells. Cells were cultured for 72 h in medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium without FBS containing either vehicle or LPS (0.1 or 1.0 μg/ml). After medium change, cells were incubated for 24, 48, or 72 h, and the number of cells was measured. Each value is the mean ± SEM of six experiments. *$P < 0.01$, compared with the control value. White bars, wild-type cells; hatched bars, LPS (0.1 μg/ml); black bars, LPS (1.0 μg/ml).

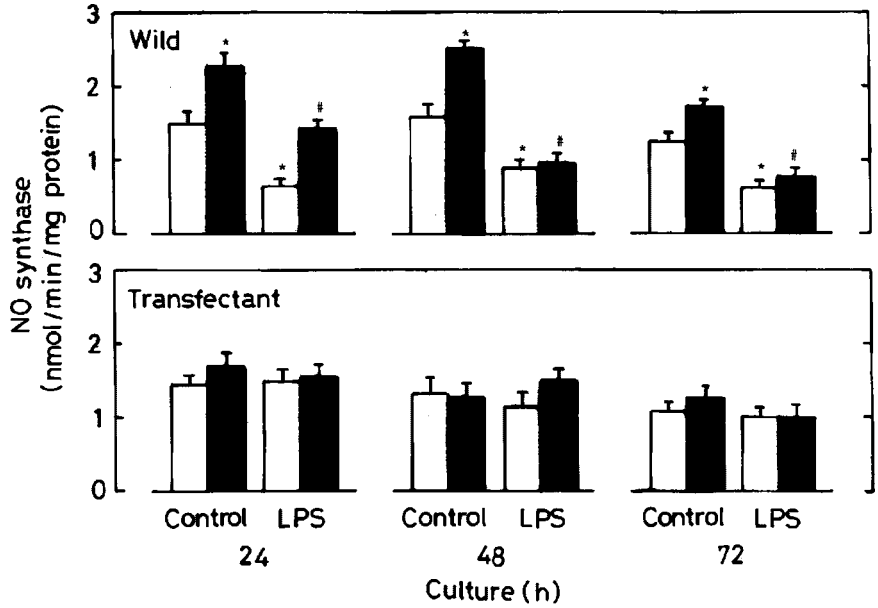

Fig. 2. Effect of LPS on nitric oxide (NO) synthase activity in the cloned rat hepatoma H4-II-E cells (wild-type) or RC/pCXN2-transfected cells. Cells were cultured for 72 h in medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium without FBS containing either vehicle or LPS (1.0 μg/ml). After medium change, cells were incubated for 24, 48, or 72 h, and NO synthase activity in cell lysate was measured. Enzyme reaction mixture contained either vehicle or calcium chloride (10 μM) plus calmodulin (2.5 μg/ml). Each value is the mean ± SEM of six experiments. $^{*}P < 0.01$, compares with the control value without calcium and calmodulin addition. $^{\#}P < 0.01$, compared with the value with calcium and calmodulin addition of wild-type cells cultured with LPS. White bars, none; black bars, calcium chloride (10 μM) plus calmodulin (2.5 μg/ml).

tive concentration on enzyme activity. The effect of LPS (0.1 or 1.0 μg/ml) in decreasing cell number of hepatoma cells (wild-type) was not seen in the presence of caspase inhibitor (10^{-8} M) (Fig. 3). The presence of caspase inhibitor did not have a significant effect on cell number in wild-type cells or transfectants without or with LPS (0.1 or 1.0 μg/ml).

