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Overexpression of Regucalcin Suppresses Cell Death and Apoptosis in Cloned Rat Hepatoma H4-II-E Cells Induced by Insulin or Insulin-Like Growth Factor-I

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Abstract The role of regucalcin, a regulatory protein in intracellular signaling pathway, in cell death was investigated by using the cloned rat hepatoma H4-II-E cells overexpressing regucalcin. The hepatoma cells (wild-type) and stable regucalcin/pCXN2 transfectants were cultured for 72 h in a medium containing 10% fetal bovine serum (FBS) to obtain subconfluent monolayers. After culture for 72 h, cells were further cultured for 24–72 h in a medium containing either vehicle, insulin (10^{-8} or 10^{-7}M) or insulin-like growth factor-I (IGF-I; 10^{-9} or 10^{-8}M) in the absence of FBS. The number of wild-type cells was significantly decreased by culture for 24, 48, or 72 h in the presence of insulin (10^{-8} or 10^{-7}M) or IGF-I (10^{-9} or 10^{-8}M). Agarose gel electrophoresis showed the presence of low-molecular-weight deoxyribonucleic acid (DNA) fragments of adherent wild-type cells cultured with insulin or IGF-I. The effect of insulin or IGF-I in stimulating cell death and DNA fragmentation in hepatoma cells (wild-type) was significantly prevented in transfectants overexpressing regucalcin. Meanwhile, epinephrine (10^{-6} or 10^{-5}M) or transforming growth factor-β1 (10^{-13} or 10^{-12}M) did not cause cell death of hepatoma cells. Insulin-induced decrease in the number of wild-type cells was significantly prevented by culture with caspase-3 inhibitor (10^{-8}M), although the effect of IGF-I was not inhibited. The effect of insulin or IGF-I in inducing the death of hepatoma cells (wild-type) was significantly prevented in the presence of *N* ω-nitro-L-arginine methylester (NAME), an inhibitor of nitric oxide synthase. Genistein (10^{-6}M), an inhibitor of protein tyrosine kinase, or vanadate (10^{-5}M), an inhibitor of protein tyrosine phosphatase, caused a significant decrease in the number of hepatoma cells (wild-type). The effect of insulin in inducing the death of wild-type cells was not seen in the presence of genistein or vanadate. The effect of IGF-I on the death of wild-type cells was observed in the presence of genistein or vanadate. The effect of genistein on cell death was significantly prevented in transfectants. Such effect was not seen with vanadate. This study demonstrates that insulin or IGF-I stimulates cell death and apoptosis in the hepatoma cells, and that overexpression of regucalcin has a suppressive effect on cell death induced by insulin or IGF-I that is mediated through different signaling pathway. J. Cell. Biochem. 96: 145–154, 2005. © 2005 Wiley-Liss, Inc.

Key words: regucalcin; insulin; IGF-I; cell death; apoptosis; caspase-3; hapatoma 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; Thiselton 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 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, 2005;

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in reviews]. Recent study has demonstrated that regucalcin has a suppressive effect on cell proliferation. Regucalcin has been shown to inhibit the enhancement of protein kinase activity, protein phosphatase activity, deoxyribonucleic acid (DNA) and RNA synthesis in the nucleus of 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 be a downregulated gene in line with findings for hepatocellular carcinomas in the liver of rats treated with diethylnitrosamine and then 2-acetylaminofluorene combined with partial hepatectomy [Suzuki et al., 2004]. Regucalcin has been shown to translocate to liver nucleus, and the localization of regucalcin is enhanced in regenerating rat liver [Tsurusaki et al., 2000; Tsurusaki and Yamaguchi, 2002b]. Nuclear localization of regucalcin has also been found to increase in the liver of growth hormone-administered rats [Laz et al., 2004]. Moreover, overexpression of regucalcin suppresses cell proliferation and DNA synthesis in the cloned rat hepatoma H4-II-E cells [Misawa et al., 2002; Tsurusaki and Yamaguchi, 2003]. Regucalcin may have a role as suppressor in the regulation of proliferation of liver cells due to inhibiting nuclear function.

