Calcitonin Gene-related Peptide Inhibits Tumor Cell Proliferation of Hepatocellular Carcinoma Cells Through the Ras/MEK/ERK Pathway

Masaki Hara¹, Yuko Takeba², Minoru Watanabe³, Yuki Ohta²,
Takehito Ohtsubo⁴, Toshio Kumai¹, and Naoki Matsumoto²

(Received for Publication: September 17, 2015)

Abstract

Calcitonin gene-related peptide (CGRP) is widely distributed in the central and peripheral nervous systems and regulates physiological functions. Several neuropeptides are involved in the development and progression of hepatocellular carcinoma (HCC), although the role of CGRP in HCC pathogenesis is unclear. This study attempted to clarify the effects of CGRP on tumor progression in HCC cells. CGRP and its receptors, calcitonin receptor-like receptor and receptor activity-modifying protein-1, were expressed in HCC tissues and HCC cell lines. When Huh7 cells were cultured with CGRP 10^{-10} and 10^{-9} M for 24 h, cell proliferation was significantly inhibited. In addition, the total and phosphorylated protein levels of Ras and mitogen-activated protein kinase (MAPK) family proteins, MAP kinase (MEK) 1/2 and extracellular signal-regulated kinase (ERK) 1/2, were also inhibited by CGRP 10^{-10} M incubation. CGRP significantly increased the protein levels of cAMP response element binding protein (CREB) and its phosphorylated form in the nuclei of Huh 7 cells. Furthermore, pretreatment of CGRP receptor antagonist CGRP_{8–37} abolished the increases in CREB and pCREB protein levels in the nuclei of Huh7 cells. In addition, pretreatment of CGRP_{8–37} and cAMP inhibitor Rp-cAMP tended to reverse the ERK inhibition in Huh 7 cells cultured with CGRP. These results suggest that CGRP inhibits HCC cell proliferation via CREB activation and Ras/MEK/ERK pathway. CGRP may play an important role in the amelioration of cancer progression.

Key words

calcitonin-gene related peptide, hepatocellular carcinoma, Huh7, ERK, CREB

Introduction

The release of biochemical mediators such as cortisol, catecholamines, and neuropeptides is induced by a variety of physiological changes and enhances the immune response^{1,2}. Although such mediators generally exert beneficial effects, they often trigger pathological processes and contribute to the development and progression of various diseases, including cancer³. Several neuropeptides show antiproliferative activity in tumor models and cell lines³. In addition, direct antitumor effects are mediated through their receptors⁴. Thus, some mediators and receptors can become novel targets for antitumor therapy.

Hepatocellular carcinoma (HCC) is the most common cause of cancer-related death, and its incidence is increasing worldwide. Due to the known risk factors, mainly hepatitis B and C viruses (HBV and HCV), there is a rationale for a chemopreventive ap-

1 Department of Genomics, St Marianna University Graduate School of Medicine

2 Department of Pharmacology, St Marianna University School of Medicine

3 Institute for Animal Experimentation, St Marianna University Graduate School of Medicine

4 Division of Gastroenterological Surgery, St Marianna University Graduate School of Medicine

proach to treat HCC⁵). Chemotherapeutic agents currently used to treat advanced solid tumors are associated with significant toxicity. If neuropeptides secreted in vivo could be used as antitumor agents, those adverse effects might be reduced.

Therefore, the relationship between neuropeptide activity and HCC development must be clarified. To elucidate the roles of neuropeptides in the pathogenesis of HCC, we focused on the activity of calcitonin gene-related peptide (CGRP) as a novel target for antitumor therapy. The 37-amino acid neuropeptide CGRP is a potent vasodilator and mitogen involved in cardiovascular and neuronal physiology and neurogenic inflammation⁶).

The rat hepatobiliary tract is innervated by CGRP- and CGRP/SP/TK-immunoreactive fibers⁷). However, the expression and roles of CGRP have not yet been studied in patients with HCC. Receptors for neuropeptides are overexpressed in several human tumors⁸). Somatostatin analogues are used in the treatment of HCC due to the presence of somatostatin receptors in HCC cells^{8,9}). The calcitonin peptide family, including CGRP and adrenomedullin, are calcitonin receptor-like receptor (CLR) and receptor activity-modifying proteins (RAMPs)¹⁰). The cellular distribution of CGRP receptors in hepatocytes and HCC cells is still unknown.

In this study, we characterized the expression of CGRP and its receptor in HCC. In addition, we explored the effects of CGRP in HCC development or progression.

