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Suppressive role of regucalcin in liver cell proliferation: involvement in carcinogenesis

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Abstract

Regucalcin (RGN/SMP30) was discovered in 1978 and is a unique calcium-binding protein contains no EF-hand motif calcium-binding domain. Its name, regucalcin, was proposed as it suppresses activation of enzymes related to calcium signalling. The regucalcin gene (*rgn*) is localized on the X chromosome. Regucalcin plays its role of suppressor protein in intracellular signalling pathways, including of protein kinases and protein phosphatase activities, protein synthesis, and DNA and RNA synthesis in liver cells. Overexpression of endogenous regucalcin has a suppressive effect on cell proliferation in modelled rat hepatoma H4-II-E cells, which are induced by various signalling stimulations *in vitro*. This suppressive effect is independent of apoptosis. Endogenous regucalcin plays a suppressive role on overproduction of proliferating cells in regenerating rat liver *in vivo*. Regucalcin mRNA expression is uniquely down-regulated in development of carcinogenesis in liver of rats *in vivo*. Regucalcin mRNA and protein expressions are also depressed in human hepatoma HepG2 cells, MCF-7 breast cancer cells, and prostate cancer LNCaP cells. Depression of regucalcin expression may be associated with activity progression of carcinogens. Regucalcin may be a key molecule suppressor protein in cell proliferation and carcinogenesis.

Introduction

Regucalcin was discovered in 1978 and is a calcium-binding protein that contains no EF-hand motif as calcium-binding domain, found in many calcium-binding proteins (1–3). The name, regucalcin, was proposed as it suppresses activity of various enzymes activated by Ca^{2+} or Ca^{2+}/calmodulin (2,3). Regucalcin gene is localized on the X chromosome and consists of seven exons and six introns (4,5). Regucalcin (RGN) and its gene (*rgn*) are identified in over 15 species; the regucalcin family and gene species are highly conserved in vertebrates (6–8). Senescence marker protein-30, a senescence marker is identical to regucalcin, and was reported after discovery of regucalcin (9,10).

Regucalcin gene expression is regulated through various transcription factors (including AP-1, NF1-A1, RGPR-p117, β-catenin, SP1 and others), which are identified as enhancers and suppressors, and expression is regulated by hormonal stimulation in physiological states (8,11–16). Regucalcin has been demonstrated to play a multifunctional role of regulation in various tissues and cell types (reviewed in Refs. 3,17–19). Regucalcin has a role in maintaining intracellular Ca^{2+} levels due to activating various Ca^{2+} pump enzymes (17). Cytoplasm regucalcin localizes to the nucleus and suppresses nuclear Ca^{2+}-dependent protein kinase and protein phosphatase, Ca^{2+}-activated DNA fragmentation, and DNA and RNA synthesis (17,18) in various phenotypes. Regucalcin has been proposed to be pivotal as a suppressor protein in various types of signal transduction, to maintain cell homoeostasis stimuli (17,18).

There is growing evidence that endogenous regucalcin is a protein suppressor of cell proliferation, and is mediated through a variety of signalling pathways, in cloned normal rat kidney proximal tubular epithelial NRK52E cells and cloned rat hepatoma H4-II-E cells. Suppression of regucalcin gene expression may lead to promotion of cell proliferation and carcinogenesis. This review will discuss regucalcin as suppressor of liver cell proliferation and its involvement in carcinogenesis.

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Role of regucalcin in nuclear signalling regulation

Regulation of signalling pathway-related enzymes

The pathway of cell signalling following hormonal stimulation is mediated through Ca^{2+}, cyclic adenosine monophosphate (cyclic AMP), nitric oxide (NO) and their related proteins, in various cell types. Ca^{2+}/calmodulin-dependent protein kinase is important in response of cells to calcium signalling (20). Protein kinase C, a diacylglycerol-activated Ca^{2+}, and phospholipid-dependent protein kinase, is capable of phosphorylating many cytoplasmic proteins in (21). cAMP is degraded by cAMP phosphodiesterase activated through Ca^{2+}/calmodulin (22). NO, produced by NO synthase, requires Ca^{2+}/calmodulin, and acts as a messenger or modulator molecule in many biological systems (23). Regucalcin has been shown to have depressive effects on activation of Ca^{2+}/calmodulin-dependent protein kinase, protein kinase C, cAMP phosphodiesterase and NO synthase in various cells and tissues (17,18).

Protein phosphorylation–dephosphorylation is a universal mechanism by which numerous cell events are regulated (24). There may exist many phosphatases that, like kinases, are elaborately and rigorously controlled (25). Protein phosphatase occurs in intracellular signal transduction due to hormonal stimulation. Calcineurin, a calmodulin-binding protein, has been shown to have Ca^{2+}-dependent and calmodulin-stimulated protein phosphatase activity (25) and regucalcin has been found to inhibit calcineurin activity in cell cytoplasm and nuclei; it is a unique protein having inhibitory effects on protein tyrosine phosphatase and protein serine/threonine phosphatases (17,18).

