The suppression of regucalcin gene expression may lead to hepatocarcinogenesis

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Abstract
Regucalcin, which was discovered in 1978 as a calcium-regulatory protein, plays a multifunctional role as a suppressor in signal transduction in various types of cells and tissues. The regucalcin gene (rgn) is localized on the X chromosome. Regucalcin was found to suppress nuclear deoxyribonucleic acid and ribonucleic acid synthesis in liver cells. Overexpression of endogenous regucalcin revealed suppressive effects on proliferation in the modeled rat hepatoma cells due to regulating the gene expression of various proteins that are involved in cell cycle and carcinogenesis. Regucalcin mRNA expression was uniquely downregulated in the development of carcinogenesis in the liver of rats in vivo. The expressions of regucalcin mRNA was found to depress in human hepatoma tissues. The suppressed regucalcin gene expression was found to be associated with progression of hepatocarcinogenesis by proteosome analysis. The regucalcin gene may be a target in the therapy of hepatocarcinoma.

Introduction
Regucalcin was discovered in 1978 as a Ca\(^{2+}\)-binding protein that contains no EF-hand motif of Ca\(^{2+}\)-binding domain [1-4]. The regucalcin gene is localized on the X chromosome [5,6]. The organization of regucalcin gene consists of seven exons and six introns [7]. Regucalcin and its gene (rgn) are identified in over 15 species consisting of regucalcin family in vertebrate and invertebrate species [4,8,9]. Various transcription factors have been shown to regulate transcription activity of the regucalcin gene expression that is mediated through Ca\(^{2+}\) and other signal systems [9].

Regucalcin plays a multifunctional role in cell regulation of various types of cells and tissues; regulation of intracellular Ca\(^{2+}\) homeostasis and suppressions of various signal transductions, protein synthesis, cell proliferation and apoptosis [10-13]. Regucalcin, which is present in the cytoplasm, is translocated into the nucleus of various types of cells, and it plays a pivotal role in the regulation of nuclear function including suppression of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis, inhibition of various protein kinases and phosphatases activities, and regulation of the gene expression of various proteins [14]. Regucalcin is proposed to play a pivotal role in maintaining cell homeostasis for stimulation of various factors including hormones and cytokines [10,11].

Moreover, regucalcin is found to be involved in various pathophysioligic states including liver disease, kidney disorder, heart failure, brain disease, osteoporosis, and lipid metabolism [15-19]. Interestingly, the expressions of regucalcin mRNA was found to depress in human hepatoma tissues [12]. Suppression was associated with progression of hepatocarcinogenesis by proteosome analysis [20,21]. The regucalcin gene may be a target in the therapy of hepatocarcinoma. This review will discuss the recent findings regarding to involvement of regucalcin in hepatocarcinogenesis.

Regucalcin regulates liver nuclear function
Regucalcin is greatly in the cytoplasm of liver cells, and the protein is translocated into the nucleus through protein kinase C-dependent signaling mechanism [14]. Nuclear translocation of regucalcin was not regulated through adenosine 5’-triphosphate and guanosine 5’-triphosphate that are required for nuclear import of proteins [22], and it was not changed with the lectin wheat germ agglutinin that suppresses transport of nuclear protein [23]. Nuclear translocation of regucalcin was not related to nuclear localization signal that is responsible for selection for intranuclear active transport. Regucalcin may be passively transported to the nucleus through nuclear pore in cells, since the molecular weight of regucalcin is about 33 kDa [4]. Regucalcin has also been shown to localize in the nuclei of the cloned normal rat kidney proximal tubular epithelial NRK52E cells with immunocytochemical analysis [24]. This nuclear localization of regucalcin was found to enhance through hormonal Ca\(^{2+}\)-signaling dependent process that is involved protein kinase C [24].