The effect of regucalcin on cell death and apoptosis, however, has not been fully clarified. Overexpression of regucalcin has been shown to have a suppressive effect on cell death and apoptosis induced by stimulation of tumor necrosis factor-α, lypoplysaccharide, thapsigargin, an inhibitor of Ca^{2+}-ATPase in the endoplasmic reticulum (Ca^{2+} store) in cells, or Bay K 8644, an agonist of cellular Ca^{2+} entry, in the cloned rat hepatoma H4-II-E cells [Izumi and Yamaguchi, 2004a,b]. Regucalcin may have a suppressive effect on various signaling pathways that mediate cell death and apoptosis. The suppressive effect of regucalcin on cell death and apoptosis may be related to the inhibitory effect on the activation of caspase-3, nitric oxide (NO) synthase or Ca^{2+}-dependent endonuclease and the activatory effect on Bcl-2.

This study, moreover, was undertaken to determine whether insulin and IGF-I, which act on liver cells, induce cell death and apoptosis in the cloned rat hepatoma H4-II-E cells and overexpression of regucalcin suppresses their effects on cell death in the hepatoma cells. We found that overexpression of regucalcin has a preventive effect on cell death and apoptosis induced by insulin or IGF-I in the cloned rat hepatoma H4-II-E cells.

MATERIALS AND METHODS

Chemicals

α-Minimun essential medium (α-MEM) and penicillin-streptomycin solution (5,000 U/ml penicillin; 5,000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Isoland, NY). Fetal bovine serum (FBS), insulin (from bovine pancreas), insulin-like growth factor-I (human IGF-I), transforming growth factor-β1 (human TGF-β1), epinephrine, Bay K 8644, and genistein were obtained from Sigma Chemical Co. (St. Louis, MO). Caspase-3/CPP 32 inhibitor W-1 (caspase-3 inhibitor), *N* ω-nitro-L-arginine methylester (NAME), vanadate 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 experiment 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 *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/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 regucalcin/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 regucalcin/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; the increase in regucalcin in transfectants was 12.6-fold of wild-type cells. 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 phosphate-buffered saline (PBS), and the cells were incubated for 24–72 h in α-MEM without 10% FBS in the presence or absence of insulin (10^{-8} or 10^{-7}M) or IGF-I (10^{-9} or 10^{-8}M). Cells were washed three times with PBS after culture, and the number of cells was counted.

Cell Counting

After trypsinization of each of the culture dishes using 0.2% trypsin plus 0.02% EDTA in Ca^{2+}/Mg^{2+}-free 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.

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 presence or absence of insulin (10^{-7}M) or IGF-I (10^{-9}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 an UV transilluminator (Funakoshi Co. Ltd., Tokyo, Japan). DNA content in the cell lysate was determined by the method of Cerriotti [1955].

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 Insulin or IGF-I on Hepatoma Cells Overexpressing Regucalcin

The hepatoma cells (wild-type), pCXN2 transfected cells (mock type), or stable regucalcin/pCXN2 transfectants were cultured for 72 h in a 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 presence or absence of insulin (10^{-8} or 10^{-7}M) or IGF-I (10^{-9} or 10^{-8}M), and further cultured for 24, 48 or 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]. The number of wild-type cells was significantly decreased by culture of 24, 48, or 72 h in the presence of insulin (10^{-8} or 10^{-7}M) (Fig. 1) or IGF-I (10^{-9} or 10^{-8}M) (Fig. 2). The effect of insulin (10^{-8} or 10^{-7}M) or IGF-I (10^{-9} or 10^{-8}M) in decreasing the number of wild-type cells was not observed in stable regucalcin/pCXN2-transfected cells cultured for 24, 48, or 72 h (Figs. 1 and 2).

Hepatoma wild-type cells and transfectants with subconfluent monolayers were cultured for 48 h in a medium containing either vehicle, insulin (10^{-7}M) or IGF-I (10^{-9}M) in the absence of FBS (Fig. 3). Adherent cells were collected, and then the lysate of cell was separated by electrophoresis in agarose gel. Culture with insulin or IGF-I caused a remarkable DNA fragmentation in wild-type cells. The effect of insulin or IGF-I on DNA fragmentation was significantly suppressed in transfectants.