Intracellular Ca^{2+} may be required for maintenance of high rates of protein synthesis in cells (26,27); regucalcin has been found to have suppressive effects on this due to inhibiting aminoacyl-tRNA synthase activity, suggesting that endogenous regucalcin may be a suppressor of protein synthesis (28). This effect of regucalcin is independent of Ca^{2+}/calmodulin in liver cells; regucalcin may bind aminoacyl (leucyl)-tRNA synthetase (28) and Ca^{2+}-activated protease (calpains) are also implicated in signal transduction (29,30). Regucalcin has been found to activate cysteinyl proteases including calpains in cytoplasm of rat hepatocytes and cells of the renal cortex (17,30). Regucalcin may be important in regulation of signal transduction involved in proteases, thus, it may help regulate protein turnover due to inhibiting protein synthesis and activating protein degradation, suggesting its suppressive role in enhancement of protein production in hepatocytes.

Regulation of nuclear function

Calmodulin exists in rat liver cell nuclei and stimulates DNA synthesis mediated through α-adrenergic stimulation (31,32) and regucalcin has been found to localize in nuclei binding to calmodulin (33,34). Nuclear translocation of regucalcin has been shown to be independent of any nuclear localization signal responsible for selection for intranuclear active transport (34). The molecular weight of regucalcin, in the region of 33 kDa (6), indicates that it may be passively transported to the nucleus through nuclear pores in hepatocytes. It has also been shown by immunocytochemical analysis, to localize in nuclei of cloned normal rat kidney proximal tubular epithelial NRK52E cells (35). This translocation is stimulated through signalling pathway of protein kinase C (35). Nuclear regucalcin also binds to proteins and DNA in isolated rat liver nuclei (36); it seems to be important in regulation of hepatocyte nuclear function.

Isolated rat liver nuclei display DNA endonuclease activity depending upon Ca^{2+} and Ca^{2+} results after extensive DNA hydrolysis (37). DNA fragmentation in rat liver nuclei is stimulated through Ca^{2+}-calmodulin existing within the nuclei (37). Such endogenous endonuclease activity may be responsible for DNA fragmentation occurring during programmed cell death (apoptosis) (37). Regucalcin has been shown to have suppressive effects on Ca^{2+}-activated DNA fragmentation in isolated rat liver nuclei (38). Presence of regucalcin (0.5–2.0 μM) completely suppresses activation of liver nuclear DNA fragmentation with presence of 10 μM Ca^{2+} in the reaction mixture (38), suggesting that regucalcin depresses DNA endonuclease activation mediated through Ca^{2+}/calmodulin.

Processes of signal transduction from cytoplasm to nuclei in liver cells is mediated through various protein kinases and protein phosphatases, related to gene expression and cell proliferation. Regucalcin has been shown to suppress activity of Ca^{2+}-dependent protein kinase and protein phosphatases in isolated liver nuclei (33,34).

Regucalcin has inhibitory effects on DNA (39,40) and RNA (41,42) synthesis isolated from rat liver nuclei; this was not seen, however, in the presence of α-amanitin, an inhibitor of RNA polymerases II and III, suggesting that the suppressive effect of regucalcin partly resulted from its inhibitory action on the RNA polymerases (42). Regucalcin also suppresses stimulating effects of Ca^{2+} on nuclear RNA synthesis (41,42). By reducing RNA synthesis, regucalcin may be partly involved in its inhibitory action on the activities of both RNA polymerases II and III and Ca^{2+}-dependent protein kinase; regucalcin can directly bind DNA (36), although which base pairs of DNA bind regucalcin

remain to be elucidated. Regucalcin binding DNA may reveal suppressive effects on DNA and RNA synthesis, suggesting it as a transcriptional factor in the liver cell nucleus.

Thus, regucalcin may be suppressive in signal transduction from cytoplasm to nucleus in cell regulation, mediated through phosphorylation and dephosphorylation of many cell proteins (17,18) and it may suppress protein in various cell signalling pathways.

Regucalcin suppresses hepatocyte proliferation *in vitro*

Regucalcin has been found to suppress enhancement of hepatoma cell proliferation after serum stimulation, its mRNA and protein are expressed in cloned rat hepatoma H4-II-E cells and human hepatoma HepG2 cells (43–45). These expressions, however, are at low levels as compared to normal rat liver (43–45), suggesting an involvement of regucalcin in hepatoma cells.