Materials and Methods

Materials

CGRP and the CGRP receptor antagonist CGRP₈₋₃₇ were purchased from Sigma (St. Louis, MO, USA) and the cAMP antagonist Rp-cAMPS was obtained from the Biology Life Science Institute (Bremen, Germany). Antibodies (Abs) used in this study were: rabbit anti-extracellular signal-regulated kinase (ERK) polyclonal Ab, mouse anti-phosphorylated (p)ERK monoclonal Ab, rabbit anti-mitogen-activated protein kinase (MEK)-1 polyclonal Ab, goat anti-pMEK polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-Ras monoclonal Ab, rabbit anti-cAMP responsive element binding protein (CREB) monoclonal Ab, rabbit anti-pCREB polyclonal Ab (Abcam, Tokyo, Japan), and mouse anti- α -tubulin monoclonal Ab (Wako, Tokyo, Japan).

Human liver tissues

Human HCC tissues were obtained from patients undergoing partial hepatectomy in St. Marianna University School of Medicine Hospital after written informed consent was obtained from each patient prior to surgery. The tissues were immediately frozen in liquid nitrogen after resection. Normal hepatic tissues were obtained from the organs of Caucasian and Hispanic transplantation donors and supplied by the National Disease Research Interchange (Philadelphia, PA, USA) through the Biomedical Research Institute, Human and Animal Bridging Research Organization (Chiba, Japan). This study protocol was approved by the St. Marianna University School of Medicine Ethics Committee (approval number 1207).

Immunohistochemistry

Normal liver tissues and HCC tissues fixed in 10% formalin were embedded in paraffin and then sliced 3 μ m thick. Paraffin-embedded samples were deparaffinized in xylene and rehydrated in graded ethanol. Immunoreactivity in sections was demonstrated using a Dako Envision system (Carpinteria, CA, USA) according to the manufacturer's instructions.

RNA extraction and reverse-transcription polymerase chain reaction

Total RNA was extracted using a commercial kit (RNeasy Mini, Qiagen, Germantown, MD, USA). The cDNA was synthesized from 1 μ g of total RNA using a RETROscript kit (Ambion Inc., Austin, TX, USA). cDNA was amplified in the polymerase chain reaction (PCR) on a thermal cycler (Life Tech, Foster City, CA, USA). The PCR reactions were performed in a Bio-Rad PCR System PTC-1152 thermal cycler (Tokyo, Japan) under the following conditions: 95°C for 30 s, followed by 35 cycles of denaturation at 95°C for 30 s; annealing at 55°C for 60 s; extension at 72°C for 60 s; and inactivation at 72°C for 10 min. The amplified products were resolved in a 2% agarose gel and visualized by Ultra Power DNA staining (Gellix International Ltd., Tokyo, Japan).

The primers for CGRP type-1 receptor were 5'-GACATCCAGCAAGCAACAGA-3' (sense) and 5'-CATGCCAAGCAATGGCACC-3' (antisense) and the primers for RAMP-1 were 5'-CTGCCAGGAGGCTAACTACG-3' (sense) and 5'-CCTCAGTGCGCTTGCTCT-3' (antisense). *GAPDH* was used as a housekeeping gene. The primers for

GAPDH were 5'-GACAACTTTGGTATCGTGGA-3' (sense) and 5'-TACCAGGAAATGAGCTTGAC-3' (antisense).

Cell lines and cell culture

The HCC Huh7 and hepatoblastoma HepG₂ cell lines were obtained from the Riken Gene Bank (Tsukuba, Japan). The Huh7 and HepG₂ cells were cultured with RPMI-1640 medium (Sigma, Osaka, Japan) containing 10% fetal calf serum (Invitrogen, Grand Island, NY, USA), with streptomycin 100 µg/ml and penicillin 100 units/ml at 37°C under a humidified atmosphere of 5% CO₂ and 95% O₂. After seeding, the cells were incubated for 24 h. Then, the cells were cultured with CGRP (10⁻¹²–10⁻⁸ M).

Cell proliferation assay

Huh7 cells (4 × 10⁴) were cultured with CGRP for 4, 16, and 24 h in 96-well flat Costar plates. (Corning International, Tokyo, Japan) in 200 µl of RPMI-1640 medium. After incubation, 20 µl of [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS)] substrate was added to each well. Cell proliferation was evaluated using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) following the manufacturer's instructions. Absorbance was measured at 490 nm with a microplate reader (Multiscan, Thermo Labsystems, Ventaa, Finland). The proliferation rate was expressed as the ratio of optical density of the treated to the control cells.