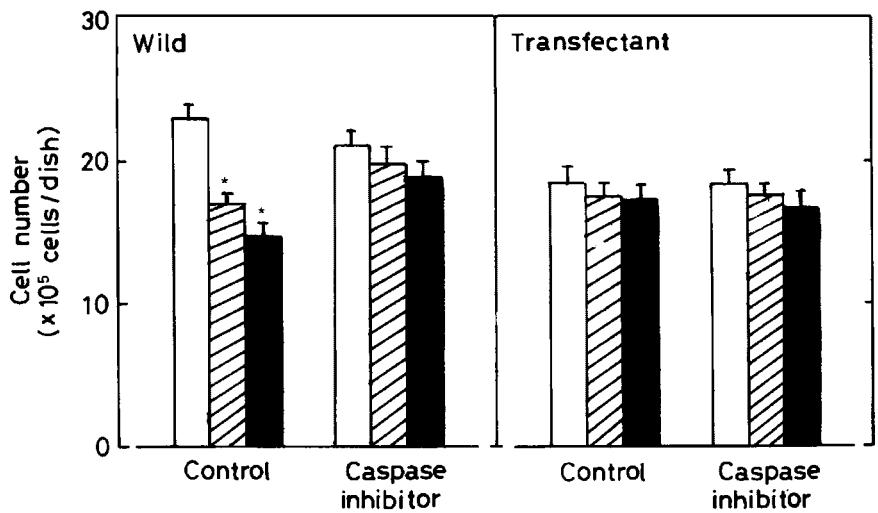

Fig. 3. Effect of caspase inhibitor on LPS-induced decrease in the number of the cloned rat hepatoma H4-II-E cells (wild-type) or RC/pCXN2-transfected cells. Cells were cultured for 72 h in medium containing 10% FBS to obtain subconfluent monolayers, and exchanged to culture medium without FBS containing either vehicle or LPS (0.1 or 1.0 μg/ml) in the absence or presence of caspase inhibitor (10 nM). After medium change, cells were incubated for 24 h, and the number of cells was measured. Each value is the mean ± SEM of six experiments. $^{*}P < 0.01$, compared with the control value obtained from cells cultured without LPS. White bars, none; hatched bars, LPS (0.1 μg/ml); black bars, LPS (1.0 μg/ml).

The effect of inhibitor of protein kinases on cell death of the hepatoma cells is shown in Figure 4. The number of hepatoma cells (wild-type) was significantly decreased by culture with PD 98059 (10^{-6} M), dibucaine (10^{-6} M), or staurosporine (10^{-6} M). The effect of LPS (1.0 μg/ml) in decreasing the number of hepatoma cells (wild-type) was not seen in the presence of PD 98059 or dibucaine. Culture with staurosporine caused a significant decrease in cell number of hepatoma cells (wild-type) in the presence of LPS. Meanwhile, the effect of PD 98059 or dibucaine in decreasing the number of hepatoma cells was not seen in transfectants. The effect of staurosporine on cell number was also seen in transfectants cultured in the absence or presence of LPS. In this experiments, there was potential loss of cell attachement by culture with staurosporine.

Effect of PMA on Hepatoma Cells Overexpressing Regucalcin

The effect of PMA, an activator of protein kinase C, on the number of hepatoma cells is shown in Figure 5. Hepatoma cells (wild-type) and transfectants with subconfluent monolayers were cultured for 24 h in medium without FBS containing either vehicle or PMA (10^{-6} M) in the absence or presence of LPS (1.0 μg/ml). The number of wild-type cells or transfectants was not significantly altered by culture with PMA. The effect of LPS in decreasing the number of wild-type cells was weakened in the presence of PMA.

Effect of Bay K 8644 on Hepatoma Cells Overexpressing Regucalcin

The effect of Bay K 8644, an agonist of Ca^{2+} entry in cells, on the number of hepatoma cells is shown in Figure 6. Hepatoma cells (wild-type) and transfectants with subconfluent monolayers were cultured for 24 h in medium without FBS containing either vehicle or Bay K 8644 (2.5×10^{-6} M) in the absence or presence of LPS (0.1 or 1.0 μg/ml). Cell number of wild-type cells was significantly decreased by culture with Bay K 8644. This decrease was not seen in transfectants. The effect of LPS (0.1 or 1.0 μg/ml) in decreasing the number of wild-type cells was not observed in the presence of Bay K 8644.

Hepatoma wild-type cells (W) and transfectants (T) with subconfluent monolayers were cultured for 24 h in medium without FBS containing either vehicle or Bay K 8644 (2.5×0^{-6} M) in the absence of LPS (Fig. 7). Adherent cells were lysed, and then the lysate was separated by electrophoresis in agarose gel. Culture with Bay K 8644 caused DNA fragmentation in wild-type cells. The effect of Bay K 8644 on DNA fragmentation was significantly suppressed in transfectants. The same results were also observed using equal amount of total DNA in wild-type cells or transfectants.