Suppression of protein kinase activity enhanced with cell proliferation

When H4-II-E cells were cultured for 6–72 h in the presence of foetal bovine serum (FBS; 1 or 10%), numbers of cells and protein kinase activity in 5500 g supernatant of cell homogenate were elevated 24 and 48 h after culture with FBS (1 or 10%); culture in 10% FBS had a potent effect compared to that of 1% FBS (46). Culture with FBS produced higher protein kinase activity and corresponding elevation in number of H4-II-E cells (46). Increase in protein kinase activity preceded significant elevation in cell number (46), suggesting that serum factors (including growth factors, cytokines and hormones) stimulate cell proliferation that is partly mediated through cascades of various protein kinases. Serum-stimulated protein kinase activity in H4-II-E cells was enhanced after addition of calmodulin or dioctanoylglycerol in the presence of calcium chloride in the enzyme reaction mixture (46). This increase was inhibited in the presence of each inhibitor for Ca^{2+}/calmodulin-dependent protein kinase, protein kinase C, and protein tyrosine kinase (46). Various protein kinases may be involved in enhancement of hepatocyte proliferation after serum stimulation. Endogenous regucalcin has been shown to be suppressive in enhancement of protein kinase activity in the cytoplasm in H4-II-E cells with cell proliferation using anti-regucalcin monoclonal antibody (46). Regucalcin may have a suppressive effect on overexpression of cell proliferation due to inhibiting various protein kinases in cytoplasm and nuclei of hepatocytes.

Suppression of protein phosphatase activity enhanced with cell proliferation

Regucalcin has been shown to have a suppressive effect on protein phosphatase activity in cytoplasm and nuclei of normal rat liver cells (33,34); Ca^{2+}/calmodulin-dependent protein tyrosine phosphatases have been found to be present in H4-II-E cells (47,48). Regucalcin had inhibitory effects on Ca^{2+}/calmodulin-dependent protein tyrosine phosphatase activity in the cells (47,48). Culture with Bay K 8644, an agonist of Ca^{2+} channels, caused elevation of protein tyrosine phosphatase in H4-II-E cells, while dibutyryl cAMP did not have any such effect (47,48). Endogenous regucalcin, increased after culture with Bay K 8644, was found using anti-regucalcin monoclonal antibody, to be suppressive to Ca^{2+}/calmodulin-activated protein tyrosine phosphatase activity in proliferating cells (47,48). Processes reversibly controlled by protein phosphorylation require not only protein kinase but also protein phosphatase (49). Target proteins are phosphorylated at specific sites by one or more protein kinases and these phosphoproteins are removed by specific protein phosphatase (49). Regucalcin may be an important suppressor for enhancement of cell proliferation due to inhibiting activities of various protein kinases and protein phosphatases enhanced in proliferation of H4-II-E cells.

Suppression of DNA synthesis enhanced with cell proliferation

Endogenous regucalcin has been shown to have suppressive effects on enhancement of DNA synthesis in nuclei of H4-II-E, with cell proliferation (50,51). Numbers of cells cultured for 6–96 h in a medium containing FBS (1 or 10%), were increased between 24 and 96 h; cell proliferation was markedly stimulated after culture with 10% FBS compared to 1% FBS (50). DNA synthesis *in vitro* was elevated at 6 h after culture with 10% FBS and its elevation was remarkable by 12 and 24 h (50). Increase in DNA synthesis preceded elevation in numbers of H4-II-E cells cultured with FBS (1 or 10%) (50). Increase in DNA synthesis at 12 and 24 h after culture with FBS was inhibited in the presence of PD98059, an inhibitor of extracellular signal-related kinase, staurosporine, an inhibitor of protein kinase C, and trifluoperazine (TFP), an antagonist of Ca^{2+}/calmodulin-dependent protein kinase, in the reaction mixture (50). Increase in DNA synthesis after serum stimulation may be partly mediated by action of various protein kinases in nuclei of H4-II-E cells, however, serum-stimulated increase in DNA synthesis may not be related to protein phosphatases (50).

Presence of regucalcin in the reaction mixture caused reduction in DNA synthesis H4-II-E nuclei cultured with FBS (50). DNA synthesis became elevated in the presence of anti-regucalcin monoclonal antibody in the reaction mixture of H4-II-E cell nuclei cultured for 24 h in 10% FBS (50) and this elevation was depressed after addition of various protein kinase inhibitors (50). These findings support the view that endogenous regucalcin suppresses DNA synthesis enhanced through mechanisms by which it depresses nuclear protein kinases, and that it has a directly suppressive effect on DNA synthesis in nuclei of H4-II-E cells, with proliferation. Thus, regucalcin may have a suppressive effect on enhancement of DNA synthesis in proliferating cells, and it may be suppressive on overexpression of cell proliferation. This has been further supported using H4-II-E cells overexpressing regucalcin stably.

Regucalcin-overexpressing cells have been generated, in which regucalcin content of regucalcin/pCXN2-transfected cells was 19.7-fold higher compared to parental wild-type H4-II-E cells and pCXN2 vector-transfected cells (mock-type) (51). When regucalcin/pCXN2 vector-transfected cells (transfectants) were cultured for 72 h in the presence of FBS (10%), cell numbers and DNA synthesis in the transfectants were found to be suppressive compared to those of wild- and mock-type cells. This finding supports the view that overexpression of endogenous regucalcin has a suppressive effect on cell proliferation (51). Presence of anti-regucalcin monoclonal antibody in the reaction mixture caused elevation in DNA synthesis in nuclei of wild-type H4-II-E cells, mock-type cells and transfectants with overexpression of regucalcin (51). However, augmentation of DNA synthesis was remarkable in the transfectants (51), supporting the view that endogenous regucalcin has a highly suppressive effect on DNA synthesis.