Western blot analysis

Whole proteins of Huh7 cells were extracted with cell lysing buffer (Tris-HCl 50 mM, pH 7.4, EGTA 1 mM, 0.001% leupeptin), and the nuclear proteins in the cells were separated using a commercial kit (NE-PER nuclear and cytoplasmic extraction kit, Pierce Biotechnology, Rockford, IL, USA). The protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). The samples (25 µg) were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Thereafter, the membrane was blocked with 5% skim milk in Tris-buffered saline containing Tween 20 (NaCl 150 mM, Tris-HCl 100 mM, pH 7.5, Tween 0.5%) overnight at 4°C. The blots were allowed to stand with the first Ab for 2 h at room temperature. Anti- α -tubulin polyclonal Ab (Wako, Osaka, Japan) was used as the loading control. Reactive proteins

were viewed under enhanced chemiluminescence (ImmunoStar, Wako). The intensity of the detected bands was analyzed using the C-DiGit Chemiluminescent Western Blot Scanner (LI-COR, Lincoln, NE, USA). Quantitative results of proteins detected were determined by densitometric analysis. Protein levels are expressed as the percentage of the values in untreated cells (controls).

Statistical analysis

All results are expressed as mean ± SEM. Statistical analysis was performed using Kyplot software (version 5.0, Kyeus Lab, Tokyo, Japan). Statistical differences were determined using the Dwass-type Steel multiple-comparison method. A *p* value of less than 0.05 was assumed to represent a statistically significant difference.

Results

Immunohistochemistry of CGRP in HCC tissues

We first studied the localization of CGRP protein in the hepatic tissues from patients with HCC. Etiological factors of HCC include HBV or HCV infection, and HCC is negative for both hepatitis B surface antigen and HCV antibody (non-B, non-C). We also examined liver tissues from patients with metastatic colorectal cancer and normal liver for comparison with HCC tissue.

Although marked localization of CGRP in normal liver tissue and that from patients with metastatic colorectal cancer was not observed, CGRP immunoreactivity was abundant in HCC tissue (Fig. 1A). Notable CGRP immunoreactivity was seen in both tumorous and nontumorous regions and was particularly high on the periphery of intrahepatic blood vessels in HCC tissues. In addition, these results indicate that CGRP is produced by HCC cells (Fig. 1B).

mRNA expression of CGRP receptors, CLR, and RAMP-1 in HCC tissue and cells

CGRP exerts its effects through a heterodimeric receptor comprised CLR and RAMP-1⁽¹¹⁾. CLR mRNA expression was seen in normal liver, the nontumorous region of HCC tissue, and Huh7 and HepG2 cells. However, CLR mRNA expression in the tumorous region of HCC tissue was relatively weak. Similar results on RAMP-1 mRNA expression were obtained (Fig. 2).

Figure 1. Immunohistochemical staining of calcitonin gene-related peptide (CGRP) in hepatocellular carcinoma (HCC) tissues with various etiologic factors. **(A)** CGRP expression in normal liver (**a**), colorectal cancer liver metastasis (**b**), hepatitis B virus-positive HCC (**c**), hepatitis C virus-positive HCC (**d**) and non-B, non-C HCC (**e**). Original magnification $\times 200$. The brown staining in the tissues indicates CGRP positivity. HCC tissues were obtained adjacent to the tumorous tissue. MC, metastatic carcinoma. **(B)** Immunohistochemical staining for CGRP in the tumorous and nontumorous regions of non-B, non-C HCC tissues. Original magnification $\times 200$ (**f** and **h**) and $\times 400$ (**g** and **i**). The nontumorous samples were taken at least 10 cm from the tumorous part of the liver and demonstrated pathologically to be free of tumor cells. All data are representative of tissues from 5 individual patients.

Cell proliferation in Huh7 and HepG2 cells cultured with CGRP

The MTS assay was used to examine the proliferation of Huh7 and HepG2 cells after culture with CGRP at concentrations ranging from 10^{-12} M to 10^{-8} M for 24 h. CGRP 10^{-10} M ($77.2 \pm 3.9\%$) and 10^{-9} M ($80.8 \pm 4.5\%$) inhibited the proliferation of Huh7 cells compared with controls ($100.0 \pm 1.0\%$). There was a significant difference in the proliferation of Huh7 cells cultured with CGRP 10^{-12} M ($95.2 \pm 1.9\%$) and 10^{-10} M. However, significant inhibition

of HepG2 cell proliferation ($92.2 \pm 3.7\%$) compared with controls ($100.0 \pm 1.6\%$) was only seen when CGRP 10^{-10} M was added to the culture medium (**Fig. 3**).