The effect of insulin (10^{-8} or 10^{-7}M) in decreasing the number of hepatoma (wild-type)

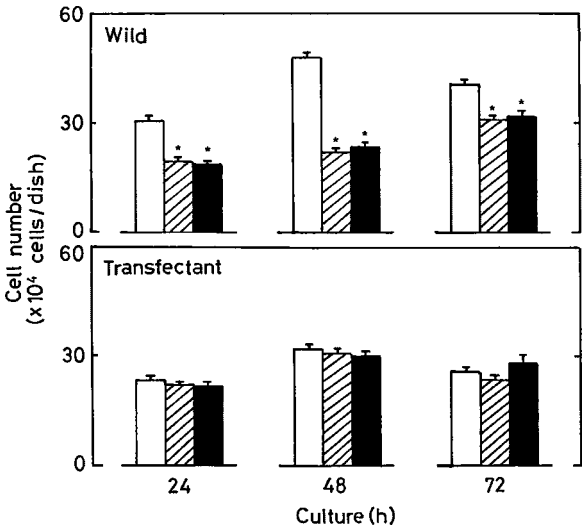

Fig. 1. Effect of insulin on the number of the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells. Cells were cultured for 72 h in a medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium containing either vehicle or insulin (10^{-8} or 10^{-7}M) in the absence of FBS. 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 (none) value. White bars, control; hatched bars, insulin (10^{-8}M); black bars, insulin (10^{-7}M).

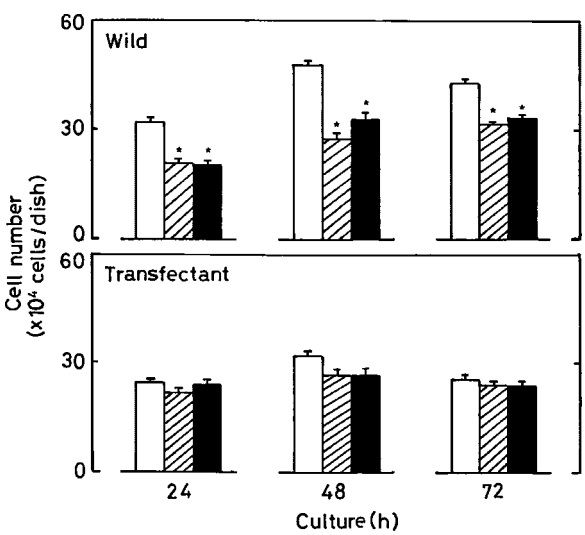

Fig. 2. Effect of IGF-I on the number of the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells. Cells were cultured for 72 h in a medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium without FBS containing either vehicle or IGF-I (10^{-9} or 10^{-8}M). 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 (none) value. White bars, control; hatched bars, IGF-I (10^{-9}M); black bars, IGF-I (10^{-8}M).

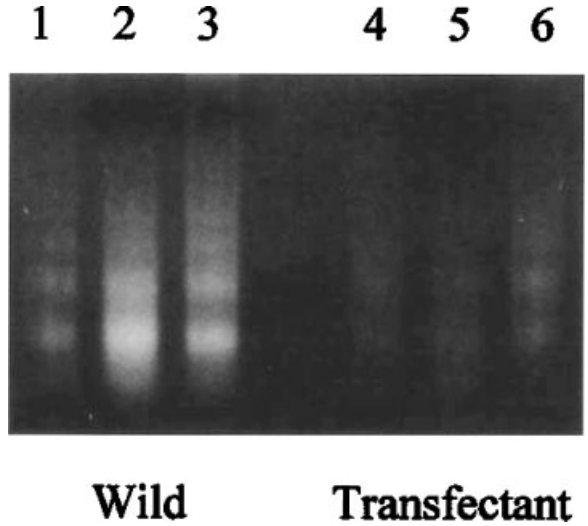

Fig. 3. Effect of insulin or IGF-I on DNA fragmentation in the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells. Cells were cultured for 72 h in a medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium without FBS containing either vehicle or insulin (10^{-7}M) or IGF-I (10^{-9}M). After medium change, cells were incubated for 48 h, and the cells lysate (containing 1.8 μg DNA) was applied to agarose gel. The figure shows one of four experiments with separate samples. **Lanes 1** and **4**, control (none); **lanes 2** and **5**, insulin; **lanes 3** and **6**, IGF-I.

cells was not significantly enhanced in the presence of IGF-I (10^{-9}M) (Fig. 4). The combination of insulin (10^{-8} or 10^{-7}M) and IGF-I (10^{-9}M) did not cause a significant decrease in the number of transfectants, although the higher concentration of IGF-I may have an additional effect on cell death (Fig. 4).