Expression of regucalcin mRNA has been shown through a Ca^{2+}-signalling mechanisms to stimulate H4-II-E cells (43). Nuclear regucalcin inhibits nuclear protein kinase and protein phosphatase activities in H4-II-E cells (46,47) and suppresses DNA synthesis in them while proliferating (50,51).

Regulation of cell cycle-related gene expression

Regucalcin has been shown to regulate gene expression of cell cycle-related proteins in proliferating hepatoma cells (52). Hepatocyte proliferation was suppressed in the transfectants cultured for 24–72 h (52) and proliferation of H4-II-E cells (wild-type and transfectants) was reduced in the presence of PD98059, dibucaine, staurosporine, or genistein (that inhibits various protein kinases) (52). The effect of regucalcin in suppressing hepatoma cell proliferation may partly be related to its inhibitory effect on mitogen-activated protein kinase (MAPK), Ca^{2+}/calmodulin-dependent kinase, protein kinase C, and protein tyrosine kinase in H4-II-E cells (52). Proliferation of H4-II-E cells (wild-type) was inhibited in the presence of wortmannin, an inhibitor of PI3 kinase, or vanadate, an inhibitor of protein tyrosine phosphatase (52); these effects were not observed in the transfectants (52). Endogenous regucalcin, overexpressed in the cells, may inhibit activity of PI3 kinase and protein tyrosine phosphatase, and these effects may partly contribute to suppression of H4-II-E cell proliferation.

Overexpression of endogenous regucalcin has been found to regulate effects of various factors which induce cell cycle arrest, on the proliferation of H4-II-E cells (wild-type) (52). Roscovitine can arrest them in G1 and their accumulation in G2 (53). This effect of roscovitine, a potent and selective inhibitor of cyclin-dependent kinases (CDK) (cdc2, CDK2m and CDK5) (53), or sulphoraphane, which can induce G2/M phase cell cycle arrest (54), inhibiting proliferation of wild-type cells, was not observed in the transfectants (52). Sulphoraphane at higher concentrations has caused reduction in cell number of transfectants, suggesting that this reagent induces cell death/apoptosis (54). Butyrate induces inhibition of G1 progression (55) and has caused inhibition of proliferation of wild-type cells and transfectants (52). The inhibitory effect of roscovitine or sulforaphane on cell proliferation is not seen in transfectants, as regucalcin has a suppressive effect on the identical pathway used by roscovitine or sulforaphane thus has inhibitory effects on cell proliferation. Regucalcin may cause G1 and G2/M phase cell cycle arrest in H4-II-E cells.

Expression of p21 mRNA has been found to be enhanced in transfectants overexpressing endogenous regucalcin, although expressions of cdc2a and chk2 (checkpoint-kinase 2) mRNAs were not changed in transfectants (52). p21 is an inhibitor of CDK (55) and regucalcin may enhance p21 expression and inhibit G1 progression in H4-II-E cells. This cannot exclude the possibility, however, that regucalcin directly inhibits CDK activity in the cells.

In addition, overexpression of endogenous regucalcin has suppressed expression of IGF-I mRNA in H4-II-E cells (52); IGF-I is a growth factor for cell proliferation. Regucalcin may have a suppressive effect on IGF-I expression in H4-II-E cells and suppression of IGF-I expression may lead to retardation of cell proliferation.

Regulation of tumour-related gene expression

c-*myc,* c-*fos*, c-*jun* and Ha-*ras* are tumour-stimulator genes (56); *p53* and *Rb* are tumour-suppressor genes

and *c-src* is an oncogene (57). Expression of c-*myc*, Ha-*ras* or c-*src* mRNAs has been found to suppress in transfectants overexpressing regucalcin (58). Expression of *p53* and *Rb* mRNAs is markedly enhanced in transfectants (52) from *p53* has also been known to stimulate expression of the gene coding for p21, the inhibitor of cell cycle-related protein kinases, that induces cell cycle arrest. Suppression of expressions of c-*myc*, Ha-*ras* and *c-src* mRNAs and enhancement of expressions of *p53* and *Rb* mRNAs in transfectants overexpressing regucalcin may lead to retardation of proliferation of hepatoma H4-II-E cells.

Mechanisms by which regucalcin regulates expression of many genes related to carcinogenesis remains to be elucidated. However, regucalcin, translocated to the nucleus, may bind DNA and modulate nuclear transcriptional activity. Regucalcin possibly binds to promoter regions of tumour-related genes, and suppresses expression of tumour-stimulating genes, or stimulates expression of tumour-suppressor genes in H4-II-E cells overexpressing endogenous regucalcin. As a result, overexpression of endogenous regucalcin may suppress proliferation of hepatoma cells. Interestingly, expression of regucalcin is reduced in cloned rat hepatoma H4-II-E cells compared to those of normal rat liver (43). Down-regulation of regucalcin gene expression in hepatoma cells may lead to stimulation of cell proliferation with changes in various tumourigenesis-related gene expressions.