Protein levels of CREB and ser-133 pCREB in Huh7 cells cultured with CGRP

The action of neuropeptides is mediated by the binding to specific membrane-associated neuropeptide receptors. Most of these receptors belong to the G-protein-coupled receptor family¹². cAMP/CREB

Figure 2. mRNA expression of CGRP receptors, CGRP type 1 receptor (CLR), and receptor activity-modifying protein (RAMP)-1 in HCC tissue and HCC cell lines. RNA extraction and RT-PCR were performed in normal liver and HCC tissues and in Huh7 and HepG2 cells. Details are described in Materials and Methods. All data are representative of tissues from 5 individual patients.

activation requires the phosphorylation of CREB on ser-133, and CREB is essential for the response of the CGRP gene¹³). After Huh7 cells were cultured with CGRP for 15 min, we examined the protein levels of total CREB and pCREB in the nuclei using Western blotting. As shown in **Fig. 4**, CREB protein levels in cells cultured with CGRP 10^{-10} M ($270.9 \pm 55.0\%$) were significantly higher than those in control cells ($100.0 \pm 33.6\%$). The protein levels of pCREB also significantly increased in cells cultured with CGRP 10^{-10} M ($352.9 \pm 67.1\%$) compared with those of the controls ($100.0 \pm 38.8\%$).

Effects of the CGRP receptor antagonist CGRP₈₋₃₇ on CREB activation in Huh7 cells cultured with CGRP

To examine whether CREB activation in CGRP-treated cells mediates the CGRP receptor, Huh7 cells were precultured with the CGRP receptor antagonist CGRP₈₋₃₇ for 1 h, followed by incubation with CGRP 10^{-10} M for 15 min. CGRP₈₋₃₇ 10^{-11} M ($91.7 \pm 17.7\%$) and 10^{-10} M ($85.0 \pm 18.2\%$) significantly inhibited the increases in CREB protein levels in Huh7 cells incubated with CGRP 10^{-10} M ($161.1 \pm 11.8\%$). CGRP₈₋₃₇ 10^{-11} M ($107.4 \pm 22.3\%$) also significantly inhibited the increases in pCREB protein levels in cells incubated with CGRP ($257.3 \pm 55.7\%$). The results seen in pCREB protein levels were similar to those of CREB protein levels. CGRP₈₋₃₇ alone was confirmed to have no effect on CREB activation (**Fig. 5**). These results

Figure 3. Cell proliferation in HCC cells cultured with CGRP. The cells (4×10^4) were cultured with CGRP in a 96-well plate for 24 h. Cell proliferation was evaluated in the MTS assay. MTS was added to the medium after 2 h incubation with CGRP. Cell survival was detected by measuring absorbance at 490 nm. Cell proliferation was expressed as the ratio of the optical density of CGRP-cultured cells to that of controls. Data are expressed as mean $\pm$ SEM. $p < 0.05$ versus controls.

Figure 4. Protein levels of cAMP response element binding protein (CREB) and phosphorylated CREB (pCREB) in Huh7 cells cultured with CGRP. Huh7 cells were 80% confluent at the time of culture with CGRP (10^{-11} M, 10^{-10} M, and 10^{-9} M) for 15 min, and then their nuclei were analyzed using Western blotting. Details are described in Materials and Methods. Data are expressed as mean $\pm$ SEM. CGRP 10^{-10} M significantly increased CREB and pCREB protein levels in the nuclei of Huh7 cells. $p < 0.05$ versus controls.

indicate that CGRP stimulates CREB activation through its receptor.

Total and phosphorylated protein levels of Ras, MEK-1, and ERK in Huh7 cells cultured with CGRP

CGRP is known to regulate mitogen-activated protein kinase (MAPK) signaling¹³. To clarify the mechanisms of the inhibition of HCC cell proliferation, protein levels of Ras, MEK-1, and ERK1/2 in Huh7 cells cultured with CGRP for 24 h were analyzed using Western blotting. CGRP 10^{-10} M significantly inhibited the protein levels of Ras ($38.6 \pm 11.6\%$), MEK-1 ($66.4 \pm 11.2\%$), and ERK1/2 (60.8

Figure 5. Effects of the CGRP receptor antagonist CGRP₈₋₃₇ on protein levels of CREB and pCREB in Huh7 cells cultured with CGRP. Huh7 cells were precultured with CGRP₈₋₃₇ (10^{-11} M and 10^{-10} M) for 1 h, followed by the addition of CGRP (10^{-10} M) for 15 min, and the nuclei of cells were analyzed using Western blotting. Data are expressed as mean $\pm$ SEM. $p < 0.05$ versus CGRP 10^{-10} M-cultured cells.

