Journal of Cellular Biochemistry 92:296–306 (2004)

Overexpression of Regucalcin Suppresses Cell Death in Cloned Rat Hepatoma H4-II-E Cells Induced by Tumor Necrosis Factor-α or Thapsigargin

Takako Izumi and Masayoshi Yamaguchi*

Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan

Abstract The role of regucalcin, which is a regulatory protein in intracellular signaling pathway, in the regulation of 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. The proliferation of the cells was significantly suppressed in transfectants cultured for 72 h, as shown previously (Tsurusaki and Yamaguchi [2003]: J Cell Biochem 90:619–626). After culture for 72 h, cells were further cultured for 24–72 h in medium without FBS containing either vehicle, tumor necrosis factor-α (TNF-α; 0.1, 1, or 10 ng/ml) or thapsigargin (10^{-7}–10^{-5} M). The number of wild-type cells was significantly decreased by culture for 42 or 72 h in the presence of TNF-α (0.1, 1, or 10 ng/ml) or thapsigargin (10^{-7}–10^{-5} M). The effect of TNF-α (0.1 or 1 ng/ml) or thapsigargin (10^{-7} or 10^{-6} M) in decreasing the number of hepatoma cells was significantly prevented in transfectants overexpressing regucalcin. The presence of TNF-α (10 ng/ml) or thapsigargin (10^{-5} M) caused a significant decrease in cell number of transfectants. Ca^{2+}/calmodulin-dependent nitric oxide (NO) synthase activity in wild-type cells was significantly increased by culture with TNF-α (10 ng/ml) for 48 or 72 h. This increase was significantly prevented in transfectants. Culture with thapsigargin (10^{-5} M) caused a significant increase in Ca^{2+}/calmodulin-dependent NO synthase activity in wild-type cells or transfectants. TNF-α-induced decrease in the number of wild-type cells was significantly prevented by culture with *N*ω-nitro-L-arginine (10^{-4} M), an inhibitor of caspase. Agarose gel electrophoresis showed the presence of low-molecular-weight deoxyribonucleic acid (DNA) fragments of adherent wild-type cells cultured with thapsigargin (10^{-6} M), and this DNA fragmentation was not suppressed by culture with caspase inhibitor. Thapsigargin-induced DNA fragmentation was significantly suppressed in transfectants cultured with or without caspase inhibitor. This study demonstrates that overexpression of regucalcin has a suppressive effect on cell death induced by TNF-α or thapsigargin. J. Cell. Biochem. 92: 296–306, 2004.

Key words: regucalcin; cell death; apoptosis; NO synthase; TNF-α; thapsigargin; hepatoma cells

Regucalcin, which 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], has been demonstrated to play a multifunctional role as an inhibitory protein on intracellular signaling process in cells [Yamaguchi, 2000a,b; reviews] in recent years. 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 messenger ribonucleic acid (mRNA) and its protein are greatly present in liver and kidney cortex [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993]. The expression of regucalcin mRNA is mediated through Ca^{2+}-signaling mechanism [Murata and Yamaguchi, 1999; Yamaguchi and Nakajima, 1999]. AP 1 and NFI-A1 have been found to be transcriptional factors for the enhancement of regucalcin gene promoter activity [Murata and Yamaguchi, 1999; Misawa and Yamaguchi, 2002].

*Correspondence to: Masayoshi Yamaguchi, PhD, Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan.
E-mail: yamaguch@u-shizuoka-ken.ac.jp

Received 26 November 2003; Accepted 6 January 2004
DOI 10.1002/jcb.20056

Regucalcin plays a role in the maintenance of intracellular Ca^{2+} homeostasis, the inhibitory regulation of various Ca^{2+}-dependent protein kinase and thyrosine kinases, protein phosphatases, nitric oxide (NO) synthase, and the control of the enhancement of nuclear DNA and RNA syntheses in proliferative cells [Yamaguchi, 2000a,b; Tsurusaki and Yamaguchi, 2002a,b; Izumi et al., 2003]. Regucalcin may play a pivotal role in the regulation of cell function.

Regucalcin has been shown to translocate to the nucleus of rat liver [Tsurusaki et al., 2000], and it has been demonstrated to regulate nuclear function in regenerating rat liver with proliferative cells, suggesting that the protein have a suppressive effect on the overexpression of proliferation of liver cells. Recent study have demonstrated that 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]. Moreover, endogenous regucalcin has been shown to inhibit the enhancement of protein kinase [Inagaki and Yamaguchi, 2001] and protein phosphatase activities [Inagaki et al., 2000] in the cloned rat hepatoma H4-II-E cells with proliferation. Thus, 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. The present study was undertaken to determine whether regucalcin has a role in the regulation of cell death and apoptosis in the cloned rat hepatoma H4-II-E cells overexpressing regucalcin. We found that endogenous regucalcin has a suppressive effect on cell death and apoptosis induced by stimulation of tumor necrosis factor-α (TNF-α) or thapsigargin.

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 Island, NY). Fetal bovine serum (FBS), TNF-α, thapsigargin, arginine, β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), citrulline, calmodulin (52,000 U/mg protein from bovine brain), Z-Asp-CH_2-DCB, and *N*ω-nitro-L-arginine methylester (NAME) were obtained from Sigma Chemical Co. (St. Louis, MO). Calcium chloride 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

The cDNA encoding rat regucalcin was isolated and cloned into the pBluscript vector [Shimokawa and Yamaguchi, 1993]. The regucalcin cDNA contains *Pst* I site downstream of the translational stop codon, and a *Pst* 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/pCNX2 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 [Yamaguchi and Nakajima, 1999; 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 absence or presence of TNF-α (0.1, 1, or 10 ng/ml) or thapsigargin (10^{-7}–10^{-5} M).