In addition, regucalcin has been shown to have a suppressive effect on cloned normal rat kidney proximal tubular epithelial NRK52E cells (58). Overexpression of endogenous regucalcin has been demonstratd retard their proliferation inducing G1 and G2/M arrest; this consequence may be mediated through reduction in various Ca^{2+} signalling-dependent protein kinases and PI3 kinase activities and suppression of *c-jun* and *chk2* mRNA expression, or enhancement of *p53* mRNA (58). Thus, the mechanism by which regucalcin modulates cell proliferation may be based similar modes in normal NRK52E cells and hepatoma H4-II-E cells. Regucalcin aids physiological control of cell over-proliferation.

Effects of regucalcin on cell proliferation may not be related to apoptosis. Overexpression of endogenous regucalcin has been shown to have a suppressive effect on this mechanism of cell death induced by various factors (including tumour necrosis factor-α and lipopolysaccharide) in cloned rat hepatoma H4-II-E cells (59–61).

Molecular mechanisms by which regucalcin suppresses cell proliferation are summarized in Fig. 1. They may be related to suppression of calcium-dependent signalling factors, various protein kinases and protein phosphatases activities, protein synthesis, DNA and RNA synthesis, IGF-I expression and various tumourigenesis-related types of gene expression.

Regucalcin reduces cell proliferation in regenerating liver *in vivo*

Regucalcin has been shown to regulate cell proliferation in regenerating rat liver *in vivo*; this tissue is a good model for the purpose. Adult rat hepatocytes are normally quiescent *in vivo*, however, 20–30 h after partial hepatectomy (HP) (in the order of 70% removal), they

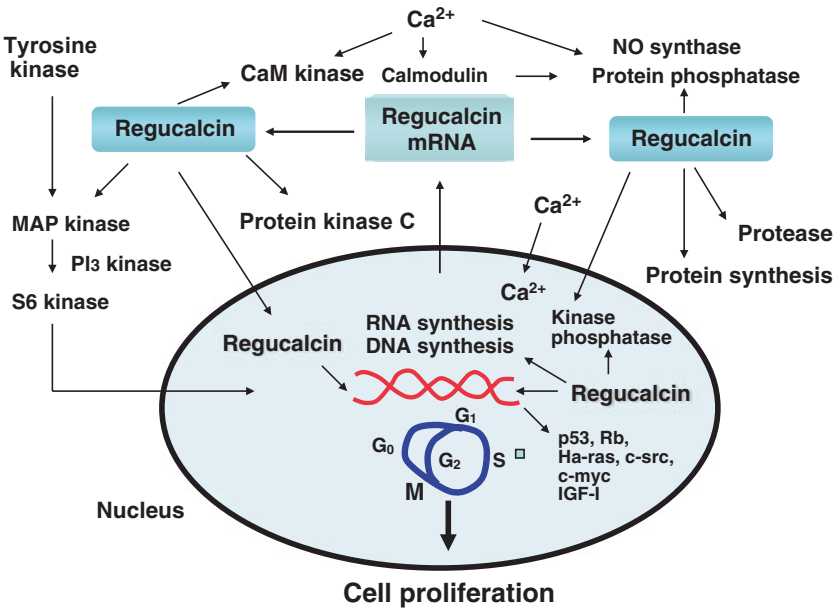

Figure 1. Regucalcin suppresses promotion of cell proliferation; it is expressed *via* signalling factors that stimulate cell proliferation and translocation to the nucleus by mechanisms mediated through protein kinase C. Regucalcin inhibits activities of various Ca^{2+}/calmodulin-dependent enzymes, protein kinases and protein phosphatases in cytoplasm and nuclei; it inhibits DNA and RNA synthesis. Regucalcin has a suppressive effect on expression of *c-myc*, *Ha-ras*, and *c-src* mRNAs, tumour-stimulator genes and also causes expression of *p53* and *Rb* mRNAs - tumour-suppressor genes. Moreover, regucalcin inhibits protein synthesis and stimulates protein degradation due to activating cysteinyl protease. Thus, suppressive effects of regucalcin on cell proliferation are mediated through regulation of many signalling processes. Regucalcin may cause G1 and G2/M phase cell cycle arrest in liver cells.

undergo a synchronous wave of DNA synthesis and cell division and continue to divide until the original liver mass is regenerated 3–7 days later (62). Liver weight 1 day after HP increases by around 50% over sham-operated rats and regains identical levels as sham operated animals by 3 days after partial HP, indicating a burst of cell proliferation (63).