Hepatoma wild-type cells and transfectants with subconflueent monolayers were cultured for 72 h in a medium containing either vehicle, epinephrine (10^{-6} or 10^{-5}M) or TGF-β1 (10^{-13} or 10^{-12}M) in the absence of FBS (Fig. 5). The number of wild-type cells or transfectant was not significantly changed in the presence of epinephrine or TGF-β1.

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 a medium without FBS containing either vehicle, insulin (10^{-7}M) or IGF-I (10^{-9}M) in the presence or absence of Bay K 8644 (2.5×10^{-6}M). The

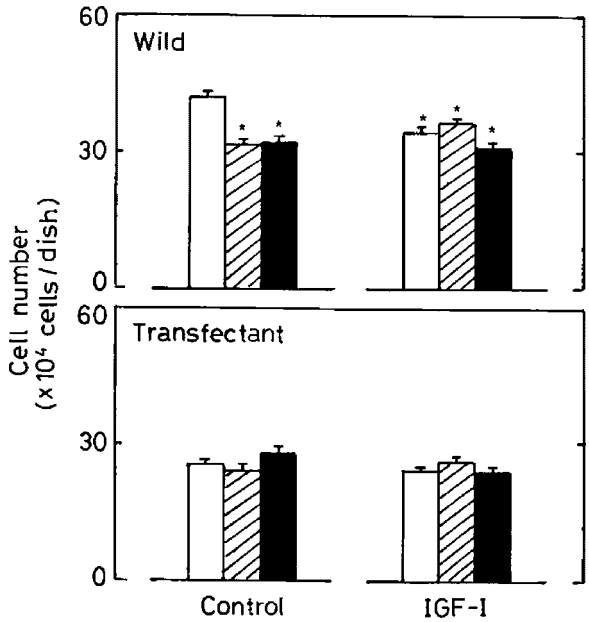

Fig. 4. Effect of insulin on the number of the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells in the presence of IGF-I. Cells were cultured for 72 h in a medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium without FBS containing either vehicle or insulin (10^{-8} or 10^{-7}M) in the presence or absence of IGF-I (10^{-9}M). After medium change, cells were incubated for 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 (none) value. White bars, control; hatched bars, insulin (10^{-8}M); black bars, insulin (10^{-7}M).

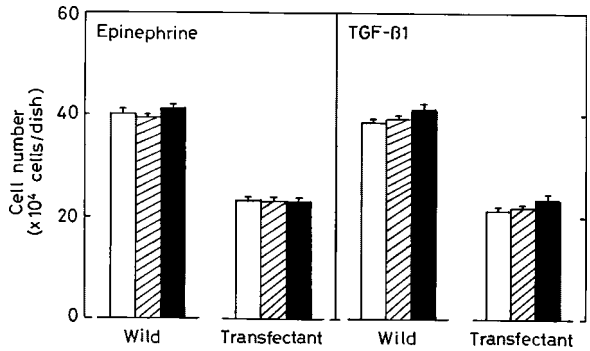

Fig. 5. Effect of epinephrine or TGF-β1 on the number of the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells. Cells were cultured for 72 h in a medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium without FBS containing either vehicle epinephrine (10^{-6} or 10^{-5}M) or TGF-β1 (10^{-13} or 10^{-12}M). After medium change, cells were incubated for 72 h, and the number of cells was measured. Each value is the mean ± SEM of six experiments. Epinephrine: white bars, control (none); hatched bars, 10^{-6}M; black bars, 10^{-5}M. TGF-β1: white bars, control; hatched bars, 10^{-13}M; black bars, 10^{-12}M.

number of wild-type cells was significantly decreased by culture with Bay K 8644. The decrease was not seen in transfectants, as shown previously [Izumi and Yamaguchi, 2004a,b]. The effect of IGF-I (10^{-9}M) in decreasing the number of wild-type cells was significantly enhanced in the presence of Bay K 8644. Such effect was not observed in the case of insulin. The number of transfectants was significantly decreased in the presence of Bay K 8644. This decrease was not significantly modulated in the presence of insulin or IGF-I.