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 mitochondria. The 5,500*g* supernatant was pooled to assay NO synthase activity and to analyze regucalcin protein with Western blot [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].

Determination of Cell Numbers

After trypsinization using 0.2% trypsin plus 0.02% ethylenediamine-tetraacetic acid in Ca^{2+}/Mg^{2+}-free PBS, cell numbers were determined by electronic particle counter.

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 TNF-α (10 ng/ml) or thapsigargin (10^{-5} 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 TNF-α 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, and further cultured for 24, 48, or 72 h (Fig. 1). The number of wild-type cells and mock-type cells was significantly increased by culture of 48 and 72 h. A significant suppression of cell proliferation of transfectants was maintained during 24–72 h of culture. 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 24, 48, or 72 h in medium without FBS containing either vehicle or TNF-α (0.1, 1, or 10 ng/ml) (Fig. 2). The number of wild-type cells was significantly decreased by culture of 24–72 h in the presence of TNF-α (1 or 10 ng/ml). With culture of 48 or 72 h, the cell number of wild-type was significantly decreased in the presence of 0.1 ng/ml of TNF-α. The effect of TNF-α (0.1 or 1 ng/ml) in decreasing the number of hepatoma cells (wild-type) was not observed in stable RC/pCXN2-transfected cells cultured for 24, 48, or 72 h. With the highest concentration (10 ng/ml) of TNF-α used, cell number of transfectants was significantly decreased. However, the effect of TNF-α (10 ng/ml) decreasing

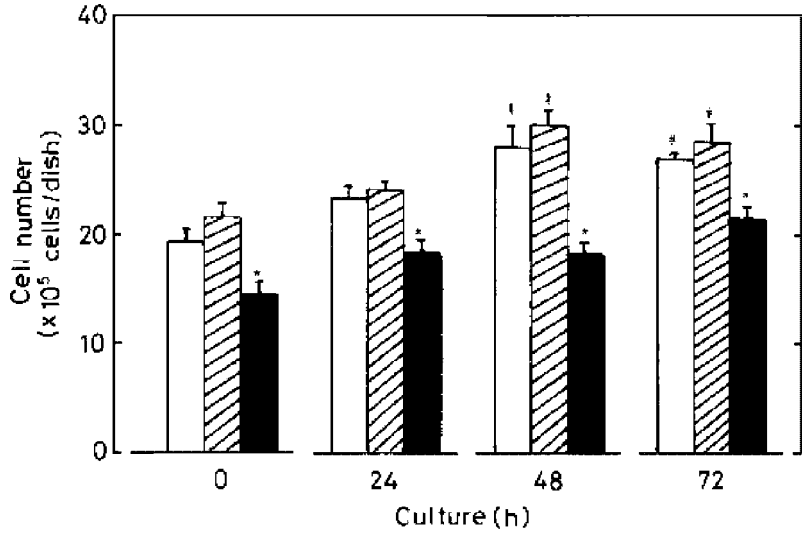

Fig. 1. Alteration in cell number of the cloned rat hepatoma H4-II-E cells (wild-type), pCXN2-transfected cells (mock-type), or stable RC/pCXN2-transfected cells after subconfluent monolayers. Cells were cultured for 72 h in medium containing 10% fetal bovine serum (FBS) to obtain without FBS. After 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 value of wild-type cells or mock-type cells obtained at zero time. $^{*}P < 0.01$, compared with the value of wild-type cells obtained at 48 or 72 h of culture. Open bars, wild-type cells; hatched bars, mock-type cells; black bars, transfectant.

cell number was significantly ($P < 0.01$) weakened in transfectants.

NO synthase activity is shown to increase in the cloned rat hepatoma cells (H4-II-E) with the proliferation, and the increase is suppressed in stable RC/pCXN2-transfected cells [Izumi et al., 2003]. 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 TNF-α (10 ng/ml) (Fig. 3). The cell lysate of wild-type cells obtained with culture for 24, 48, or 72 h was used to assay of 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 TNF-α for 48 or 72 h of wild-type cells

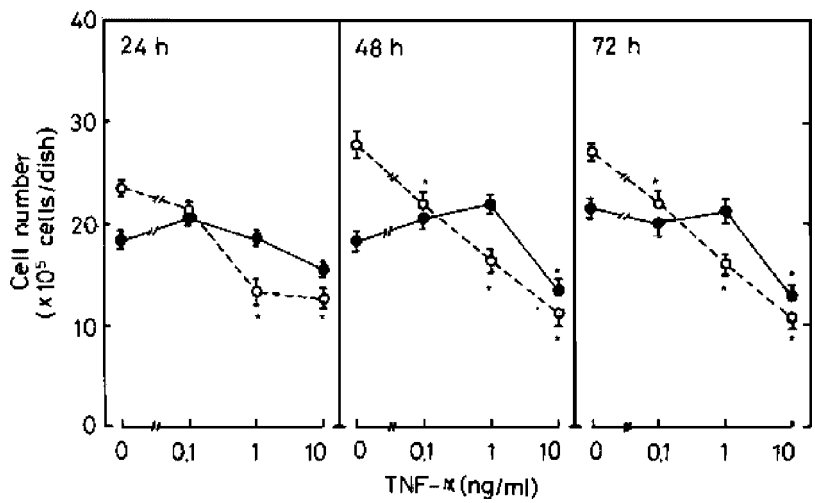

Fig. 2. Effect of tumor necrosis factor-α (TNF-α) on cell number of the cloned rat hepatoma H4-II-E cells (wild-type) or stable RC/pCXN2-transfected cells. Cells were cultured for 72 h in medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium without FBS containing either vehicle or TNF-α (0.1, 1, or 10 ng/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. Open circles, wild-type cells; closed circles, transfectants.