Nuclear Ca^{2+} signalling in regenerating liver

Hepatocyte growth factor, which promotes regenerating liver after partial HP (64), has been shown to increase calcium concentration in rat hepatocytes (65); calmodulin synthesis in regenerating rat liver is increased by 8 h after HP (66). A Ca^{2+} transport system exists in liver cell nuclei (67); Ca^{2+}-ATPase activity there between 1 and 5 days after partial HP is enhanced in regenerating rat liver (68). Hepatocyte nuclear Ca^{2+}-ATPase is related to Ca^{2+} uptake and the cation accumulates in nuclear matrix (68). Increase in nuclear Ca^{2+}-ATPase activity in regenerating liver causes corresponding augmentation of nuclear calcium content (63), elevated between 1 and 5 days after partial HP (63). Calcium, increased in cytoplasm of regenerating liver, may be transported into nuclei in association with an enhancement of nuclear Ca^{2+}-ATPase activity. Protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase exist in the liver nuclei (34) where Ca^{2+}-ATPase activity is reduced by addition of protein kinase inhibitor into an enzyme reaction mixture (63). High nuclear Ca^{2+}-ATPase activity in regenerating liver may be partly activated through Ca^{2+}-dependent protein kinases. Such DNA content increases from 1 day after HP, while DNA fragmentation reduces (63); this is not altered in the presence of EGTA, a Ca^{2+} chelator (63). Increased nuclear calcium in regenerating liver may be involved in regulation of DNA synthesis rather than in activation of DNA fragmentation.

Endogenous regucalcin suppresses enhancement of nuclear protein kinase and protein phosphatase in regenerating liver

Regucalcin mRNA expression has been shown to increase in regenerating rat liver 1–5 days after partial HP compared to that of sham-operated animals, the same longer time, 5 days, of activation of liver proliferation (69). Expression of regucalcin mRNA in regenerating liver may be mediated through Ca^{2+}/calmodulin, as hepatocyte growth factor, promoting regeneration after partial HP (64), increases calcium concentration in rat hepatocytes (65). Increase in regucalcin mRNA expression may be independent of decomposition of mRNA (69) and processes transcribing regucalcin mRNA may be stimulated. Increase in regucalcin expression may contribute to suppression of cell proliferation here.

Regucalcin has been found to have be suppressive to enhancement of Ca^{2+}/calmodulin-dependent protein kinase and protein kinase C in nuclei of regenerating rat liver (70,71); protein kinase activity increases in hepatocyte nuclei obtained 6–48 h after partial HP (70), suggesting that protein kinases, which are involved in Ca^{2+} signalling, participate in enhancement of nuclear protein phosphorylation. Protein phosphorylation in nuclei of regenerating liver cells is elevated in presence of anti-regucalcin antibody in any reaction mixture (71). This stimulation is completely negated after addition of exogenous regucalcin (71), indicating that endogenous regucalcin is involved in inhibition of protein kinase there. Net increase in protein kinase activity after anti-regucalcin antibody addition has been shown to be in the order of 2-fold higher compared to normal hepatocytes (71). Endogenous regucalcin, which is enhanced in regenerating liver, may be translocated to nuclei and regulates nuclear functions. Protein phosphatase activity on phosphotyrosine is also found in liver cell nuclei, whereas nuclear enzymes on phosphoserine and phosphothreonine are only minimally expressed (33). Protein phosphotyrosine phosphatase in cytoplasm and nuclei of hepatocytes increases in regenerating rat liver (72) and this is enhanced in the presence of anti-regucalcin monoclonal antibody in any enzyme reaction mixture (72).

Thus, endogenous regucalcin has an inhibitory effect on Ca^{2+}/calmodulin-dependent protein kinases, protein kinase C and protein phosphatases in regenerating rat liver. It may participate in regulation of cell function due to suppression of phosphorylation and dephosphorylation of various proteins in proliferating hepatocytes *in vivo*; this may be important in reduction of such overproliferation.

Endogenous regucalcin suppresses enhancement of protein, DNA and RNA synthesis in regenerating liver

Hepatic protein synthesis has been shown to be enhanced when the organ regenerates, this induces hepatocyte proliferation after partial HP (73) and the enhancement is remarkable at 24 and 48 h after PH. Regucalcin has been found to suppress protein synthesis in regenerating liver (73); hepatocyte protein synthesis is further enhanced in the presence of anti-regucalcin monoclonal antibody in any reaction mixture (73). This elevation is completely eliminated by addition of regucalcin (73). Endogenous regucalcin may have this effect on enhancement of protein synthesis in proliferating hepatocytes *in vivo*.

Liver cell DNA synthesis stimulates regenerating rat liver (39,40) and is markedly enhanced 1 and 3 days after partial HP (39). Regucalcin has been found to suppressive it in regenerating liver (39,40). This does not result from DNA hydrolysis, as regucalcin inhibits Ca^{2+}-activated DNA fragmentation (38). DNA synthesis in regenerating rat liver is enhanced in the presence of anti-regucalcin monoclonal antibody in any reaction mixture (40), supporting the view that endogenous regucalcin suppresses enhancement of DNA synthesis here.