Effect of Various Inhibitors on Hepatoma Cells Overexpressing Regucalcin

Hepatoma cells (wild-type) and transfectants with subconfluent monolayers were cultured for 24 h in a medium without FBS containing either vehicle, insulin (10^{-7}M) or IGF-I (10^{-9}M) in the presence or absence of various inhibitors with an effective concentration. The effect of insulin in decreasing the number of wild-type cells was significantly prevented in the presence of caspase-3 inhibitor (10^{-8}M) (Fig. 7). The preventive effect of caspase-3 inhibitor was not observed in the presence of IGF-I. In transfectants, the effect of IGF-I in decreasing cell number was not revealed in the presence of caspase-3 inhibitor (Fig. 7).

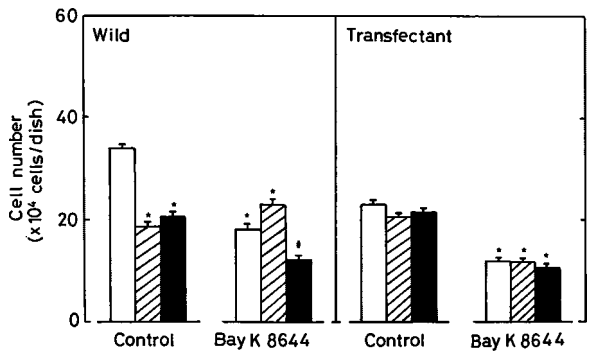

Fig. 6. Effect of insulin or IGF-I on the number of the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells in the presence of Bay K 8644. Cells were cultured for 72 h in a medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium without FBS containing either vehicle, insulin (10^{-7}M) or IGF-I (10^{-9}M) in the presence or absence of Bay K 8644 (2.5×10^{-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 (none) value. #$P < 0.01$, compared with the value obtained from Bay K 8644 alone. White bars, control; hatched bars, insulin; black bars, IGF-I.

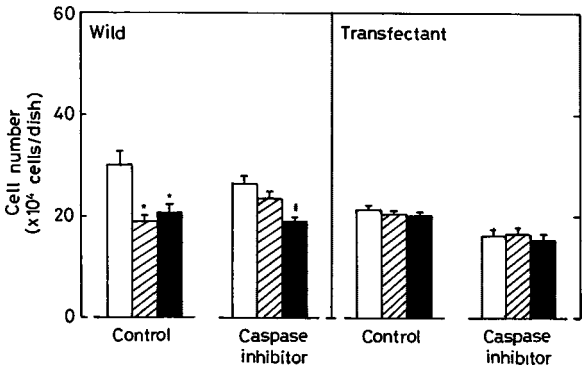

Fig. 7. Effect of caspase-3 inhibitor on insulin- or IGF-I-induced decrease in the number of the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells. Cells were cultured for 72 h in a medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium without FBS containing either vehicle, insulin (10^{-7}M) or IGF-I (10^{-9}M) in the presence or absence of caspase inhibitor (10^{-8}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 (none) value. #$P < 0.01$, compared with the value obtained from caspase inhibitor alone. White bars, control; hatched bars, insulin; black bars, IGF-I.

The effect of NAME, an inhibitor of NO synthase activity, on insulin- or IGF-I-induced decrease in the number of hepatoma cells (wild-type) is shown in Figure 8. After subconfluent monolayers, cells were cultured for 24 h in a medium containing either vehicle, insulin (10^{-7}M) or IGF-I (10^{-9}M) in the presence or absence of NAME (10^{-5}M). The decrease in the number of wild-type cells induced by insulin or IGF-I was significantly blocked in the presence of NAME. The number of transfectants cultured with insulin or IGF-I was not significantly changed in the presence of NAME.