$\pm 7.7\%$) compared with those of the respective controls (Ras, $100.0 \pm 3.1\%$; MEK-1, $100.0 \pm 7.1\%$; ERK, $100.0 \pm 9.8\%$). The phosphorylation of MEK-1 ($56.7 \pm 11.0\%$) and ERK1/2 ($58.6 \pm 10.5\%$) was inhibited in Huh7 cells cultured with CGRP 10^{-10} M compared with that in controls (pMEK-1, $100.0 \pm 3.7\%$; pERK, $100.0 \pm 15.8\%$) (Fig. 6).

Effects of CGRP₈₋₃₇ and Rp-cAMP on ERK inhibition in Huh7 cells cultured with CGRP

To determine whether the inhibition of the CGRP receptor or CREB activation causes the reverse ERK inhibition by CGRP, Huh7 cells were pre-cultured with CGRP₈₋₃₇ or the cAMP inhibitor Rp-cAMP for 1 h and then exposed to CGRP for 24 h. Pretreatment with CGRP₈₋₃₇ 10^{-11} M ($101.48 \pm 7.14\%$) and 10^{-10} M ($101.5 \pm 7.13\%$) or Rp-cAMP 10^{-7} M ($82.4 \pm 12.4\%$) tended to reverse ERK inhibition ($71.4 \pm 10.22\%$) in cells cultured with CGRP, suggesting that CGRP inhibits the MAP pathway through cAMP-dependent activation (Fig. 7).

Figure 6. Total and phosphorylated protein levels of Ras, ERK, and MEK-1 in Huh7 cells cultured with CGRP. Huh7 cells were cultured with CGRP for 24 h and then the protein levels of Ras, MEK-1, and ERK1/2 were analyzed using Western blotting. (A) Images shown are from a representative Western blotting experiment. (B) Data are expressed as the percentage of CGRP-cultured cells to that of controls. $p < 0.05$ versus controls.

Discussion

This study showed that CGRP inhibited cell proliferation through the Ras/MEK/ERK pathway in HCC cell lines. In addition, CGRP exerts its activity via the CGRP receptor and CREB activation. The rationale for this study was based on reports that the liver is densely innervated, including abundant CGRP-immunoreactive neurons of spinal afferent origin⁷. CGRP and its receptor are also expressed in hepatocytes and nonparenchymal hepatic cells¹⁴. CGRP was reported to contribute to systemic vasodilatation in cirrhosis and in cholangiocyte proliferation during cholestasis^{15,16}. CGRP is closely involved in the pathogenesis of liver disease. On the other hand, CGRP reduces the production of proinflammatory cytokines and prevents inflammatory liver damage¹⁷. Therefore, CGRP mediates both pro- and anti-inflam-

matory activities. Neuropeptides are known to play important roles in cancer such as tumor cell proliferation, antiapoptotic effects, and migration of tumor cells for invasion and metastasis^{18,19}. Although the roles of CGRP in liver disease have been investigated previously, the relationship between CGRP and HCC pathogenesis remains unknown.

We first studied the expression of CGRP and its receptor in HCC tissues with various etiological factors, i.e., HBV, HCV, and non-B, non-C infection. Nerve fibers containing CGRP are present in the perivascular nerve bundles running through the large interlobular hepatic blood vessels²⁰. Although prominent localization of CGRP in the tissues of normal liver and liver metastasis of colorectal cancer was not observed, CGRP immunoreactivity was high in the HCC tissues examined. CGRP immunoreactivity was seen in both tumorous and nontumorous regions and

Figure 7. Effects of CGRP₈₋₃₇ and the cAMP/CREB inhibitor Rp-cAMPS on protein levels of ERK in Huh7 cells cultured with CGRP. Huh7 cells were precultured with CGRP₈₋₃₇ (10⁻¹¹ M and 10⁻¹⁰ M) or Rp-cAMPS (10⁻⁸ M or 10⁻⁷ M) for 1 h, followed by 24 h incubation with CGRP, and then the cells were analyzed using Western blotting.