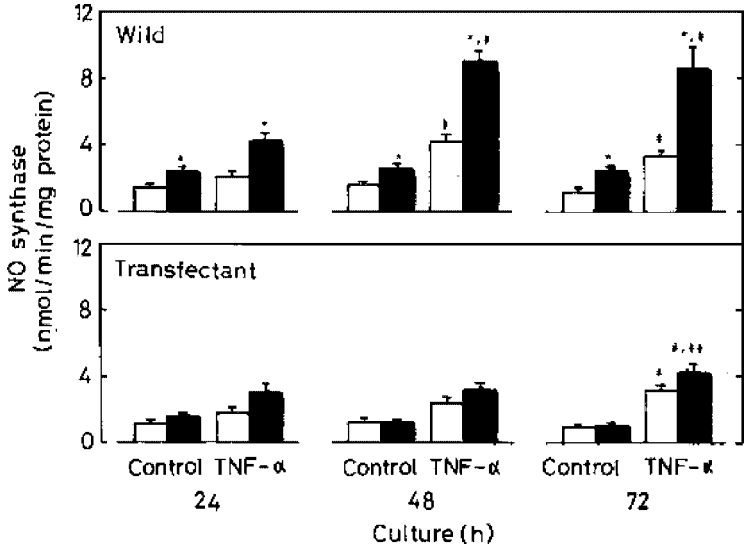

Fig. 3. Effect of TNF-α on nitric oxide (NO) synthase activity in the cloned rat hepatoma H4-II-E cells (wild-type) or stable RC/pCXN2-transfected cells. Cells were cultured for 72 h in medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium without FBS containing either vehicle or TNF-α (10 ng/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. $^{*}P < 0.01$, compared with the control value without calcium and calmodulin addition. $^{\#}P < 0.01$, compared with the control value obtained from cells cultured without TNF-α. $^{\#\#}P < 0.01$, compared with the value with calcium and calmodulin addition of wild-type cells cultured with TNF-α for 72 h. White bars, none; black bars, calcium chloride (10 μM) plus calmodulin (2.5 μg/ml).

caused a significant enhancement of NO synthase activity without or with Ca^{2+}/calmodulin addition. Meanwhile, the effect of Ca^{2+}/calmodulin addition in increasing NO synthase activity was abolished in stable RC/pCXN2-transfected cells cultured for 24–72 h. The enhancement of Ca^{2+}/calmodulin-dependent NO synthase activity induced by culture with TNF-α for 48 or 72 h was significantly suppressed in transfectants.

The effect of NAME, an inhibitor of NO synthase activity, on TNF-α-induced decrease in the number of hepatoma cells (wild-type) is shown in Figure 4. After subconfluent monolayers, cells were cultured for 48 h medium containing either vehicle or TNF-α (10 ng/ml) in the absence or presence of NAME (10^{-4} M). The decrease in cell number (Fig. 4A) and the increase in NO synthase activity (Fig. 4B) without or with Ca^{2+}/calmodulin addition induced by TNF-α was significantly inhibited in the presence of NAME.

Effect of Thapsigargin on Hepatoma Cells Overexpressing Regucalcin

Hepatoma cells (wild-type) and stable RC/pCXN2-transfected cells with subconfluent monolayers were cultured for 48 h in medium without FBS containing either vehicle or thapsigargin (10^{-7}–10^{-5} M), an inhibitor of endoplasmic reticulum calcium ion pump enzyme (Ca^{2+}-ATPase) (Fig. 5). The presence of thapsigargin (10^{-7}–10^{-5} M) caused a significant decrease in cell number of wild-type cells. The effect of thapsigargin (10^{-7} or 10^{-6} M) in decreasing cell number was not seen in transfectants. The effect of thapsigargin (10^{-5} M) in decreasing cell number was significantly ($P < 0.05$) weakened in transfectants.

NO synthase activity in wild-type cells and transfectants was not significantly altered by culture with thapsigargin (10^{-7} or 10^{-6} M) (Fig. 6). With 10^{-5} M thapsigargin, Ca^{2+}/calmodulin-dependent NO synthase activity was significantly enhanced in wild-type cells and transfectants.

Effect of TNF-α or Thapsigargin on DNA Fragmentation of Hepatoma Cells Overexpressing Regucalcin

Hepatoma cells (wild-type; W) and stable RC/pCXN2-transfected cells (T) with subconfluent monolayers were cultured for 48 h medium without FBS containing either vehicle, TNF-α