Regucalcin has been shown to bind protein and DNA in the nucleus [25]. Regucalcin regulated various enzyme activities in the nucleus. Endonuclease is responsible for DNA fragmentation occurring during programmed cell death (apoptosis) and certain forms of chemically induced cell killing [26]. Regucalcin revealed suppressive effects on Ca\(^{2+}\)-activated DNA fragmentation due to inhibiting endonuclease activity in isolated rat liver nuclei [27]. Small GTPase Ran (ras-related nuclear protein) is required for protein export from the nucleus and protein import into the nucleus [28]. Regucalcin inhibited GTPase activity in rat liver nucleus [22]. Process of signal transduction from the cytoplasm to nucleus in liver cells is mediated through various protein

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kinases and protein phosphatases. Regucalcin suppressed the activities of tyrosine kinase, protein kinase C and Ca2+/calmodulin-dependent protein kinase, which are enhanced in the cytoplasm and nucleus obtained from regenerating rat liver with proliferating cells in vivo [29]. The activity of nuclear Ca2+-dependent protein kinases was increased in the presence of anti-regucalcin monoclonal antibody in the enzyme reaction mixture, and such increases are completely depressed with addition of regucalcin [29]. Nuclear endogenous regucalcin played a suppressive role in the regulation of protein tyrosine phosphatases using anti-regucalcin monoclonal antibody in the reaction mixture [30]. Regucalcin was demonstrated to play a pivotal role in the regulation of the activity of various enzymes in the nucleus.

Regucalcin was found to reveal suppressive effects on DNA and RNA synthesis activity in the nuclei of normal rat liver and regenerating rat liver in vivo [31-34]. Regucalcin may suppress the enhancement of nuclear DNA and RNA synthesis in proliferating liver cells in vivo. The presence of anti-regucalcin monoclonal antibody in the reaction mixture causes an increase in nuclear DNA synthesis activity [32]. This increase was completely depressed in the presence of regucalcin [32]. Endogenous regucalcin was found to reveal a suppressive effect on DNA synthesis in the nuclei of rat liver. The effect of regucalcin in decreasing nuclear RNA synthesis activity in normal rat liver was not seen in the presence of α-amanitin, an inhibitor of RNA polymerase II and III [33,34], suggesting that this suppressive effect is partly resulted from the inhibitory action on RNA polymerase II and III. Thus, regucalcin was demonstrated to reveal direct inhibitory effects on nuclear DNA and RNA polymerase activity.

As described above, regucalcin, which is translocated into the nucleus, plays a pivotal role in the regulation of liver nuclear function.

**Overexpression of regucalcin suppresses hepatoma cell proliferation**

Overexpression of endogenous egucalcin has been shown to regulate nuclear function in proliferating cells using cloned hepatoma H4-II-E cells, which were cultured in the presence of fetal bovine serum (FBS). Culture with FBS enhanced an increase in cell number and a corresponding elevation of various kinase activities, which are related to Ca2+/calmodulin-dependent protein kinase, protein kinase C, protein tyrosine kinase and protein phosphatase activity in H4-II-E cells [35-37]. These enzymes may contribute to the enhancement of hepatoma cell proliferation after serum stimulation. The presence of anti-regucalcin monoclonal antibody in the enzyme reaction mixture using H4-II-E cells cultured with FBS stimulation was found to increase the activities of protein kinase and protein phosphatase [35,36]. Such an effect was depressed by addition of exogenous regucalcin in the enzyme reaction mixture. Regucalcin may play an important role as a suppressor protein in the enhancement of cell proliferation due to inhibiting the activities of various protein kinases and protein phosphatases in the cytoplasm and nucleus [35-37].

Nuclear DNA synthesis activity has been shown to increase at 6 hours after culture with FBS, which is preceded an elevation of the number of H4-II-E cells cultured with FBS [38,39]. The presence of regucalcin in the reaction mixture suppressed nuclear DNA synthesis activity in the cells [38,39]. This effect was partly mediated through pathway of various protein kinases in H4-II-E cells. Endogenous regucalcin suppressed nuclear DNA synthesis activity through mechanism by which inhibits protein kinases in the nuclei of proliferating H4-II-E cells using anti-regucalcin monoclonal antibody [38]. To determine the role of endogenous regucalcin in the regulation of nuclear DNA synthesis, moreover, regucalcin/pCXN2-transfected cells, which H4-II-E cells overexpress regucalcin stably, was generated [39]. The increase in cell number and DNA synthesis activity in transfectants was found to suppress as compared with those of wild- and mock-type, indicating that overexpression of endogenous regucalcin has suppressive effects on cell proliferation [39]. The presence of anti-regucalcin monoclonal antibody in the reaction mixture caused increases in nuclear DNA synthesis activity in the nuclei obtained from wild-type H4-II-E cells, mock-type cells, and transfectants with overexpression of regucalcin, although such an increase was remarkable in the transfectants [39]. This finding supports the view that the augmentation of endogenous regucalcin has great suppressive effects on nuclear DNA synthesis activity enhanced in proliferating hepatoma cells. Regucalcin may play a suppressive role for the over-proliferation of liver cells.