The effect of genistein, an inhibitor of protein tyrosine kinase, or vanadate, an inhibitor of protein tyrosine phosphatase, on insulin- or IGF-I-induced decrease in the number of hepatoma cells (wild-type) is shown in Figures 9 and 10. Cells with subconfluency were cultured for 24 h in a medium containing either vehicle, insulin (10^{-7}M) or IGF-I (10^{-9}M) in the presence or absence of genistein (10^{-6}M) or vanadate (10^{-5}M). Culture with genistein (Fig. 9) or vanadate (Fig. 10) caused a significant decrease in the number of wild-type cells. Such effect was not seen in transfectants. The effect of insulin in decreasing the number of wild-type cells was not observed in the presence of genistein (Fig. 9) or vanadate (Fig. 10). Meanwhile, IGF-I caused a significant decrease in the number of wild-type cells in the presence of genistein (Fig. 9) or vanadate (Fig. 10).

Cell number of transfectants was not significantly altered by the combination of insulin and genistein or IGF-I and genistein (Fig. 9). Vanadate had a suppressive effect on the cell number of transfectants (Fig. 10). In the presence of vanadate, IGF-I caused a significant

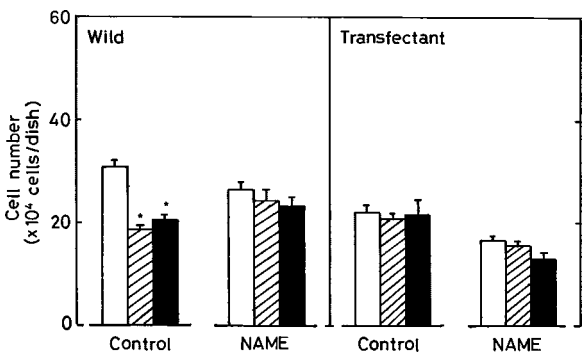

Fig. 8. Effect of NAME on insulin- or IGF-I-induced decrease in the number of the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells. Cells were cultured for 72 h in a medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium without FBS containing either vehicle, insulin (10^{-7}M) or IGF-I (10^{-9}M) in the presence or absence of NAME (10^{-5}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 (none) value. White bars, control; hatched bars, insulin; black bars, IGF-I.

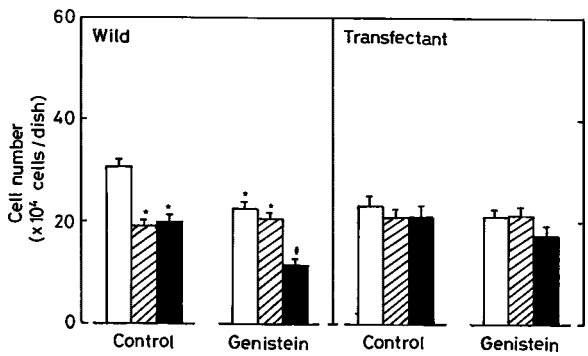

Fig. 9. Effect of genistein on insulin- or IGF-I-induced decrease in the number of the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells. Cells were cultured for 72 h in a medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium without FBS containing either vehicle, insulin (10^{-7}M) or IGF-I (10^{-9}M) in the presence or absence of genistein (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 (none) value. #$P < 0.01$, compared with the value obtained from genistein alone. White bars, control; hatched bars, insulin; black bars, IGF-I.

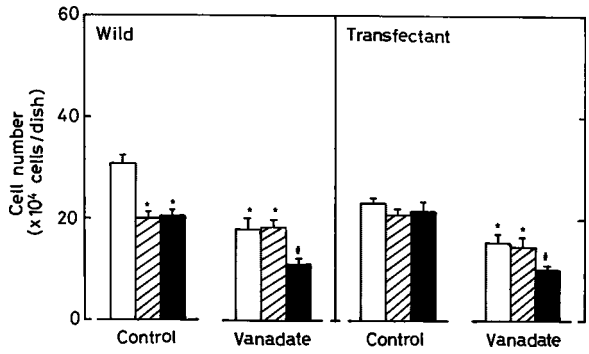

Fig. 10. Effect of vanadate on insulin- or IGF-I-induced decrease in the number of the cloned rat hepatoma H4-II-E cells (wild-type) or regucalcin/pCXN2-transfected cells. Cells were cultured for 72 h in a medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium containing either vehicle, insulin (10^{-7}M) or IGF-I (10^{-9}M) in the presence or absence of vanadate (10^{-5}M) without FBS. 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 (none) value. $^{\#}P < 0.01$, compared with the value obtained from vanadate alone. White bars, control; hatched bars, insulin; black bars, IGF-I.

decrease in the cell number of transfectants, although such effect was not observed with insulin (Fig. 10).