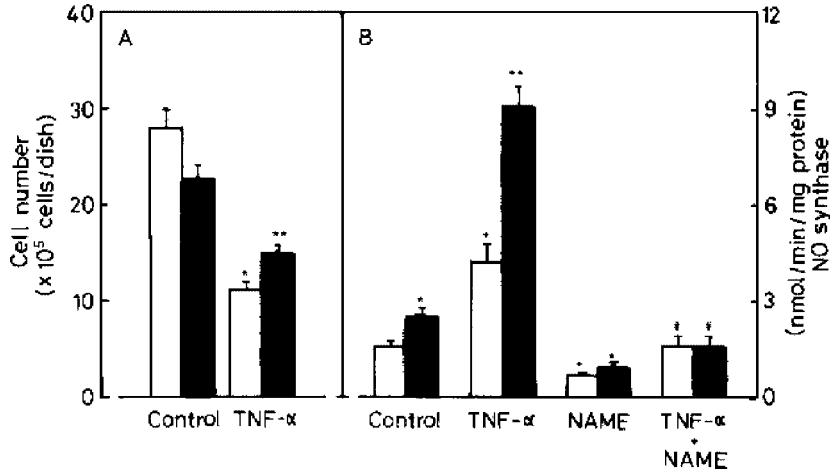

Fig. 4. Effect of *N*ω-nitro-L-arginine methylester (NAME), an inhibitor of NO synthase activity, on TNF-α-induced decrease in cell number and TNF-α-induced increase in NO synthase activity in the cloned rat hepatoma H4-II-E cells (wild-type). Cells were cultured for 72 h in medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium without FBS containing either vehicle, TNF-α (10 ng/ml), NAME (10^{-4} M), or TNF-α (10 ng/ml) plus NAME (10^{-4} M). After medium change, cells were incubated for 48 h and the number of cells (**A**) or NO synthase activity (**B**) 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. $^{*}P < 0.01$, compared with the control value obtained from cells cultured without TNF-α. $^{**}P < 0.01$, compared with the control value obtained from cells cultured with TNF-α. $^{\#}P < 0.01$, compared with the control value obtained from cells cultured with TNF-α in the absence of NAME. A: White bars, none; black bars, culture with NAME. B: White bars, none; black bars, calcium and calmodulin addition.

(10 ng/ml) or thapsigargin (10^{-6} M) in the absence or presence of Z-Asp-CH_2-DCB, an inhibitor of caspase (Figs. 7 and 8). The decrease in cell number induced by TNF-α was significantly prevented in the presence of caspase inhibitor in wild-type cells or transfectants, while the effect of caspase inhibitor was not seen in the presence of thapsigargin (Fig. 7).

The effect of TNF-α or thapsigargin on DNA fragmentation in hepatoma cells (wild-type; W) and stable RC/pCXN2-transfected cells (T) in the absence or presence of caspase inhibitor is

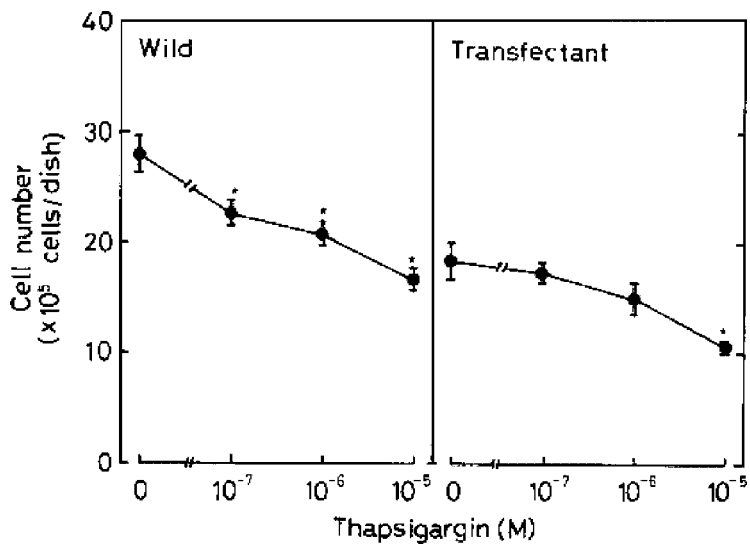

Fig. 5. Effect of thapsigargin on cell number of the cloned rat hepatoma H4-II-E cells (wild-type) or stable RC/pCXN2-transfected cells. Cells were cultured for 72 h in medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium without FBS containing either vehicle or thapsigargin (10^{-7}–10^{-5} M). After medium change, cells were incubated for 48 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 thapsigargin.

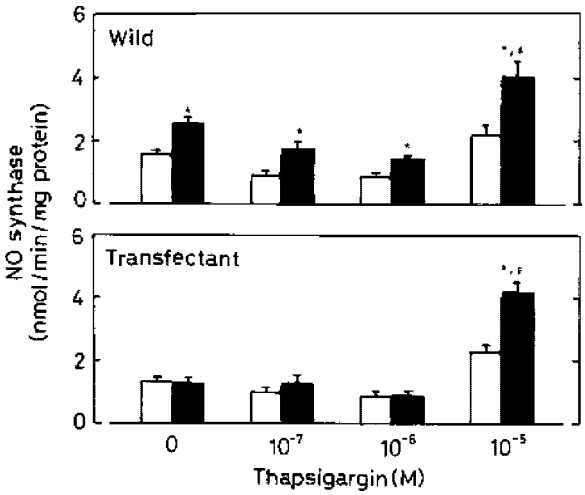

Fig. 6. Effect of thapsigargin on NO synthase activity in the cloned rat hepatoma H4-II-E cells (wild-type) or stable RC/pCXN2-transfected cells. Cells were cultured for 72 h in medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium without FBS containing either vehicle or thapsigargin (10^{-7}–10^{-5} M). After medium change, cells were incubated for 48 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. $^{*}P < 0.01$, compared with the control value without calcium plus calmodulin addition. $^{\#}P < 0.01$, compared with the control value of calcium and calmodulin addition. White bars, control; black bars, calcium plus calmodulin.

shown in Figure 8. Cells were cultured for 48 h in medium containing either vehicle, TNF-α (10 ng/ml) or thapsigargin (10^{-6} M). Adherent cells were lysed, and then the lysate was separated by electrophoresis in agarose gel. The presence of TNF-α did not cause DNA fragmentation in wild-type cells or transfectants cultured in the absence or presence of caspase inhibitor. Thapsigargin induced DNA fragmentation in wild-type cells in the absence or presence of caspase inhibitor. The effect of thapsigargin on DNA fragmentation was suppressed in transfectants. The suppressive effect in transfectants was remarkable in the presence of caspase inhibitor. The same results were also observed using equal amount of total DNA in wild cell or transfectants.