Data are expressed as mean ± SEM and representative of 7 independent experiments. **p* < 0.05 versus CGRP 10⁻¹⁰ M-cultured cells. *p* < 0.05 versus control.

was strong on the periphery of the intrahepatic blood vessels of HCC tissues. In addition, we found that CGRP was produced by HCC cells. CGRP has specific functional receptors. The mRNA expression of CLR and RAMP-1 was confirmed using RT-PCR in HCC cells and the HCC cell line in the present study. Both CLR and RAMP-1 mRNA were expressed in normal liver, the nontumorous region of HCC liver tissue, Huh7 cells, and HepG2 cells, although the expression in the tumorous region of HCC tissue was relatively weak. CGRP receptor mRNA levels are not altered by physiological stimulation such as inflammation^{21,22}. One reason is considered to be the presence of necrotic tissue within the tumorous region in HCC samples. Another reason is that expressions of CGRP receptors are decreased in the presence of liver cancer. Further study is needed concerning whether the expressions of CGRP receptors decrease in hepatic carcinogenesis of animal models. CGRP increased the production of proinflammatory cytokines in murine macrophages and splenocytes^{23,24}. On the other hand, CGRP also inhibits nuclear factor κB activation, thereby down-modulating the production of tumor necrosis factor-α²⁵. Umeda et al. repor-

ted the inhibition of mitogen-stimulated T lymphocyte proliferation by CGRP²⁶. CGRP thus exerts both antiinflammatory and pro-inflammatory effects.

To clarify the effects of CGRP on HCC cell proliferation, Huh7 and HepG2 cells were cultured for 24 h with CGRP at concentrations ranging from 10⁻¹² M to 10⁻⁸ M. Maximal inhibitory effects on cell proliferation occurred at the physiological concentration of CGRP 10⁻¹⁰ M. Bell-shaped concentration-response curves were obtained after culture with CGRP at high concentrations of 10⁻⁹ M and 10⁻⁸ M. This may be attributable to receptor desensitization due to internalization and depletion of the second messenger. Because greater inhibition of Huh7 cells was observed compared with HepG2 cells, subsequent experiments focused on Huh7 cells.

CGRP can upregulate CREB and its phosphorylation on ser-133 in the rat brain with ischemic neurons²⁷. Our data showed that the protein levels of total CREB and pCREB in the nuclei of Huh7 cells cultured with 10⁻¹⁰ M CGRP were significantly higher than those in control cells, consistent with previous reports. Activated CGRP receptors stimulate adenylyl cyclase activity and phospholipase C, stimulating cAMP production and the activation of protein kinase A (PKA), respectively. CRE is essential for the response of the calcitonin/CGRP gene promoter^{12,13}. Villa et al. reported that CGRP enhances PKA activity in human osteoblast-like cells and inhibits osteoprotegerin production through the CGRP receptor²⁸. To examine whether CREB activation in cells cultured with CGRP mediates the CGRP receptor, Huh7 cells were precultured with the CGRP receptor antagonist CGRP₈₋₃₇, followed by culture with CGRP 10⁻¹⁰ M. CGRP₈₋₃₇ 10⁻¹¹ and 10⁻¹⁰ M abolished the increases in CREB and pCREB protein levels in CGRP 10⁻¹⁰ M-cultured cells. These results demonstrate that CGRP stimulates CREB activation through the CGRP receptor in HCC cells. One target of cAMP/CREB associated with tumor cell proliferation is the MAPK cascade²⁹. cAMP/CREB activation inhibited cell mitogenesis, and the phenomenon was dependent on ERK-MAPK signaling in a rat HCC model³⁰. Ras/Ras families of oncogenes are transforming genes, and the Ras/Raf/MEK/ERK cascade has important roles in cell proliferation, differentiation, malignant transformation, and apoptosis^{31,32}. MAPK signaling is critical in HCC tumorigenesis³³. The anticancer drug 5-fluorouracil combined with the multikinase inhibitor sorafenib inhibits MAPK and STAT3 signaling in HCC cells³⁴. Our data showed

that CGRP 10^{-10} M significantly decreased Ras protein levels. In addition, the decreases in MEK and ERK protein levels were accompanied by their reduced phosphorylation. These results suggest that CGRP exerts antitumor effects by inhibiting the MAPK cascade in HCC.

Our results also showed that culture with CGRP₈₋₃₇ 10^{-11} M and 10^{-10} M or with the cAMP inhibitor Rp-cAMP 10^{-7} M tended to reverse the ERK inhibition in cells cultured with CGRP, suggesting that CGRP inhibits the MAPK cascade through the CGRP receptor and cAMP-dependent activation.

In conclusion, our results demonstrated the inhibitory mechanisms of CGRP on tumor cell proliferation in HCC. CGRP blocks tumor cell proliferation by targeting the CREB-dependent Ras/MAPK cascade.