Total nuclear function in regenerating rat liver may be stimulated *via* many signalling factors. Increase in DNA synthesis is reduced in the presence of staurosporine, TFP and PD98059, all protein kinase inhibitors, in any reaction mixture *in vitro* (40). This observation suggests that DNA synthesis in proliferating hepatocytes is partly stimulated by signalling pathways related to protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase, and MAPK kinase in nuclei. Effects of anti-regucalcin monoclonal antibody in enhancing DNA synthesis is reduced in the presence of staurosporine, TFP and PD98059 (40). This finding suggests that the suppressive effect of endogenous regucalcin on DNA synthesis may be partly mediated through inhibitory action of regucalcin on various protein kinases. In addition, regucalcin may directly inhibit DNA synthesis in nuclei of liver cells.

Regucalcin has been shown to inhibit RNA synthesis in nuclei isolated from control rat liver (41) as well as from regenerating rat liver (42). Presence of anti-regucalcin monoclonal antibody in the reaction mixture causes significant increase in RNA synthesis in nuclei of both (42), suggesting that endogenous regucalcin suppresses enhancement of RNA synthesis in regenerating rat liver. Enhancement of RNA synthesis in regenerating rat liver is partly dependent on activation of protein kinases and protein phosphatases involved in intracellular signalling pathways of hormones and growth factors (42). Effects of anti-regucalcin monoclonal antibody enhancing RNA synthesis in nuclei of regenerating rat liver reduces in presence of α-amanitin, PD98059, staurosporine, or vanadate (42). Enhancement of RNA synthesis in regenerating liver may be suppressed *via* various signalling pathways; endogenous regucalcin depresses RNA polymerases II and III, MAPK kinase, protein kinase C and protein phosphatases in liver nuclei, thus, regucalcin has been found to reduce RNA synthesis here. Regucalcin may be a transcription-related factor, which suppresses gene expression, in liver cell nuclei. Whether regucalcin itself binds directly to the gene promoter region however, at present is unknown.

As mentioned above, regucalcin mRNA expression in regenerating liver cells is stimulated through pathways of signalling mechanisms concerning protein kinases and protein phosphatases elevated in proliferating cells of regenerating liver, *in vivo*. Endogenous regucalcin, increased in hepatocytes, suppresses synthesis of protein, DNA, and RNA, mediated through various signalling pathways. Regucalcin may reduce hepatocyte overexpression *in vivo*.

Involvement of regucalcin in liver carcinogenesis

Regucalcin is regulatory at maintaining intracellular Ca^{2+} homoeostasis, suppression of cell proliferation, depression of oncogene expression and stimulation of tumour suppressor gene expression in liver cells. The reduced regucalcin in cancer cells may lead to disorder of regucalcin function in malignant tissues, which may relate to development of carcinogenesis.

In general, expression of various gene mRNAs is changed in various cancer cell types; activation of genes involved in cell population growth are stimulated, instead of being suppressed as in differentiated phenotypes (74). Expression of regucalcin gene has been found to be reduced in liver tumours of rats *in vivo* (75). Rat hepatomas have been induced after continuous feeding of basal diet containing 0.06% 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) (75). After 35 weeks feeding, non-malignant plus tumours of livers were removed (75) and in individual rats, regucalcin mRNA levels of tumourous were lower compared to normal counterparts (75). *c-myc* mRNA was specifically higher in induced hepatomas (75) and genomic DNA extracted from liver tumours was digested using restriction enzymes (*Eco*RI, *Bam*HI and *Hin*dIII) before analysis by Southern blotting (75). Distinct changes were not found in regucalcin genes of the tumourous (75); this suggests that the gene was not modified during the tumourigenesis, although suppression of regucalcin mRNA expression may participate in development of tumourigenesis with feeding this chemical to the rats.

Interestingly, new markers for liver pre-neoplastic foci in rats treated with diethylnitrosamine then 2-acetylaminofluorene, combined with partial HP, (which induces increase in proliferating cells), have been investigated (76). Transaldolase, aflatoxin B1 aldehyde reductase and gamma- glutamylcysteine synthetase have found to have up-regulated coding genes, and regucalcin gene was found to be down-regulated; this is in line with findings for hepatocellular carcinomas (76). Suppression of regucalcin gene expression was seen at early stages in development of carcinogenesis (76).

Thus, reduction of regucalcin expression may be related to development of liver carcinogenesis. If chemical feeding-induced reduction in regucalcin expression

accelerates enhancement of various signalling pathways, this may generate possible circumstances for initiation of carcinogenesis. In this aspect, such suppression of the regucalcin gene by specific chemical feeding *in vivo*, may be a pathophysiological step in carcinogenesis.