DISCUSSION

Overexpression of regucalcin has been shown to have a suppressive effect on cell 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 TNF-α

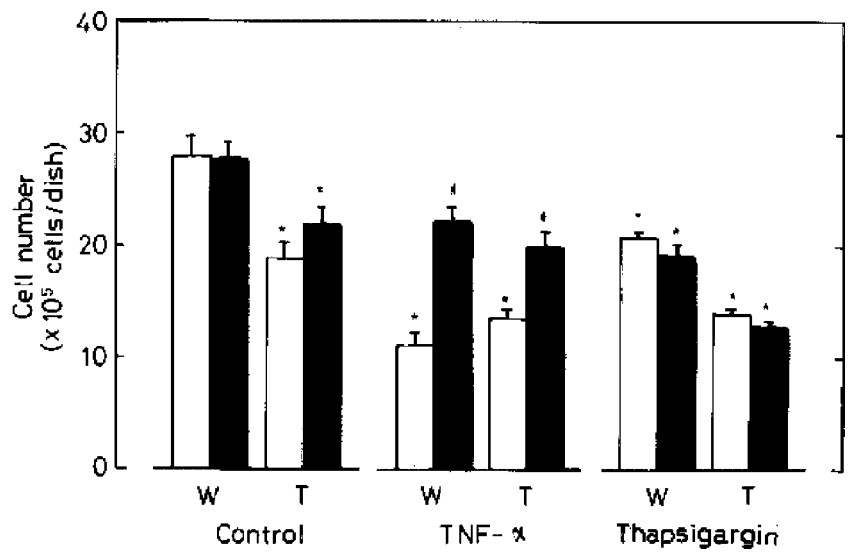

Fig. 7. Effect of caspase inhibitor (Z-Asp-CH_2-DCB) on cell number of the cloned rat hepatoma H4-II-E cells (wild-type; W) or stable RC/pCXN2-transfected cells (T) cultured with TNF-α or thapsigargin. Cells were cultured for 72 h in medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium without FBS containing either vehicle, TNF-α (10 ng/ml) or thapsigargin (10^{-6} M) in the absence or presence of caspase inhibitor (10^{-4} M). After medium change, cells were incubated for 48 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 wild-type cells cultured without caspase inhibitor in the absence of TNF-α or thapsigargin. $^{\#}P < 0.01$, compared with the control value obtained from cells cultured without caspase inhibitor in the presence of TNF-α. White bars, without caspase inhibitor; black bars, caspase inhibitor.