The decrease of adverse effects is an important problem in the multidisciplinary opinions including combination chemotherapeutic agents for HCC treatment. Because CGRP is a biological active substance in a living body, CGRP seem to have few pharmacological side effects and enables long term administration for cancer treatment. CGRP 10^{-10} M significantly inhibited cell proliferation in HCC cell line although the action was weak. One idea for future study is the combined use of CGRP and anticancer agents.

However, it is necessary to clarify whether CGRP is effective in modulating the progression of HCC in human primary cultured cancer cells and animal models in vivo in the future. These findings may lead to promising new pharmacological strategies to inhibit tumor development and progression.

References

- 1) Chrousos GP, Gold PW. The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. *JAMA* 1992; 267: 1244–1252.
- 2) Seeman TE, Singer BH, Rowe JW, Horwitz RI, McEwen BS. Price of adaptation--allostatic load and its health consequences. *MacArthur studies of successful aging. Arch Intern Med* 1997; 157: 2259–2268.
- 3) McEwen BS, Stellar E. Stress and the individual. Mechanisms leading to disease. *Arch Intern Med*. 1993; 153: 2093–2101.
- 4) Cochaud S, Chevrier L, Meunier AC, Brillet T, Chadéneau C, Muller JM. The vasoactive intestinal peptide-receptor system is involved in human glioblastoma cell migration. *Neuropeptides* 2010; 44: 373–383.
- 5) Epstein RJ, Leung TW. Reversing hepatocellular carcinoma progression by using networked biological therapies. *Clin Cancer Res* 2007; 13: 11–17.
- 6) Geppetti P, Capone JG, Trevisani M, Nicoletti P, Zagli G, Tola MR. CGRP and migraine: neurogenic inflammation revisited. *J Headache Pain* 2005; 6: 61–70.
- 7) Goehler LEI, Sternini C. Calcitonin gene-related peptide innervation of the rat hepatobiliary system. *Peptides* 1996; 17: 209–217.
- 8) Reubi JC. Neuropeptide receptors in health and disease: the molecular basis for in vivo imaging. *J Nucl Med* 1995; 36: 1825–1835.
- 9) Borbath I, Leclercq IA, Abarca-Quinones J, Desaegeer C, Lebrun V, Moulin P, Sempoux C, Horsmans Y. Inhibition of early preneoplastic events in the rat liver by the somatostatin analog lanreotide. *Cancer Sci* 2007; 98: 1831–1939.
- 10) Verhoef C, van Dekken H, Hofland LJ, Zonderman PE, de Wilt JH, van Marion R, de Man RA, IJzermans JN, van Eijck CH. Somatostatin receptor in human hepatocellular carcinomas: biological, patient and tumor characteristics. *Dig Surg* 2008; 25: 21–26.
- 11) Cottrell GS, Padilla B, Pikios S, Roosterman D, Steinhoff M, Grady EF, Bunnett NW. Post-endocytic sorting of calcitonin receptor-like receptor and receptor activity-modifying protein 1. *J Biol Chem* 2007; 282: 12260–12271.
- 12) Burbach JP, Meijer OC. The structure of neuropeptide receptors. *Eur J Pharmacol* 1992; 227: 1–18.
- 13) Freeland K, Liu YZ, Latchman DS. Distinct signalling pathways mediate the cAMP response element (CRE)-dependent activation of the calcitonin gene-related peptide gene promoter by cAMP and nerve growth factor. *Biochem J* 2000; 345: 233–238.
- 14) Bracq S, Clement B, Pidoux E, Moukhtar MS, Jullienne A. CGRP is expressed in primary cultures of human hepatocytes and in normal liver. *FEBS Lett* 1994; 351: 63–66.
- 15) Møller S, Bendtsen F, Schifter S, Henriksen JH. Relation of calcitonin gene-related peptide to systemic vasodilatation and central hypovolaemia in cirrhosis. *Scand J Gastroenterol* 1996; 31: 928–933.
- 16) Glaser SS, Ueno Y, DeMorrow S, Chiasson VL, Katki KA, Venter J, Francis HL, Dickerson IM,