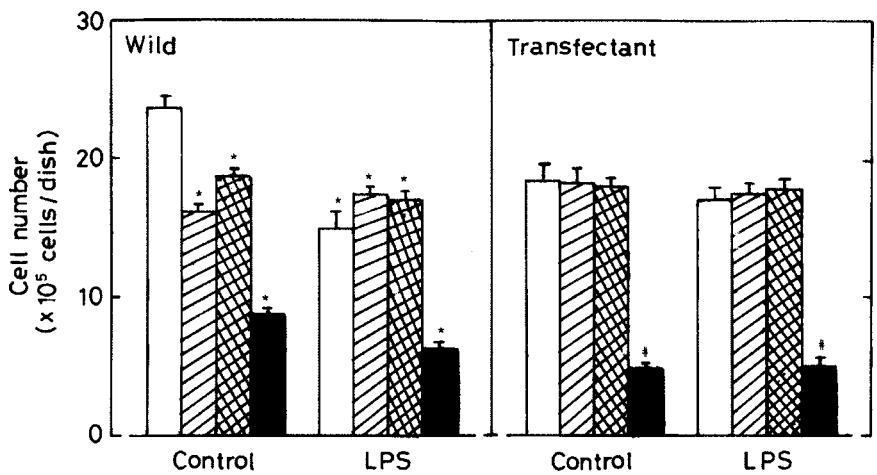

Fig. 4. Effect of protein kinase inhibitors on the number of the cloned rat hepatoma H4-II-E cells (wild-type) or RC/pCXN2-transfected cells. Cells were cultured for 72 h in medium containing 10% FBS to obtain subconfluent monolayers, and exchange to culture medium without FBS containing either vehicle or LPS (1.0 μg/ml) in the absence or presence of PD 98059 (10^{-6} M), dibucaine (10^{-6} M), or staurosporine (10^{-6} M). After medium change, cells were incubated for 24 h and the number of cells was measured. Each value is the mean ± SEM of six experiments. $^{*}P < 0.01$, compared with the control value without the addition of protein kinase inhibitor. $^{\#}P < 0.01$, compared with the control value from transfectants. White bars, control; hatched bars, PD 98059; double hatched bar, dibucaine; black bars, staurosporine.

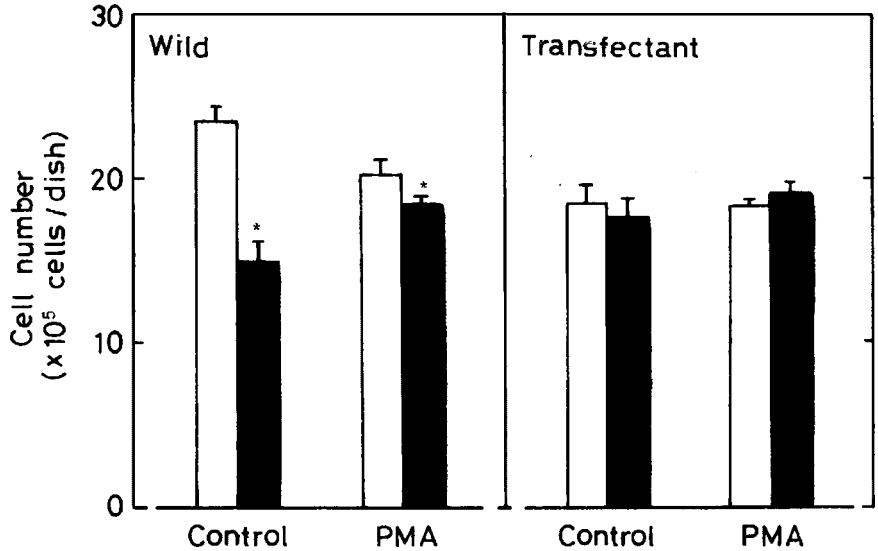

Fig. 5. Effect of PMA, an activator of protein kinase C, on the number of the cloned rat hepatoma H4-II-E cells (wild-type) or RC/pCXN2-transfected cells. Cells were cultured for 72 h in medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium without FBS containing either vehicle or LPS (1.0 μg/ml) in the absence or presence of PMA (10^{-6} M). After medium change, cells were incubated for 24 h, and the number of cells was measured. Each value is the mean ± SEM of six experiments. *$P < 0.01$, compared with the control value without LPS. White bars, without LPS; black bars, with LPS.