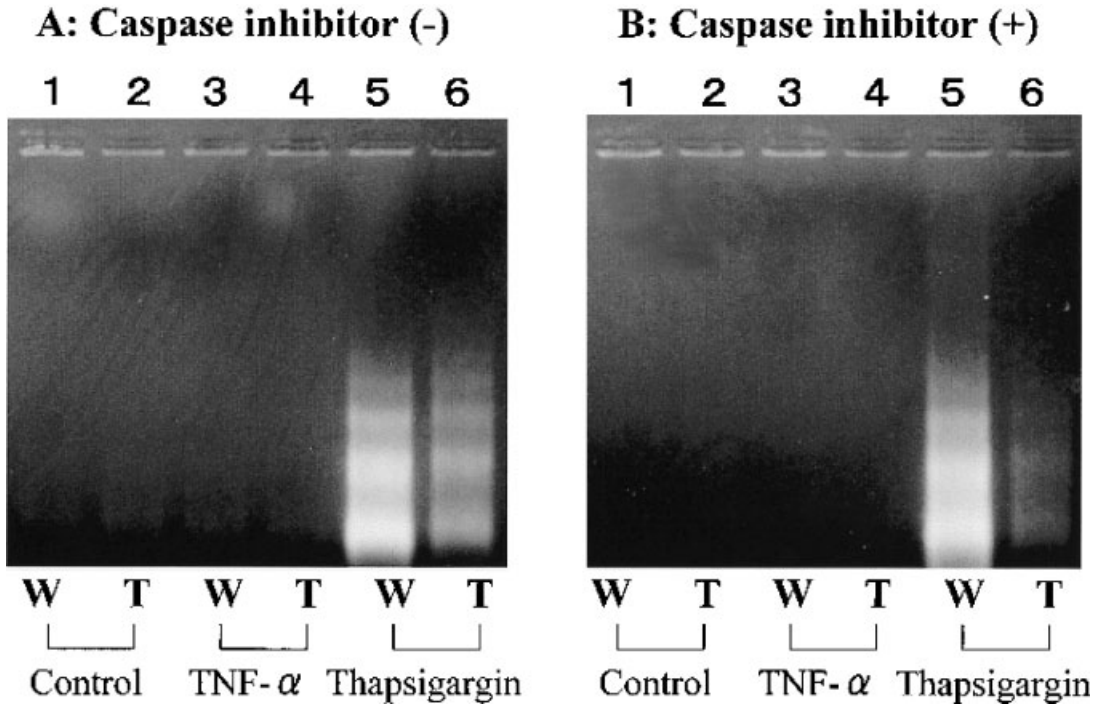

Fig. 8. Effect of caspase inhibitor (Z-Asp-CH_2-DCB) on deoxyribonucleic acid (DNA) fragmentation in the cloned rat hepatoma H4-II-E cells (wild-type; W) or stable RC/pCXN2-transfected cells (T) cultured with TNF-α or thapsigargin. Cells were cultured for 72 h in medium containing 10% FBS to obtain subconfluent monolayers, and then exchanged to culture medium without FBS containing either vehicle, TNF-α (10 ng/ml) or thapsigargin (10^{-6} M) in the absence (**A**) or presence (**B**) of caspase inhibitor (10^{-4} M). After medium change, cells were incubated for 48 h, and cell lysate was applied to agarose gel. The figure shows one of four experiments with separate samples.

or thapsigargin. TNF-α (0.1, 1, or 10 ng/ml) or thapsigargin (10^{-7}–10^{-5} M) caused a significant decrease in the number of hepatoma H4-II-E cells (wild-type), inducing cell death. The effect of TNF-α (0.1 or 1 ng/ml) or thapsigargin (10^{-7} or 10^{-6} M) in decreasing cell number was found to be prevented significantly in the hepatoma cells (transfectants) overexpressing regucalcin. Transfectants significantly prevented cell death induced with the higher concentration of TNF-α (10 ng/ml) or thapsigargin (10^{-5} M). These observations were also seen in transfectants with other clones (data not shown). The present finding demonstrates that overexpression of regucalcin has a suppressive effect on cell death induced by stimulation of TNF-α or thapsigargin.

NO may be important as a signaling factor in many cells [Lowenstein et al., 1994] and plays a role in apoptosis of hepatoma cells [Liu et al., 2000]. TNF-α and NO mediate apoptosis by D-galactosamine in a primary culture of rat hepatocytes [Abou-Elella et al., 2002]. Culture with TNF-α (10 ng/ml) caused a significant increase in Ca^{2+}/calmodulin-dependent NO synthase activity in rat hepatoma H4-II-E cells (wild-type). TNFα-induced enhancement of NO synthase activity was found to be suppressed significantly in the hepatoma cells overexpressing regucalcin. Culture with NAME, an inhibitor of NO synthase, had a significant preventive effect on cell death induced by TNF-α. Regucalcin has been shown to inhibit Ca^{2+}/calmodulin-dependent NO synthase activity in rat liver cytosol [Yamaguchi et al., 2003] and rat hepatoma cells [Izumi et al., 2003]. From these observations, it is speculated that the suppressive effect of regucalcin on cell death is partly resulted from the inhibition of NO production stimulated by TNF-α in the hepatoma cells. It is possible, however, that regucalcin may also act on other signaling pathways by which TNF-α mediates cell death in the hepatoma cells.

Calcium channel blockers, the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin and calcium ionophores are potent to lead several cell types to apoptosis [Martikainen et al., 1991; Balakuraman et al., 1996; Christensen et al., 1999]. Culture with thapsigargin caused a significant decrease in the number of the

hepatoma cells (wild-type). Thapsigargin-induced cell death was found to suppress significantly in the hepatoma cells overexpressing regucalcin. NO synthase activity in the hepatoma cells (wild-type) and transfectants was not significantly altered by culture with thapsigargin (10^{-7} or 10^{-6} M). Higher concentration (10^{-5} M) of thapsigargin significantly increased Ca^{2+}/calmodulin-dependent NO synthase activity in wild-type cells, and this increase was also seen in transfectants. These results suggest that thapsigargin-induced cell death is not related to NO production in the hepatoma cells.

Thapsigargin is an inhibitor of Ca^{2+}-ATPase in the endoplasmic reticulum (Ca^{2+} store) in cells. Treatment with thapsigargin causes an elevation of sustained Ca^{2+} concentration in cells and induces apoptosis in the hepatoma cells [Jiang et al., 1994; Kaneka and Tsukamoto, 1994; Tombal et al., 2000]. Experiments on nuclei isolated from thymocytes clearly demonstrate the induction of a Ca^{2+}-dependent endonuclease activity during triggering apoptosis events [Cohen and Duke, 1984]. A nuclear Ca^{2+}/Mg^{2+} dependent endonuclease, which is able to digest chromatin in situ into mononucleosomal and oligonucleosomal fragments, has been purified from human spleen cells [Ribeiro and Carson, 1993]. Rises in intracellular Ca^{2+} concentration are believed to activate this nuclease and to mediate DNA cleavages into oligonucleosome fragments [Pereira et al., 2002]. It has been shown that regucalcin inhibits Ca^{2+}-activated DNA fragmentation in the nuclei isolated from rat liver [Yamaguchi and Sakurai, 1991], suggesting that the protein has an inhibitory effect on apoptosis in liver cells. Moreover, regucalcin has been demonstrated to activate Ca^{2+} pump enzymes in the plasma membranes, mitochondria, and endoplasmic reticulum of rat liver cells [Takahashi and Yamaguchi, 1997, 1999, 2000], suggesting that regucalcin plays a role in the regulation of Ca^{2+} homeostasis in the cells. Presumably, regucalcin has a suppressive effect on thapsigargin-mediated cell death due to preventing the rise in intracellular Ca^{2+} concentration in the hepatoma cells.