DISCUSSION

Regucalcin has been shown to translocate to liver nucleus, and the localization is enhanced in regenerating rat liver with proliferative cells [Tsurusaki et al., 2000; Tsurusaki and Yamaguchi, 2002b]. Overexpression of regucalcin suppresses cell proliferation and nuclear DNA synthesis in the cloned rat hepatoma H4-II-E cells [Misawa et al., 2002; Tsurusaki and Yamaguchi, 2003]. The suppression of cell proliferation was not resulted from cell death, since DNA fragmentation was not observed in regucalcin-overexpressing transfectants. The removal of transfectants from culture dish was not caused by PBS washing for adherent transfectants. In addition, the number of cloned rat hepatoma H4-II-E cells (wild-type) was not significantly changed by culture with regucalcin addition for 72 h (data not shown). Overexpression of regucalcin did not cause cell death in the cloned rat hepatoma H4-II-E cells, although it suppressed cell proliferation.

Overexpression of regucalcin has been shown to have a suppressive effect on cell death and apoptosis of the cloned rat hepatoma H4-II-E cells induced by tumor necrosis factor-α, lypopolysaccharide, thapsigargin, or Bay K 8644 [Izumi and Yamaguchi, 2004a,b]. The present study demonstrates, moreover, that insulin or IGF-I induces cell death or apoptosis in the hepatoma H4-II-E cells, and that their effects are suppressed in the hepatoma cells overexpressing regucalcin. This finding further supports the view that regucalcin has a suppressive effect on various signaling mechanisms by which induce cell death and apoptosis in the cloned rat hepatoma H4-II-E cells.

The effect of insulin or IGF-I on cell death and apoptosis in the hepatoma cells has not been fully clarified. Insulin or IGF-I was found to induce cell death and apoptosis in the cloned rat hepatoma H4-II-E cells. The effect of insulin in decreasing the number of hepatoma H4-II-E cells was prevented in the presence of caspase-3 inhibitor. The effect of IGF-I on cell death, however, was also observed in the presence of caspase-3 inhibitor. These observations suggest that the effect of insulin on cell death is involved in activation of caspase-3, and that the effect of IGF-I is not dependent on caspase-3 in the hepatoma H4-II-E cells. The effect of IGF-I in inducing cell death in the presence of caspase-3 inhibitor was completely blocked in transfectants overexpressing regucalcin, suggesting that regucalcin inhibits signaling pathway of IGF-I-induced cell death which is not mediated through caspase-3 in the cloned rat hepatoma H4-II-E cells.

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]. The effect of insulin or IGF-I in inducing cell death and apoptosis of the cloned rat hepatoma H4-II-E cells was not observed in the presence of NAME, an inhibitor of NO synthase. This result suggests that insulin- or IGF-I-induced cell death is partly involved in production of NO in the hepatoma H4-II-E cells. Overexpression of regucalcin has been shown to have a suppressive effect on activation of Ca^{2+}/calmodulin-dependent NO synthase in the cloned rat hepatoma H4-II-E cells [Izumi et al., 2003].

Bay K 8644, an agonist of calcium entry into cells, induces cell death and apoptosis in the cloned rat hepatoma H4-II-E cells and

transfectants, although the effect is partially suppressed by overexpression of regucalcin [Izumi and Yamaguchi, 2004b]. The effect of IGF-I in inducing cell death of the hepatoma H4-II-E cells was also observed in the presence of Bay K 8644. Such effect was not seen in the case of insulin. These results suggest that the mode of IGF-I action differs from that of insulin. The effect of IGF-I in inducing cell death in the presence of Bay K 8644 was not observed in transfectants overexpressing regucalcin. From these observations, it is assumed that insulin induces cell death that is partly mediated through intracellular calcium-dependent signaling pathway in the hepatoma H4-II-E cells. IGF-I may not be mediated through calcium-dependent signaling pathway in the hepatoma H4-II-E cells.