DISCUSSION

Overexpression of regucalcin has been shown to have a suppressive effect on the proliferation of the cloned rat hepatoma H4-II-E cells when cultured to obtain subconfluent monolayers with 10% FBS [Misawa et al., 2002; Tsurusaki and Yamaguchi, 2003]. The subconfluent monolayer cells were further cultured in medium without FBS in the presence of LPS. LPS caused a significant decrease in the number of hepatoma H4-II-E cells (wild-type), inducing cell death. This decrease was completely prevented in the regucalcin cDNA-transfected hepatoma cells overexpressing regucalcin with culture for 12–48 h. These

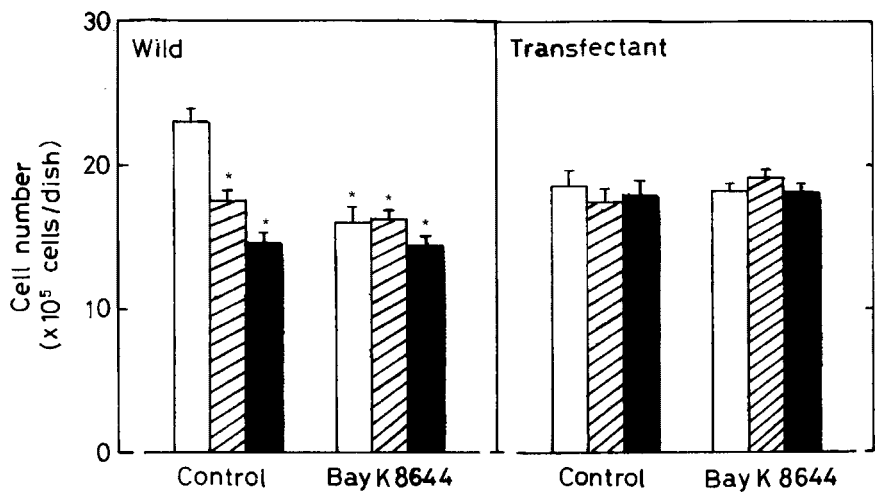

Fig. 6. Effect of Bay K 8644, an agonist of Ca^{2+} entry in cells, on the number of the cloned rat hepatoma H4-II-E cells (wild-type) or RC/pCXN2-transfected cells. Cells were cultured for 72 h in medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium without FBS containing either vehicle or LPS (0.1 or 1.0 μg/ml) in the absence or presence of Bay K 8644 (2.5×10^{-6} M). After medium change, cells were cultured for 24 h, and the number of cells was measured. Each value is the mean ± SEM of six experiments. *$P < 0.01$, compared with the control value without LPS addition. White bars, control; hatched bars, LPS (0.1 μg/ml) addition; black bars, with LPS (1.0 μg/ml).

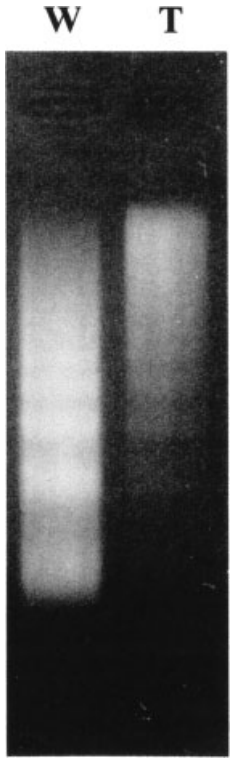

Fig. 7. Effect of Bay K 8644 on DNA fragmentation in the cloned rat hepatoma H4-II-E cells (wild-type; W) or RC/pCXN2-transfected cells (T). Cells were cultured for 72 h in medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium without FBS containing either vehicle or LPS (0.1 or 1.0 μg/ml) in the absence or presence of Bay K 8644 (2.5×10^{-6} M). After medium change, cells were cultured for 24 h, and the cell lysate was applied to agarose gel. The figure shows one of four experiments with separate samples.