The effect of caspase inhibitor on TNF-α- or thapsigargin-mediated cell death in the cloned rat hepatoma H4-II-E cells and regucalcin-overexpressing hepatoma cells was examined. TNF-α-induced cell death was significantly prevented by culture with caspase inhibitor in wild-type cells and transfectants. Such an effect was not seen in the presence of thapsigargin. These results suggest that TNF-α-induced cell death is partly involved in activation of caspases in the hepatoma cells. The effect of thapsigargin may not related to caspases in the hepatoma cells.

The presence of low-molecular-weight DNA fragments was observed in the adherent hepatoma cells cultured with thapsigargin. Results indicate that thapsigargin induces DNA fragmentation in the adherent hepatoma cells. However, DNA fragmentation was not observed in the adherent hepatoma cells cultured with TNF-α, although TNF-α induced decrease in cell number. Presumably, TNF-α may have a potent effect on apoptosis. TNF-α induces DNA fragmentation in the hepatoma cells and it caused promptly cell death. Meanwhile, thapsigargin-induced DNA fragmentation in the hepatoma cells was not altered by culture with caspase inhibitor, suggesting that thapsigargin-mediated apoptosis is independent of activation of caspases. Overexpression of regucalcin in the hepatoma cells was found to suppress DNA fragmentation induced by thapsigargin. This effect was further enhanced by culture with caspase inhibitor. Regucalcin may have an inhibitory effect on stimulation of apoptosis in the hepatoma cells.

The signaling mechanism by which TNF-α or thapsigargin mediates cell death and apoptosis may be different. Endogenous regucalcin was shown to have a suppressive effect on cell death and apoptosis induced by TNF-α or thapsigargin. Regucalcin may have the suppressive effect on many signaling pathways that mediate cell death and apoptosis. 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 overexpression of cell proliferation of regenerating rat liver [Yamaguchi, 2000b] and the cloned rat hepatoma H4-II-E cells [Tsurusaki and Yamaguchi, 2003] due to hormonal stimulation. Moreover, regucalcin has been demonstrated to have a suppressive effect on cell death induced by stimulation of TNF-α or thapsigargin in the hepatoma cells. From these observation, it is speculated that regucalcin plays 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 stimulation of TNF-α or thapsigargin.

REFERENCES

Abou-Elella AM, Siendones E, Padillo J, Montero JL, De la Meta M, Relat JM. 2002. Tumour necrosis factor-alpha and nitric oxide mediate apoptosis by D-galactosamine in a primary culture of rat hepatocytes: Exacerbation of cell death by cocultured Kupffer cells. Can J Gastroenterol 16:791–799.

Balakuraman A, Campbell GA, Molsen MT. 1996. Calcium channel blockers induce thymic apoptosis in vivo in rats. Toxicol Appl Pharmacol 139:122–127.

Bhattacharya SS, Ross MT, Hardcastle AJ, Menindl A. 2002. An integrated, functionally annotated gene map of the DXS80269-ELK1 interval on human Xp 11.3-Xp 11.23: Potential hotspot for neurogenetic disorders. Genomics 79:560–572.

Boyde TRC, Rahmatullah M. 1980. Optimization of conditions for the colorimetric determination of citrulline, using diacetylmonoxine. Anal Biochem 107:424–431.

Christensen SB, Andersen A, Kromann H, Treiman M, Tombal B, Denmeade S, Isaacs JT. 1999. Thapsigargin analogues for targeting programmed death of androgen-independent prostate cancer cells. Bioorg Med Chem 7:1273–1280.

Cohen JJ, Duke RC. 1984. Glucocorticoid activation of a calcium-dependent endonuclease in thymocytes nuclei leads to cell death. J Immunol 132:38–42.

Inagaki S, Yamaguchi M. 2001. Suppressive role of endogenous regucalcin in the enhancement of protein kinase activity with proliferation of cloned rat hepatoma cells (H4-II-E). J Cell Biochem Suppl 36:12–18.

Inagaki S, Misawa H, Yamaguchi M. 2000. Role of endogenous regucalcin in protein tyrosine phosphatase regulation in the cloned rat hepatoma cells (H4-II-E). Mol Cell Biochem 213:43–50.

Izumi T, Tsurusaki Y, Yamaguchi M. 2003. Suppressive effect of endogenous regucalcin on nitric oxide synthase activity in cloned rat hepatoma H4-II-E cells overexpressing regucalcin. J Cell Biochem 89:800–807.

Jiang S, Chow SC, Nicotera P, Orrenius S. 1994. Intracellular Ca^{2+} signals activate apoptosis in thymocytes: Studies using the Ca^{2+}-ATPase inhibitor thapsigargin. Exp Cell Res 212:84–92.

Kaneka Y, Tsukamoto A. 1994. Thapsigargin-induced persistent intracellular calcium pool depletion and apoptosis in human hepatoma cells. Cancer Lett 79:147–155.

Lee S-J, Stull JT. 1998. Calmodulin-dependent regulation of inducible and neuronal nitric-oxide synthase. J Biol Chem 273:277430–277437.

Liu S, Shia D, Liu G, Chen H, Liu S, Hu Y. 2000. Roles of Se and NO in apoptosis of hepatoma cells. Life Sci 68:603–610.

Lowenstein CJ, Dinerman JL, Snyder SH. 1994. Nitric oxide: A physiologic messenger. Ann Intern Med 120:227–237.

Lowry OH, Rosebrough NH, Farr AL, Randall RF. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–273.