- DiPette DJ, Supowit SC, Alpini GD. Knockout of alpha-calcitonin gene-related peptide reduces cholangiocyte proliferation in bile duct ligated mice. *Lab Invest* 2007; 87: 914–926.
- 17) Kroeger I, Erhardt A, Abt D, Fischer M, Biburger M, Rau T, Neuhuber WL, Tiegs G. The neuropeptide calcitonin gene-related peptide (CGRP) prevents inflammatory liver injury in mice. *J Hepatol* 2009; 51: 342–353.
 - 18) Xu XT, Xu B, Song QB, Zeng H. The role of neural-related factors in the metastasis of the gastrointestinal cancer. *J Cancer Res Ther* 2013; 9: S123–S128.
 - 19) Muñoz M, Coveñas R. Involvement of substance P and the NK-1 receptor in human pathology. *Amino Acids* 2014; 46: 1727–1750.
 - 20) Ashraf S, Crowe R, Loizidou MC, Turmaine M, Taylor I, Burnstock G. The absence of autonomic perivascular nerves in human colorectal liver metastases. *Br J Cancer* 1996; 73: 349–359.
 - 21) Clifton MS, Hoy JJ, Chang J, Idumalla PS, Fakhruddin H, Grady EF, Dada S, Corvera CU, Bhargava A. Role of calcitonin receptor-like receptor in colonic motility and inflammation. *Am J Physiol Gastrointest Liver Physiol* 2007; 293: G36–G44.
 - 22) Shi X, Wang L, Clark JD, Kingery WS. Keratinocytes express cytokines and nerve growth factor in response to neuropeptide activation of the ERK1/2 and JNK MAPK transcription pathways. *Regul Pept* 2013; 186: 92–103.
 - 23) Tang Y, Feng Y, Wang X. Calcitonin gene-related peptide potentiates LPS-induced IL-6 release from mouse peritoneal macrophages. *J Neuroimmunol* 1998; 84: 207–212.
 - 24) Ahmed AA, Mutt V, Nordlind K. Modulating effects of sensory and autonomic neuropeptides on murine splenocyte proliferation and cytokine secretion induced by *Leishmania major*. *Immunopharmacol Immunotoxicol* 1999; 21: 507–526.
 - 25) Millet I, Phillips RJ, Sherwin RS, Ghosh S, Voll R, Flavell RA, Vignery A, Rincón M. Inhibition of NF-kappaB activity and enhancement of apoptosis by the neuropeptide calcitonin gene-related peptide. *J Biol Chem* 2000; 275: 15114–15121.
 - 26) Umeda Y, Takamiya M, Yoshizaki H, Arisawa M. Inhibition of mitogen-stimulated T lymphocyte proliferation by calcitonin gene-related peptide. *Biochem Biophys Res Commun* 1988; 154: 227–235.
 - 27) Zhang ZH, Fang XB, Xi GM, Li WC, Ling HY, Qu P. Calcitonin gene-related peptide enhances CREB phosphorylation and attenuates tau protein phosphorylation in rat brain during focal cerebral ischemia/reperfusion. *Biomed Pharmacother* 2010; 64: 430–436.
 - 28) Villa I, Mrak E, Rubinacci A, Ravasi F, Guidobono F. CGRP inhibits osteoprotegerin production in human osteoblast-like cells via cAMP/PKA-dependent pathway. *Am J Physiol Cell Physiol* 2006; 291: C529–C537.
 - 29) Stork PJ, Schmitt JM. Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. *Trends Cell Biol* 2002; 12: 258–266.
 - 30) Kovach SJ, Price JA, Shaw CM, Theodorakis NG, McKillop IH. Role of cyclic-AMP responsive element binding (CREB) proteins in cell proliferation in a rat model of hepatocellular carcinoma. *J Cell Physiol* 2006; 206: 411–419.
 - 31) McCubrey JA, Steelman LS, Franklin RA, Abrams SL, Chappell WH, Wong EW, Lehmann BD, Terrian DM, Basecke J, Stivala F, Libra M, Evangelisti C, Martelli AM. Targeting the RAF/MEK/ERK, PI3K/AKT and p53 pathways in hematopoietic drug resistance. *Adv Enzyme Regul* 2007; 47: 64–103.
 - 32) Cagnol S, Chambard JC. ERK and cell death: mechanisms of ERK-induced cell death—apoptosis, autophagy and senescence. *FEBS J* 2010; 277: 2–21.
 - 33) Wiesenauer CA, Yip-Schneider MT, Wang Y, Schmidt CM. *J Am Coll Surg* 2004; 198: 410–421.
 - 34) Deng L, Ren Z, Jia Q, Wu W, Shen H, Wang Y. Schedule-dependent antitumor effects of 5-fluorouracil combined with sorafenib in hepatocellular carcinoma. *BMC Cancer* 2013; 13: 363.