Endogenous regucalcin suppressed the expression of cell cycle-related genes in proliferating liver cells. Overexpression of regucalcin induced G1 and G2/M phase cell cycle arrest in transfectants (H4-II-E cells) [40]. p21 mRNA expression was enhanced in the transfectants, although cdc2a and chk2 (checkpoint-kinase 2) mRNA levels were not changed [40]. p21 is an inhibitor of cyclin-dependent kinases (cdk). Regucalcin may inhibit G1 progression through enhancement of p21 gene expression in H4-II-E cells [40]. Overexpression of endogenous regucalcin has also been shown to suppress proliferation of cloned normal rat kidney proximal tubular epithelial NRK52E cells [41]. Endogenous regucalcin induced G1 and G2/M phase cell cycle arrest in NRK52E cells [41], c-myc, c-fos, c-jun, and Ha-ras are known as tumor stimulator genes [42]. p53 and Rb are tumor suppressor genes, and c-src is oncogene [43]. The expressions of c-myc, Ha-ras, or c-src mRNAs were o suppressed in regucalcin-overexpressing transfectants [44]. The expressions of p53 and Rb mRNAs were markedly enhanced in the transfectants [44]. Suppressed expressions of c-myc, Ha-ras and c-src mRNAs and enhanced expressions of p53 and Rb mRNAs in the transfectants may be partly involved in retardation of proliferation of hepatoma H4-II-E cells [44]. Thus, endogenous regucalcin was shown to reveal suppressive effects on cell proliferation due to regulating many gene expressions that are related to cell proliferation in hepatoma cells. Regucalcin can bind DNA and modulates nuclear transcriptional activity [44]. Regucalcin may bind to the promoter region of various genes that suppress stimulator gene expression or stimulate suppressor gene expression in cell proliferation [14]. As the result, overexpression of endogenous regucalcin may suppress cell proliferation. This suppressive effect was independent on enhancement of apoptosis [12,13]. Regucalcin may play an important role as a suppressor protein in cell proliferation.

**Regucalcin gene expression is suppressed in hepatocellular carcinoma**

Hepatocellular carcinoma (HCC), the most common primary liver cancer, is one of the most prevalent malignant diseases worldwide, and the third most common causes of cancer-related death [45-47]. Globally, there are approximately 750,000 new cases of HCC reported per year. The incidence of HCC is increasing in the United States and other developed countries. Features of HCC are an aggressive cancer with a dismal outcome largely due to metastasis and postsurgical recurrence. HCC originates on a background of cirrhosis, a chronic and diffuse hepatic disease which results from continuous liver injury and regeneration [47]. Cirrhosis is present in approximately 80-90% of HCC patients and constitutes the largest single risk factor. In cirrhotic liver, changes in fat metabolism associated with the activation
of adipocyte-like pathways are thought to be involved in neoplastic transformation [47]. Increased hepatocyte turnover, inflammation and oxidative DNA damage is implicated in the pathogenesis of the liver disease including obesity, Type 2 diabetes, insulin resistant, and nonalcoholic fatty liver disease. The prevalent risk factors for HCC are also the cause of liver cirrhosis and include viral infections (hepatitis B and C) and alcohol consumption. Further risk factors include tobacco smoking, exposure to aflatoxin B1 and vinyl chloride, diabetes, and genetic disorders, such as hemochromatosis and alpha-1 antitrypsin deficiency [48-52].

Hepatocarcinogenesis is a multistep process initiated by external stimuli that lead to genetic changes in hepatocytes or stem cells, resulting in proliferation, apoptosis, dysplasia and neoplasia. The majority of HCC cases are related to chronic viral infections. Hepatitis B virus (HBV) DNA integrates into the host genome, inducing chromosome instability and insertional mutations that may activate various oncogenes, such as cyclin A [53-56]. Viral proteins, in particular X protein (HBx), act as transactivators to upregulate several oncogenes (such as c-myc and c-jun) and transcriptional factors (such as nuclear factor-kB) [57-60]. Additionally, HBx activates promoters of genes encoding interleukin-8 (IL-8), tumor necrosis factor (TNF), transforming growth factor (TGF)-β and epidermal growth factor receptor (EGFR) [60]. HBx can also stimulate several signal transduction pathways, including the JAK/STAT, RAS/RAF/MAPK, and Wnt/β-catenin pathways [60,61]. The contributions of hepatitis C virus (HCV) to hepatocarcinogenesis are mediated through viral proteins, including core, NS3 and NS5A proteins. HCV core protein can promote apoptosis or cell proliferation through interaction with p53 or upregulation of Wnt-1 at the transcriptional level [62-64].