Martikainen P, Kyprianou N, Tucker RW, Isaacs JT. 1991. Programmed death of nonproliferating androgen-independent prostatic cancer cells. Cancer Res 51:4693–4700.

Misawa H, Yamaguchi M. 2000. The gene of Ca^{2+}-binding protein regucalcin is highly conserved in vertebrate species. Int J Mol Med 6:191–196.

Misawa H, Yamaguchi M. 2002. Identification of transcription factor in the promoter region of rat regucalcin gene: Binding of nuclear factor I-A1 to TTGGC motif. J Cell Biochem 84:795–802.

Misawa H, Inagaki H, Yamaguchi M. 2002. Suppression of cell proliferation and deoxyribonucleic acid synthesis in cloned rat hepatoma H4-II-E cells overexpressing regucalcin. J Cell Biochem 84:143–149.

Murata T, Yamaguchi M. 1999. Promotor characterization of the rat gene for Ca^{2+}-binding protein regucalcin. Transcriptional regulation by signaling factor. J Biol Chem 274:1277–1285.

Niwa H, Yamamura K, Miyazaki J. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108:193–199.

Pereira M, Millot J-M, Sebille S, Manfait M. 2002. Inhibitory effect of extracellular Mg^{2+} on intracellular Ca^{2+} dynamic changes and thapsigargin-induced apoptosis in human cancer MCF7 cells. Mol Cell Biochem 229:163–171.

Preaux A-M, D'Oratho M-P, Bralet M-P, Laperche Y, Mavier P. 2002. Apoptosis of human hepatic myofibroblasts promotes activation of matrix metalloproteinase-2. Hepatology 36:615–622.

Ribeiro JM, Carson DA. 1993. Ca^{2+}/Mg^{2+}-dependent endonuclease from human spleen: Purification, properties, and role in apoptosis. Biochemistry 32:9129–9136.

Shimokawa N, Yamaguchi M. 1992. Calcium administration stimulates the expression of calcium-binding protein regucalcin mRNA in rat liver. FEBS Lett 305:151–154.

Shimokawa N, Yamaguchi M. 1993. Molecular cloning and sequencing of the cDNA coding for calcium-binding protein regucalcin from rat liver. FEBS Lett 327:251–255.

Shimokawa N, Matsuda Y, Yamaguchi M. 1995. Genomic cloning and chromosomal assignment of rat regucalcin gene. Mol Cell Biochem 151:157–163.

Takahashi H, Yamaguchi M. 1997. Stimulatory effect of regucalcin on ATP-dependent calcium transport in rat liver plasma membranes. Mol Cell Biochem 168:149–153.

Takahashi H, Yamaguchi M. 1999. Role of regucalcin as an activator of Ca^{2+}-ATPase activity in rat liver microsomes. J Cell Biochem 74:663–669.

Takahashi H, Yamaguchi M. 2000. Stimulatory effect of regucalcin on ATP-dependent Ca^{2+} uptake activity in rat liver mitochondria. J Cell Biochem 78:121–130.

Tombal B, Weeraratna AT, Denmeade SR, Isaacs JT. 2000. Thapsigargin induces a calmodulin/calcineurin-dependent apoptotic cascade responsible for the death of prostatic cancer cells. Prostate 43:303–317.

Tsurusaki Y, Yamaguchi M. 2002a. Suppressive role of endogenous regucalcin in the enhancement of deoxyribonucleic acid synthesis activity in the nucleus of regenerating rat liver. J Cell Biochem 85:516–522.

Tsurusaki Y, Yamaguchi M. 2002b. Role of endogenous regucalcin in nuclear regulation of regenerating rat liver: Suppression of the enhanced ribonucleic acid synthesis activity. J Cell Biochem 87:450–457.

Tsurusaki Y, Yamaguchi M. 2003. Overexpression of regucalcin modulates tumor-related gene expression in cloned rat hepatoma H4-II-E cells. J Cell Biochem 90: 619–626.

Tsurusaki Y, Misawa H, Yamaguchi M. 2000. Translocation of regucalcin to rat liver nucleus: Involvement of nuclear protein kinase and phosphatase regulation. Int J Mol Med 6:655–660.

Yamaguchi M. 2000a. Role of regucalcin in calcium signaling. Life Sci 66:1769–1780.

Yamaguchi M. 2000b. The role of regucalcin in nuclear regulation of regenerating liver. Biochem Biophys Res Commun 276:1–6.

Yamaguchi M, Isogai M. 1993. Tissue concentration of calcium-binding protein regucalcin in rats by enzyme-linked immunoadsorbent assay. Mol Cell Biochem 122: 65–68.

Yamaguchi M, Nakajima M. 1999. Involvement of intracellular signaling factors in the serum-enhanced Ca^{2+}-binding protein regucalcin mRNA expression in the cloned rat hepatoma cells (H4-II-E). J Cell Biochem 74:81–89.

Yamaguchi M, Sakurai T. 1991. Inhibitory effect of calcium-binding protein regucalcin on Ca^{2+}-activated DNA fragmentation in rat liver nuclei. FEBS Lett 279: 281–284.

Yamaguchi M, Yamamoto T. 1978. Purification of calcium binding substance from soluble fraction of normal rat liver. Chem Pharm Bull 26:1915–1918.

Yamaguchi M, Takahashi H, Tsurusaki Y. 2003. Suppressive role of endogenous regucalcin in the enhancement of nitric oxide synthase activity in liver cytosol of normal and regucalcin transgenic rats. J Cell Biochem 88:1226–1234.