Protein phosphorylation-dephosphorylation is a universal mechanism by which numerous cellular events are regulated [Hunter, 1995]. Genistein has an inhibitory effect on protein tyrosine kinases [Liu et al., 1994], and it can produce cell cycle arrest and apoptosis in leukemic cells [Spinozzi et al., 1994]. Genistein was found to induce cell death of the cloned rat hepatoma H4-II-E cells, and the effect was not observed in the transfectants overexpressing regucalcin. This result suggests that genistein-induced cell death is partly mediated by inhibition of protein tyrosine kinase in the hepatoma H4-II-E cells. Regucalcin has been shown to have an inhibitory effect on protein tyrosine kinase activity in the cytoplasm and nucleus of rat liver [Katsumata and Yamaguchi, 1998; Yamaguchi and Katsumata, 1999]. Meanwhile, the effect of insulin in inducing cell death of the hepatoma H4-II-E cells was not observed in the presence of genistein. Such effect was not seen in the case of IGF-I, suggesting that IGF-I-induced cell death is not mediated through signaling pathway that is involved in protein thyrosine kinase. The effect of IGF-I on cell death in the presence of genistein was significantly prevented in transfectants overexpressing regucalcin, suggesting that regucalcin has a suppressive effect on IGF-I-related signaling pathway that is not dependent on protein tyrosine kinase.

Vanadate is an inhibitor of protein tyrosine phosphatase in cells [Hunter, 1995]. Regucalcin has been shown to have an inhibitory effect on protein tyrosine phosphatase activity in the cytoplasm and nucleus of rat liver [Omura and Yamaguchi, 1999]. Vanadate was found to induce cell death of the cloned rat hepatoma H4-II-E cells, suggesting that an inhibition of protein tyrosine phosphatase activity induces cell death of the hepatoma H4-II-E cells. In the presence of vanadate, insulin had no additive effect on the toxicity of vanadate, while IGF-I-induced cell death of the hepatoma H4-II-E cells. These results suggest that the effect of insulin on cell death is partly mediated through protein tyrosine phosphatase. However, the effect of IGF-I may not be involved in protein tyrosine phosphatase in the cloned rat hepatoma H4-II-E cells. Interestingly, vanadate induced cell death in transfectants overexpressing regucalcin, suggesting that the suppressive effect of regucalcin on cell death of the hepatoma H4-II-E cells is independent on protein phosphatase. Moreover, IGF-I had a stimulatory effect on cell death of the hepatoma H4-II-E cells overexpressing regucalcin in the presence of vanadate, suggesting that the effect of IGF-I is not mediated through protein tyrosine phosphatase in the transfectants.

The effect of insulin in inducing cell death may be partly mediated through signaling pathway which is involved in caspase-3, calcium, NO, protein tyrosine kinase, or protein tyrosine phosphatase in the hepatoma H4-II-E cells. The effect of IGF-I on cell death of the hepatoma H4-II-E cells may be mediated through NO and other molecules. Overexpression of regucalcin may have a suppressive effect on signaling mechanism by which insulin or IGF-I induces cell death of the hepatoma H4-II-E cells.

Regucalcin has been shown to have a suppressive effect on proliferation of liver cells and hepatoma H4-II-E cells [Yamaguchi, 2000b; Misawa et al., 2002; 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 apoptic responses. The suppressive effect of regucalcin on cell proliferation and apoptotic cell death may be based on its regulatory action on intracellular signaling mechanism in liver cells. Presumably, regucalcin plays a physiologic role in maintaining homeostasis of cellular response for cell stimulation.

In conclusion, it has been demonstrated that insulin or IGF-I induces cell death and apoptosis in the cloned rat hepatoma H4-II-E cells, and that overexpression of regucalcin has a suppressive effect on the insulin-or IGF-I-induced cell death and apoptosis.

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