Biomarkers associated with beginnings of hepatocellular carcinoma in CuZn superoxide dismutase (CuZn-SOD, Sod1)-deficient mice have been identified (77). Liver samples were obtained from 18-month-old 1−/− and +/+ mice. Regucalcin was shown to be divergent in the samples (77). Whereas elevated regucalcin levels were observed in −/− samples with no obvious neoplastic changes, marked reduction in regucalcin is seen in −/− samples with fully developed hepatocellular carcinoma (77). Glutathione S-transferase (GST) mutant 1 (M1) models revealed significant increases only in neoplastic regions obtained from Sod1−/− livers (77); no changes in GSTM1 were observed in surrounding normal tissues (77). Marked reduction was seen in two intracellular lipid transporters, fatty acid-binding protein 1 and major urinary protein 11 and 8 in Sod1−/− livers (77); thus, regucalcin was identified to be a biomarker associated with development of hepatocellular carcinoma in CuZn superoxide dismutase-deficient mice *in vivo* (77).

As mentioned above, regucalcin suppression has been found to be associated with development of carcinogenesis in animal models *in vivo*. Reduction in regucalcin, being a multifunctional suppressor in various signalling pathways in proliferating cells, may lead to development of carcinogenesis.

Regucalcin expression is depressed in human cancer cells

Expression, post-translational processing and targeting of gene products are frequently altered in transformed and malignant cells. Regucalcin mRNA in cloned human hepatoma (HepG2) cells has been found to be longer than that of normal human liver, using northern blot analysis (44), suggesting existence of transcript heterogeneity in the human gene for regucalcin. Regucalcin mRNA expression in HepG2 cells was suppressed compared to that of human normal liver (44). cDNA for regucalcin in human normal liver and HepG2 was cloned and characterized by rapid amplification of cDNA ends (RACE-PCR) (44). Nucleotide sequences of clones indicated that they were identical in coding regions and differed only in their 5′ untranslated zones (UTRs) (44); in particular, their 5′ UTR sequences were quite different from −17 positions, suggesting that these transcripts arose from alternative splicing (44). 5′ UTR sequence of the regucalcin gene from human normal liver (−21 to −86) had homology to rat regucalcin 5′ UTR (44). These different 5′ UTR isoforms seem to reflect transcript size variation as seen by norther blot analysis (44).

Phorbol 12-myristate 13-acetate (PMA) has been shown to stimulate regucalcin mRNA expression and to promoter activity in rat hepatocytes and HepG2 cells (78); the SP1 motif is located within −188/−180 of the promoter in HepG2 cells (78). Overexpression of SP1 reduced PMA-induced up-regulation both of internal expression of mRNA and promoter activity, whereas knockdown of SP1 had the opposite effect (78).

Triiodo L thyronine has been reported to suppress regucalcin mRNA expression in rat liver and in the MCF-7 human breast cancer cell line, by binding to thyroid response elements, which may be negative response elements for regucalcin gene promoter activity (79). This suppression may lead to apoptosis of MCF-7 cells (79); down-regulation of regucalcin gene expression by thyroid hormone may induce apoptosis in them (79). Overexpression of regucalcin has been shown to suppress various factors inducing apoptosis in cloned rat hepatoma H4-II-E cells (59–61) too.

Regucalcin may be associated with abnormal cell division in tumour tissues; both oestrogens and Ca^{2+} may be implicated in breast and prostate cancers (20,80,81). 17β-oestradiol has been shown to down-regulate regucalcin mRNA and protein expressions, which are present in rat mammary gland and prostate (80). Moreover, this activation suppressed human breast and prostate cancers (81). Expressions of regucalcin mRNA and protein in cloned MCF-7 and human prostate cancer cells (LNCaP) were suppressed after culture with 17β-oestradiol or testosterone, *in vitro* (81). Loss of regucalcin in breast and prostate cancers due to presence of sex steroid hormones may be associated with development and progression of these human tumours (81).

Future prospects

Regucalcin, localized in both cytoplasm and nuclei, plays can be a suppressor protein in intracellular signalling pathways. Endogenous regucalcin has been found to reduce enhancement of various protein kinases, protein phosphatase, protein synthesis, DNA and RNA synthesis in nuclei of proliferating liver cells *in vitro* and *in vivo*, mediated through various signalling pathways and endogenous regucalcin may depress overproduction of proliferating cells. Regucalcin mRNA is uniquely down-regulated in carcinogenesis in rat liver *in vivo*, although expression of other many genes is up-regulated, suggesting that endogenous regucalcin may be depressive in development of liver carcinogenesis. Moreover, regucalcin

mRNA and protein expression are suppressed in human hepatoma HepG2, breast cancer MCF-7 and prostate cancer LNCaP cells. Thus, activation of regucalcin is reduced in various human cancer cells, suggesting that low levels lead to development of carcinogenesis. Regucalcin may be a key suppressor protein of carcinogenesis.

Enhancement of endogenous regucalcin gene expression may play a part in reducing progression of cancer cells. Targeting the regucalcin gene may be a useful tool for treatment of cancer cells and enhancement of regucalcin gene expression using chemical factors (including natural and synthetic compounds) may be useful in treatment of cancer. This innovation and development of these tools will ameliorate adverse clinical aspects.

Author contribution and disclosures

Masayoshi Yamaguchi designed and conducted the study, collection, analysis and interpretation of data, and manuscript writing. Author has no conflicts of interest.

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