The prognosis of advanced HCC remains poor in spite of the development of novel therapeutic strategies [65]. Traditional therapies are not effective for HCC and are too toxic for patients with cirrhosis. Transarterial chemoembolization and radioembolization are the main treatments for intermediate-stage HCC. Improved knowledge of the oncogenic processes and signaling pathways that regulate tumor cell proliferation, differentiation, angiogenesis, invasion and metastasis has led to the identification of several potential therapeutic targets that have driven the development of molecularly targeted therapies [65]. An ideal cancer target meets the following criteria: the target is relatively specific for cancer cells (not expressed or expressed at very low levels in normal cells but overexpressed in cancer cells) [65]. Meanwhile, overexpression of the target is associated with malignant biological phenotypes and/or poor prognosis; the target plays an essential role in cancer initiation and progression, and inhibition of expression or activity of the target induces growth suppression and/or apoptosis in cancer cells. The target is "drugable" as an enzyme (a kinase) or cell surface molecule (a membrane-bound receptor) that can be easily screened for small-molecule inhibitors or targeted by a specific antibody [65,66]. The only systemic therapy available for advanced HCC is based on the multitarget inhibitor sorafenib [66], which is the most effective therapeutic tool for advanced nonresectable HCC, in which it can slightly improve patient survival. The survival of patients with advanced HCC treated with sorafenib depends on the absence of liver dysfunction and on the status of the patient [67]. In the past few years, the use of sorafenib in combination with transarterial chemoembolization has improved survival rates in patients with advanced HCC. Recently, new perspectives in cancer treatment have appeared with the advent of microRNAs, a novel class of noncoding small RNAs [68].

Interestingly, the suppression of regucalcin gene expression has been shown to occur at earlier periods of carcinogenesis in rats treated with diethylnitrosamine and then 2-acetylaminofluorene combined with partial hepatectomy, which induces an increase in proliferating cells [69]. The suppression of regucalcin protein expression was also identified in proteomic analysis that was differentially expressed in the livers of rats fed 5% ethanol for 1 and 3 months [70]. In addition, regucalcin mRNA expression was found to be suppressed by disorder of liver metabolism that is induced by administration of carbon tetrachloride [71], galactosamine [72], phenobarbital [73] and ethanol [74]. Liver regucalcin protein levels were also decreased in conditions of diabetes [74]. Suppression of regucalcin gene expression may lead to cirrhosis and HCC. Suppressed regucalcin gene expression may lead to the development of HCC.

Noticeably, the regucalcin gene and its protein levels have been demonstrated to specifically suppress in human HCC using analysis with integrative analysis of multiple gene expression profiles and proteomics [75-79]. Regucalcin was found to be one of novel genes in HCC developed in patients with chronic viral hepatitis C.

Regucalcin, a suppressor protein in cell signaling system, may play a pivotal role as a key molecule in the depression of cell proliferation and carcinogenesis of various types of cells and tissues, as summarized in Figure 1. Overexpression of regucalcin in cancer cells may play preventive and therapeutic roles in development of carcinogenesis. The development of a novel gene therapy with the regucalcin gene deliver system will be expected in clinical aspects.

Prospect

Regucalcin is translocated into the nucleus of various types of cells. Regucalcin binds nuclear proteins and DNA and directly suppresses...
DNA and RNA synthesis. Also, regucalcin reveals suppressive effects on nuclear DNA and RNA synthesis through inhibition of phosphorylation and dephosphorylation of various proteins that are related to transcription. Regucalcin may directly bind on the promoter region of gene and regulates the expression of various genes as a transcription factor. Moreover, regucalcin is found to inhibit protein synthesis at translational process in liver cells. Thus, regucalcin reveals a multifunctional mechanism in control of cell proliferation.

Suppression of regucalcin gene expression in liver cells may lead to carcinogenesis with overexpression of cell proliferation. The regucalcin gene may be a target in the therapy of hepatoma. Further studies will be expected in clinical aspects.

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