observations were also seen in transfectants with other clones (data not shown). This finding demonstrates that overexpression of regucalcin has a suppressive effect on cell death induced by stimulation of LPS. The effect of TNF-α or thapsigargin in stimulating cell death was previously shown to suppress in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin [Izumi and Yamaguchi, 2004]. Presumably, regucalcin has a suppressive effect on cell death that is mediated through various intracellular signaling pathways.

NO may be important as a signaling factor in many cells [Lowenstein et al., 1994], and it plays a role in apoptosis of hepatoma cells [Liu et al., 2000]. NO mediates apoptosis by D-galactosamine in a primary culture of rat hepatocytes [Abou-Elella et al., 2002]. Culture with LPS caused a significant decrease in Ca^{2+}/calmodulin-dependent NO synthase activity in the cloned rat hepatoma H4-II-E cells (wild-type). LPS-induced decrease in NO synthase activity was found to prevent significantly in the hepatoma cells (transfectants) overexpressing regucalcin. These results indicate that LPS-induced cell death is not resulted from NO production in hepatoma cells, and that the suppressive effect of regucalcin on LPS-induced cell death is not involved in NO in the cells.

LPS acts to modulate the expression of a large number of genes that favor apoptosis of fibroblastic cells that are dependent upon activation of caspase-8 [Alikhani et al., 2003]. There is evidence that LPS-induced cell death is mediated by accumulation of reactive oxygene species and activation of p38 in rat cortex and hippocampus [Nalan et al., 2003]. Moreover, STAT1 has been shown to play an essential role in LPS/D-galactosamine-induced liver apoptosis and injury in human hepatoma Hep3B cells [Kim et al., 2003]. We showed that LPS-induced cell death is significantly prevented by culture with caspase inhibitor in the cloned rat hepatoma H4-II-E cells. It is speculated that the effect of regucalcin in suppressing LPS-induced cell death is partly related to the inhibitory effect on caspase-3 in hepatoma cells.

An induction of apoptosis is partly mediated through protein kinase pathway. The death of hepatoma cells (wild-type) was found to be induced by culture with PD 98059, a ERK inhibitor, dibucaine, an inhibitor of Ca^{2+}-dependent protein kinase, or staurosporine, a potent inhibitor of serine/threonine kinases. This observation suggests that the effect of LPS on cell death is partly involved in the inhibition of protein kinases. Overexpression of regucalcin in the hepatoma cells rescued cell death with PD 98059 or dibucaine. Such an effect, however, was not observed with staurosporine. PD98059 induces apoptosis, which is in part due to the inactivation of Bcl-2 by increasing phosphorylated Bcl-2 in human prostate cancer cells [Zelivianski et al., 2003]. Dibucaine has been shown to activate various caspases, such as caspase-3, -6, -8, and -9 (-like) activities, but not caspase-1 (-like) activity, and induced mitochondrial membrane depolarization and the release of cytochrome C from mitochondria into the cytosol in promyelocytic leukemia cells (HL-60) [Arita et al., 2000]. Staurosporine induces apoptosis in Chang liver cells by a mitochondria-caspase-dependent pathway, which

was closely correlated with a decrease in Bcl-2 and Bcl-XL levels in cancer cells [Giuliano et al., 2004]. It is speculated that regucalcin partly acts the activation of Bcl-2 or the inhibition of caspases for signaling mechanism that PD98059 or dibucaine induces apoptosis.

Culture with PMA, an activator of protein kinase C, did not cause a significant increase in cell death of the hepatoma cells (wild-type) and transfectants overexpressing regucalcin. The effect of LPS in inducing the death of wild-type cells, however, was weakened by culture with PMA. PMA may have partial suppressive effect on LPS-induced cell death. It has been reported that PMA may decrease the generation of apoptosis by antileukemic agent arsenic trioxide in human promonocytic cells [Fernandez et al., 2004]. Regucalcin has been shown to have an inhibitory effect on protein kinase C activity in rat liver cytosol [Yamaguchi and Mori, 1990]. Presumably, the suppressive effect of regucalcin on cell death and apoptosis is not resulted from the inhibitory effect on protein kinase C.

Calcium entry into cells induces cell death [Cano-Abad et al., 2001; Pereira et al., 2002]. Culture with Bay K 8644, an agonist of Ca^{2+} entry in cells, caused a significant increase in the death of hepatoma H4-II-E cells (wild-type). LPS-induced cell death was not further enhanced by culture with Bay K 8644 in wild-type cells. Culture with both LPS and Bay K 8644 did not induce cell death of transfectants overexpressing regucalcin. The presence of low-molecular-weight DNA fragments was observed in the adherent hepatoma cells cultured with Bay K 8644. Results indicate that Bay K 8644 induces DNA fragmentation in the adherent hepatoma cells. Overexpression of regucalcin in the hepatoma cells was found to suppress DNA fragmentation induced by Bay K 8644. Regucalcin may have an inhibitory effect on Ca^{2+} entry-induced stimulation of apoptosis in the hepatoma cells.

It has been shown that Ca^{2+} entry causes both calcium overload and mitochondrial disruption that will lead to the release of mediators responsible for the activation of the apoptotic cascade and cell death in bovine chromaffin cells [Cano-Abad et al., 2001]. Rises in intracellular Ca^{2+} concentration are believed to activate nuclear Ca^{2+}/Mg^{2+}-dependent endonuclease and to mediate DNA cleavage into oligonucleosome fragments [Pereira et al., 2002]. Regucalcin has been demonstrated to inhibit Ca^{2+}-activated DNA fragmentation in the nuclei isolated from rat liver [Yamaguchi and Sakurai, 1991]. Moreover, regucalcin has been demonstrated to activate Ca^{2+} pump enzymes in the plasma membranes, mitochondria, and endoplasmic reticulum of rat liver cells [Yamaguchi, 2000a in review], suggesting that regucalcin plays a role in the regulation of Ca^{2+} homeostasis in the cells. Regucalcin has a suppressive effect on Ca^{2+} entry-mediated cell death due to preventing the rise in intracellular Ca^{2+} concentration in the hepatoma cells. In addition, regucalcin may suppress the effect of Ca^{2+} on DNA fragmentation in the nucleus of hepatoma cells.

Overexpression of regucalcin has been shown to have a suppressive effect on cell death and apoptosis induced by TNF-α or thapsigargin in the cloned rat hepatoma H4-II-E cells [Izumi and Yamaguchi, 2004]. Here, we found that overexpression of regucalcin rescues cell death and apoptosis induced by culture with LPS, PD 98059, dibucaine, or Bay K 8644 in the hepatoma cells. The signaling mechanisms, that TNF-α, LPS, or other factors mediate cell death and apoptosis, may be different. Regucalcin may have the suppressive effect on many signaling pathways that mediate cell death and apoptosis. From the previous and present observations, it is assumed that the suppressive effect of regucalcin on cell death and apoptosis is related to its inhibitory effect on the activities of NO synthase, caspase-3, or Ca^{2+}-dependent endonuclease and its activatory effect on Bcl-2. The present study further supports the view that regucalcin plays a role as a regulatory protein in intracellular signaling pathway in cells.

Regucalcin has been shown to have a suppressive effect on proliferation of liver cells [Yamaguchi, 2000b; Tsurusaki and Yamaguchi, 2003] due to hormonal stimulation. Moreover, regucalcin has been demonstrated to have a suppressive effect on cell death and apoptosis induced by stimulation of various factors in the hepatoma H4-II-E cells, a model for normal hepatocytes, which may have altered apoptotic responses. Regucalcin may play a physiologic role in the maintenance of homeostasis of cellular response for cell stimulation.

In conclusion, it has been demonstrated that overexpression of regucalcin suppresses cell death in the cloned rat hepatoma cells induced by culture with LPS or other intracellular signaling-related factors.

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